Posters

References for abstracts from speakers invited for plenary lectures

Thomas Langer *(Köln, Germany)*: «Quality control of membrane proteins in mitochondria» **(Abstract B-1)**

Carola Hunte and Christian Lange (Frankfurt, Germany): «Crystal structure of the yeast cytochrome bc_1 complex with its bound substrate cytochrome *c*» **(Abstract H-1)**

Martin Klingenberg (München, Germany) «Regulation of Uncoupling Proteins.» **(Abstract J-1)**

Frédéric Bouillaud *(Meudon, France)* « UCP1 and UCP2 in mice » **(Abstract K-1)**

References for abstracts from speakers invited for participants-organized sessions

Implication of K-ATP channels in physiology of the heart and cardioprotection against ischemia-reperfusion
Chairs: K.D. Garlid (Beaverton, USA) and P. Dos Santos (Bordeaux, France)

K.D. Garlid (Beaverton, USA) : "Overview"
P. Dos Santos (Bordeaux, France): "Is mitoKATP opening required for positive inotropy?"
V.A. Saks (Grenoble, France): "Factors controlling outer membrane permeability to nucleotides" **(Abstract K-33)**
A. Szewczyk (Warsaw, Polland): "Interactions of potassium channel effectors with mitochondria" **(Abstract J-22)**
F. Sztark (Bordeaux, France): "Bupivicaine as a mitoKATP opener" **(Abstract F-16)**

Mitochondrial pathologies
Chair: J. P. Mazat (Bordeaux, France) jpm@u-bordeaux2.fr

J. P. Mazat (Bordeaux, France) : "Introduction."
I. Bourges (Grenoble, France): "Characterization of the assembly pathway of human mitochondrial complex I." **(Abstract B-2)**
C. Deveaud (Bordeaux, France): "Effects of the treatment by nucleoside analogs of reverse transcriptase (NRTIs) on mitochondrial function of adipose superficial and deep tissues of rats." **(Abstract F-5)**
B. Faustin (Bordeaux, France): "Biochemical threshold effect and tissue specificity: Molecular basis and case of the adenine nucleotide translocator." **(Abstract K-10)**
F.M. Ferreira (Coimbra, Portugal): "Mitochondrial permeability transition and diabetes." **(Abstract K-11)**
F. N. Gellerich (Halle, Germany): "Genotype-phenotype relations in skeletal muscle of patients with deletions and point mutations of mtDNA." **(Abstract K-13)**
A. P. Kudin (Bonn, Germany): "Mitochondrial defect and effects of creatine on the survival of hippocampal neurons with defective mitochondria in an animal model of epilepsy." **(Abstract K-20)**
W. S. Kunz (Magdeburg, Germany): "Bioenergetic consequences of homoplasmic LHON mutations." **(Abstract G-21)**
T. Letellier (Bordeaux, France): "Biochemical threshold effect and tissue specificity: Molecular basis and case of the adenine nucleotide translocator. " and "Influence of a decrease of the mitochondrial DNA on the oxidative phosphorylation." **(Abstracts K-10, K-30)**
A. Martinuzzi (Bologna, Italy): "Antioxidant capacity in cybrids harboring the three mtDNA mutations related to complex I subunits and primarily associated with Leber's (LHON)." **(Abstract F-10)**
B. Meunier (London, UK): "Dysfunction of the respiratory chain complexes cytochrome oxidase and bc1: Studying disease-related mutations in yeast." **(Abstract F-11)**
C. Rocher (Bordeaux, France): "Influence of a decrease of the mitochondrial DNA on the oxidative phosphorylation." **(Abstract K-30)**
G. Solaini (Bologna, Italy): "Correlation between altered ATPase 6 gene and dysfunction of mitochondria from cells of patients affected by different neuropathies." **(Abstract K-40)**
J.-W. Taanman (Paris, France): "Cytochrome-c oxidase subassemblies in patients with cytochrome-c oxidase deficiency." **(Abstract B-8)**
T. Yagi (La Jolla, USA): "A single subunit NADH-quinone oxidoreductase as the rescue molecule for neurodegenerative diseases with complex I deficiency." **(Abstract G-26)**

Session «Evolution of bioenergetic enzymes by shuffling of electron transfer modules»
Chair: W. Nitschke (Marseille, France) nitschke@ibsm.cnrs-mrs.fr

T. Friedrich (Freiburg, Germany): "Modular evolution of the respiratory complex I."
S. Iwata (London, UK): "Electron transfer modules of formate dehydrogenase-N from E. coli." **(Abstract E-10)**
M. Pereira (Lisbon, Portugal): "Evolution of haem-copper oxygen reductases based on their functional and structural properties."
F. Baymann (Oxford, UK): "The Rieske protein; a versatile redox module."
S. Albracht (Amsterdam, The Netherlands): "Hydrogenases: a useful basis to better understand Complex I"
M.-T. Giudici (Marseille, France): "Membrane-integral diheme b-type cytochromes."

Session «Anaerobic respiratory membrane protein complexes and succinate dehydrogenases»
Chair : R. Lancaster (Frankfurt, Germany) (roy.lancaster@mpibp-frankfurt.mpg.de)

C.R.D. Lancaster (Frankfurt, Germany) Introduction : "Structure and mechanism of the quinol : fumarate reductase from Wolinellasuccinogenes." **(Abstract E-11)**
M. Teixeira (Oeiras, Portugal): "Functional studies on dihaemic bacterial SQR/QFR."
G. Cecchini (San Fransisco, U.S.A.): "Fumarate reductase and succinate dehydrogenase from E. coli." **(Abstract E-4)**
J. Simon (Frankfurt, Germany): "The cytochrome c nitrite reductase complex of Wolinella succinogenes." **(Abstract E-17)**
S. Iwata (London, U.K.): "Structure of formate dehydrogenase-N from E. coli." **(Abstract E-10)**

Session «Role of Mitochondria in Apoptosis»
Chairs: V.P. Skulachev (Moscow, Russia) skulach@genebee.msu.su and S. Manon (Bordeaux, France) (stephen.manon@ibgc.u-bordeaux2.fr).

V.P. Skulachev (Moscow, Russia): "General introduction."
E. Fontaine (Grenoble, France): "Regulation of PTP-induced cell death by complex I." **(Abstract K-16)**
P.J. Oliveira (Coimbra, Portugal): "Carvedilol inhibits the high conductance state of mitochondrial permeability transition: role of protein thiol groups and relevance for apoptosis." **(Abstract K-28)**
A. Vercesi (Campinas, Brasil): "Participation of reactive oxygen species in mitochondrial permeability transition." **(Abstract K-43)**
E. Milanesi (Padova, Italy): "The mitochondrial mechanisms of cell killing by Bcl-2 ligand HA14-1." **(Abstract K-53)**
S. Manon (Bordeaux, France): "Yeast as a model for studying mechanisms of cell death." **(Abstract E-10)**
V. Borutaite (Kaunas, Lithuania): "Mitochondria mediate apoptosis in ischemic myocardium."
A. Carrière (Toulouse, France): "Effect of mitochondrial ROS on the adipose tissue development.?" **(Abstract K-7)**
General Discussion.

Respiratory energy conversion from archaebacteria to cyanobacteria
Chair: G.A. Peschek (Wien, Austria) peschek@ftp.bpc.univie.ac.at

G. A. Peschek (Wien, Austria): "Respiration without oxygen? - Cyanobacteria as creators of the oxygen cataclysm."

G. Schaefer (Luebeck, Germany): “Respiratory chains in Archaea: from minimal systems to supercomplexes.”
N. Sone and J. Sakamoto (Fukuoka, Japan): “Cytochrome-c reductases in Gram positive bacteria.” **(Abstract H-9)**
O.Maneg, B. Ludwig and F. Malatesta (Frankfurt, Germany): “Electron transfer between Paracoccus denitrificans cytochrome c552 and cytochrome-c oxidase CuA soluble domains.” **(Abstract D-17)**
M. Teixeira (Lisbon, Portugal): “The oxygen-reducing pathways of anaerobic sulphate-reducers and evidence for a novel type of NO reductase.”
P. Hellwig, (Frankfurt, Germany): “FTIR spectroscopic study on protonable sites in the proton pathways of cytochrome oxidases.” **(Abstract D-5)**
S. Ferguson-Miller (East Lansing, USA): “Rhodobacter sphaeroides cytochrome c oxidase: what it tells us about electron input, proton output and respiratory control” **(Abstract D-12, D-27)**

Session «Structure and function of complex I»
Chairs: T. Friedrich (Freiburg, Germany) (tfriedri@uni-freiburg.de) and T. Ohnishi (Philadelphia, USA) ohnishi@mail.med.upenn.edu

T. Ohnishi (Philadelphia, USA): Introduction

A) Structure:
T. Friedrich (Freiburg, Germany): “A Novel, Enzymatically Active Conformation of theEscherichia coli Complex I”. **(Abstract G-7)**
L. Sazanov (Cambridge, UK): “Structural characterization of E. coli complex I by electron cryo-crystallography”. **(Abstract G-23)**

B) Energy coupling:
P. Hellwig (Frankfurt, Germany): “FT-IR spectroscopic characterization of the redox reaction of cluster N2 and the quinone binding site of complex I from E. coli”. **(Abstract G-12, G-16)**
Nick Fisher (London, UK): “ATR-FTIR difference spectroscopy of Complex I from beef heart, Yarrowia lipolytica, and E. coli”. **(Abstract G-11, G-22)**
T. Ohnishi (Philadelphia, USA): “Characterization of multi-component semiquinone species and cluster N2 in bovine heart complex I in coupled SMP and in the isolated enzyme”. **(Abstract G-28)**
F. MacMillan (Frankfurt, Germany): “Complex I studied by Pulsed EPR Spectroscopy”. **(Abstract G-23)**

C) Sodium pumping:
J. Steuber (Zürich, Switzerland): “Sodium cycle mediates energy coupling between complex I and ATP synthase”. **(Abstract G-15)**
B. Barquera (Urbana, USA): “Characterization of the flavin radical of Na+-translocating NADH:ubiquinone oxidoreductase from Vibrio cholerae”. **(Abstract G-4)**
M. Verkhovsky (Helsinski, Finland): “Kinetics of the spectral changes during reduction of the Na+-motive NADH:quinone oxidoreductase from Vibrio Harveyi.” **(Abstract G-42)**

Aging and mitochondria
Chair: T. Nyström (Goteborg, Sweden) thomas.nystrom@gmm.gu.se

T. Nyström (Goteborg, Sweden) “Introduction.”

M. Ballesteros (Goteborg, Sweden): “Respiration, mistranslation and oxidation: lessons from Escherichia coli.”
H. Aguilaniu (Goteborg, Sweden): “Proteins oxidation and senescence in Saccharomyces cerevisiae.” **(Abstracts F-1, K-55)**
N. Camougrand (Bordeaux, France): “The yeast Uth1p protein: role in mitochondrial biogenesis and in aging.” **(Abstract K-6)**
V. P. Skulachev (Moscow, Russia): “Mitochondria and Aging: an overview and personal reflection.”

Session A: ATPases

0005-2728/02/$ - see front matter
PII: S0005-2728(02)00245-1

A.1. Rebuilt 3D asymmetrical structure of chloroplast F_1-ATPase complex

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Using an original homology modelling technique, based on robust distance geometry protocols, we built a 3D model of the $alpha_3-beta_3-gamma$ CF_1-subcomplex (3200 residues), in which we introduced three different nucleotide occupancies to check their influence on the tentoxin-binding site. Simultaneous comparison of high-resolution X-ray structures of nonchloroplast F_1, performed with a local structural alignment search tool, led to characterising common structural blocks (CSB) and the distortions experienced by the complex during catalytic turnover. The CSBs were used as a starting point of the rebuilding of an asymmetrical structure of spinach CF_1. Finally, tentoxin was docked into its binding site of the reconstructed structure. The docking method was validated in the mitochondrial enzyme by its ability to relocate nucleotides into their original position in the crystal. Using X-PLOR docking facilities, tentoxin binding was found to allow alpha/beta interface associated with the empty and ADP-loaded catalytic sites, and disfavoured the interface associated with the ATP-loaded site. This shows that asymmetrical structure is essential for understanding the tentoxin effect in the full complex, and suggests a mechanism of CF_1 inhibition by a single tentoxin based on the failure of the alpha/beta interface-bearing tentoxin to pass through disallowed state.

Keywords: ATP synthase; F_0F_1-ATPase; Tentoxin; Homology modelling; Local structural alignment; Docking; Binding change mechanism

Reference

[1] C. Minoletti, J. Santolini, F. Haraux, J. Pothier, F. André, Proteins Struct. Funct. Genet. (2002) in press.

A.2. Structural studies of the membrane domain of bovine F_1F_0-ATP synthase

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In the past years, atomic resolution information on the soluble domain F_1 of the ATP synthase has allowed a better understanding of the catalytic mechanism of ATP hydrolysis and synthesis [1,2]. However, despite recent advances on the structural determination of the subunit c ring from different species [3–5], little is known about the general architecture of the membrane domain, F_0.

Here we report current progress in the two-dimensional crystallisation of bovine F_1F_0-ATP synthase. The results will allow us to get an insight not only into the subunit c ring but also its interactions with the other membrane subunits a and b.

Keywords: F-ATPase; Two-dimensional crystallization; Electron microscopy; Membrane domain

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[1] J.P. Abrahams, A.G.W. Leslie, R. Lutter, J.E. Walker, Nature 370 (1994) 621–628.
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A.3. Engineering F_1-ATPase from the thermophilic *Bacillus* PS3 sensitive to the phytophathogenic inhibitor tentoxin

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In contrast to the homologous bacterial and mitochondrial enzymes, the chloroplast F_1-ATPase (CF_1) is strongly affected by the phytopathogenic inhibitor tentoxin. Based on structural information obtained from crystals of a CF_1–tentoxin co-complex, we have predicted which amino acid residues are important for tentoxin binding. Subsequently, we replaced these crucial residues in the tentoxin-resistant thermophilic F_1-ATPase from *Bacillus* PS3 (TF_1) by the corresponding residues of CF_1 in order to render the thermophilic enzyme tentoxin-sensitive. We succeeded in conferring tentoxin-sensitivity to TF_1 with a degree of inhibition comparable to CF_1. The role of several amino acid residues for tentoxin binding as well as the feasibility and limits of structure-based protein design for this kind of inhibitor studies will be discussed.

Keywords: TF1; Tentoxin; Site-directed mutagenesis; Protein design

A.4. Stepwise rotation of the γ-subunit in reconstituted EF_0F_1 H^+ ATP synthase observed by inter-subunit single-molecule FRET

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H^+ ATP synthases catalyze the synthesis of ATP by coupling two rotary motors. To monitor inter-subunit rotation, we attached two fluorophores—Cy5 at the rotating γ-subunit and Tetramethylrhodamine at one b-subunit—and reconstituted the enzymes fully functional into liposomes. Fluorescence resonance energy transfer (FRET) was monitored in photon bursts of freely diffusing ATP synthases with a confocal setup for single molecule detection. Incubation with AMPPNP resulted in stable intensity ratios within a photon burst of one enzyme and three different FRET efficiencies. With ATP, a consecutive order of three distinguishable FRET efficiencies was observed, indicating a stepwise movement of the γ-subunit. We will present our latest results as steps towards the direct visualization of γ-subunit rotation during ATP synthesis by single-molecule FRET.

Keywords: Inter-subunit rotation; Single-molecule FRET; F_0F_1 ATP synthase

Reference

[1] M. Börsch, M. Diez, B. Zimmermann, R. Reuter, P. Gräber, Monitoring γ-subunit movement in reconstituted single EF_0F_1 ATP synthase by fluorescence resonance energy transfer, in: R. Kraayenhof, A.J.W.G. Visser, H.C. Gerritsen (Eds.), Fluorescence Spectroscopy, Imaging and Probes—New Tools in Chemical, Physical and Life Sciences, Springer-Verlag, Heidelberg, Germany, 2002.

A.5. Three-dimensional map of a plant V-ATPase based on electron microscopy

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V-ATPases pump protons into the interior of various subcellular compartments at the expense of ATP. Previous studies have shown that these pumps comprise a membrane-integrated proton translocating (V_o) and a soluble catalytic (V_1) subcomplex connected to one another by a thin stalk region. Here we present two three-dimensional maps derived from electron microscopic images of the complete V-ATPase complex from the plant *Kalanchoë daigremontiana* at a resolution of 2.2 nm. In the presence of a nonhydrolyzable ATP analogue the details of the stalk region between V_o and V_1 were revealed for the first time in their three-dimensional organisation. A central stalk was surrounded by three peripheral stalks of different sizes and shapes. In the absence of the ATP analogue, the tilt of V_o changed with respect to V_1 and the stalk region was less clearly defined, perhaps due to increased flexibility and partial detachment of some of the peripheral stalks. These structural changes corresponded to decreased stability of the complex and might be the initial step in a controlled disassembly.

Keywords: V-ATPase; Electron microscopy

A.6. Purification, characterization and reconstitution of the F_1F_0-ATP synthase from an alkaliphilic, thermophilic *Bacillus* sp. strain TA2.A1

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The F_1F_0-ATP synthase from an extremely thermophilic, alkaliphilic *Bacillus* sp. strain TA2.A1 was purified in an active form that was reconstituted into proteoliposomes. SDS-polyacrylamide gel electrophoresis of the purified enzyme revealed a typical subunit pattern for an F_1F_0-ATPase with five F_1 subunits (α, β, γ, δ, ε) and three F_0 subunits (a, b, c). The polypeptides were identified by N-terminal protein sequencing. ATPase activity was specifically activated by the detergent lauryldimethylamine oxide (LDAO) but not by Na^+ or Li^+ ions. The reconstituted ATPase was able to catalyze ATP synthesis driven by an artificial membrane potential that was sensitive to either *N*,*N*-dicyclohexyl carbodiimide or carbonyl cyanide *m*-chlorophenylhydrazone, but not monensin. Furthermore, ATP synthesis was not dependent on Na^+ ions. The nine genes that encode the ATP synthase from *Bacillus* sp. TA2.A1 were cloned and sequenced. The genes were organized in an operon with the gene order *atpIBEFHAGDC*. The deduced amino acid sequences of the subunits of this enzyme are similar to the subunits from other *Bacillus* species and the c-subunit from strain TA2.A1 revealed no signature for sodium binding. The results of this study demonstrate that the F_1F_0-ATPase from *Bacillus* sp. strain TA2.A1 employs protons for ATP synthesis and not Na^+ ions.

Keywords: *Bacillus* sp. TA2.A1; Alkaliphilic and thermophilic ATP synthase

A.7. Fluorescence resonance energy transfer (FRET) reveals a role for OSCP in stabilising the conformation of F_1F_o-ATP synthase during rotation in *Saccharomyces cerevisiae*

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We have demonstrated that two different subunits of the yeast mitochondrial ATP synthase (mtATPase), OSCP and subunit b, may be fused at the C terminus with GFP variants appropriate for fluorescence resonance energy transfer (FRET; BFP11 as the donor and YEGFP3 as the acceptor fluorophore), and assembled into the same functional mtATPase complex. This subunit fusion combination exhibits FRET in isolated mitochondria. We have now examined the properties of an OSCP variant that contains a G166N substitution. The G166N variant was previously shown to have properties consistent with conformational changes within mtATPase structure, namely, proton leakage through the proton pore and an increased propensity (compared to native OSCP) to dissociate from the mtATPase upon detergent solubilisation of mitochondrial membranes [1]. Subunit b-YEGFP3 and G166N–BFP11 fusions, when expressed in yeast cells lacking both endogenous subunits, were incorporated into functional complexes. FRET was exhibited in mitochondria isolated from such cells. However, in contrast to the enzyme containing native OSCP, FRET was lost when the enzyme containing G166N entered an active state (hydrolysis). These results suggest that residue G166 of OSCP is involved in protein–protein interactions that play a crucial role in stabilizing the catalytic sector of the complex (F_1) during periods of activity.

Keywords: mtATPase; OSCP; Subunit b; GFP; FRET

Reference

[1] G.M. Boyle, X. Roucou, P. Nagley, R.J. Devenish, M. Prescott, J. Bioenerg. Biomembranes 32 (2000) 469–481.

A.8. Mutational analysis of the dimerization domain of the *b* subunit of ATP synthase

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The *b* subunit of the ATP synthase of *Escherchia coli* forms an extended, dimeric helical structure that serves as a peripheral stalk linking the F_1 and F_0 sectors [1]. Truncation studies [2] have shown that residues Asp-53 to Lys-122 are essential for dimerization of the isolated *b* subunit, and selected sites in this region were cross-linked to $\alpha_3\beta_3$ [3].

Deletion of residue Lys-100 of *b* (Δ100) resulted in loss of ability to grow using acetate as the energy source, whereas a K100A mutation was functional. The ability to grow on acetate was retained by cells carrying deletions of four residues (Δ100–103) or of seven residues (Δ100–106), demonstrating that maintaining the helical face is more essential than length.

Mutation of residues Asn-80 to Ser-83, predicted to form a bend or to impart flexibility, also had no effect on growth. To investigate the importance of specific interactions between *b* and $\alpha_3\beta_3$, residues Leu-54 through Val-110 of *E. coli b* were replaced with the corresponding residues from *Bacillus subtilis*, which are only 25% identical. Strains carrying either of these substitutions retained the ability to grow on acetate, indicating that strong specific interactions between *b* and $\alpha_3\beta_3$ are not essential for function.

Keywords: ATP synthase; *b* subunit; Dimerization domain

References

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A.9. A novel mitochondrial genome as a tool for the genetic analysis of *S. cerevisiae* F_1-F_0 ATPsynthase

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Using a tetracycline-regulatable promoter, we found that partial depletion of the δ-subunit of yeast mitochondrial F_1.ATPase resulted in a total uncoupling of mitochondria. In such mitochondria, respiratory activity was maximal (i.e. equivalent to that of the wild-type control with CCCP) but turned back to normal state 4 values when oligomycin was added. Thus, the proton channel of F_0 can apparently be made without δ, but protons pass freely across mitochondrial inner membrane. Such proton leaks are lethal and consequently δ-less cells must inactivate their F_0 to survive. This can easily be achieved by loss of mtDNA that codes for three essential F_0 subunits (at a 10^{-2} frequency). To facilitate isolation of rare mutations (10^{-5}–10^{-8}) that specifically inactivate F_0, we have relocated in mtDNA a recoded nuclear gene (ARG8m) encoding a mitochondrial protein needed for arginine biosynthesis [1]. Consequently, when no arginine is available, the mtDNA becomes absolutely essential and δ-less cells can no longer be rescued by loss of this DNA. This makes it now possible to efficiently isolate mutations that inactivate F_0 (by restoration of δ-less cell proliferation on fermentable medium devoid of arginine). This system will be particularly useful to better understand the structure/function/assembly/regulation relationships of F_0 in mitochondria.

Keywords: Mitochondrion; F_1F_0-ATPsynthase; Uncoupling; Mitochondrial DNA; F_0 inactivation

Reference

[1] D.F. Steele, C.A. Butler, T.D. Fox, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 5253–5257.

A.10. New insights in the interaction mechanism between alpha and beta subunits of the yeast F1-ATPase

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The F1-ATPase of a *Schizosaccharomyces pombe* alpha-subunit revertant strain is characterized by the loss of negative cooperativity and a lower K_i for ADP [1]. The mutation alters the binding of a nucleotide to the regulatory site [2] and was later identified as a Gln173Leu replacement [3]. Here, the 3D-structure of the F1-ATPase [4] has been analysed to propose a structural basis for the mechanism of negative cooperativity.

AlphaGln173 (Gln172 residue in the beef heart enzyme) is a conserved residue of the Walker A-motif. The 3D structure shows that it stabilises the nucleotide bound to the noncatalytic site by a hydrogen bond. Gln172 also interacts with the adjacent betaThr354 via a second hydrogen bond at the alpha(DP)–beta(TP) interface, which forms a noncatalytic site. This interaction is also stabilized by a alphaArg171–betaAsp352 salt bridge. More interestingly, beta residues 352 to 354 are closed to betaTyr345, which stacks the adenine moiety of the nucleotide bound at the catalytic site. The beta 345–354 region thus constitutes a short link that could propagate the structural information from a noncatalytic site to the adjacent catalytic site and thereby modulate the affinity of the ADP bound to the catalytic site, which is responsible for negative cooperativity.

Keywords: Mitochondrial F1-ATPase; Alpha subunit; Regulation yeast

References

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A.11. Coupling mechanism in F_oF_1-ATP synthase of *Rhodobacter capsulatus*: influence of the catalytic F_1 part on proton conduction through F_o

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We monitored proton transport through F_oF_1-ATP synthase in chromatophores of a photosynthetic bacterium *Rhodobacter capsulatus* by using the set-up described in Ref. [1]. The protonmotive force was generated by membrane photosynthetic proteins in response to a short (10 μs) actinic flash. Electrochromic bandshift of intrinsic carotenoids [2,3] was used to follow the transmembrane proton transfer.

We compared the proton transport through coupled F_oF_1-ATP synthases with the "futile" proton transport through "bare" F_o moieties that lost the connection to F_1. The pH dependence of the extent of the proton flow revealed an apparent pK of 7.1 both for the coupled and the "futile" proton transport. The slope of the pH-dependence was much steeper in the case of the coupled proton transport, implying a joint action of >2 protonatable groups. The coupled proton transfer rate showed similar pH dependence in both cases but remained approximately two times slower in the case of the coupled proton transfer. A threshold value of the protonmotive force was found for the coupled proton flow, in contrast to the "futile" one. The data obtained are discussed in connection with possible mechanism of energy transfer between F_o and F_1 moieties of the ATP synthase.

Keywords: ATP synthase; *Rhodobacter capsulatus*; Proton flow; Coupling mechanism

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A.12. Thermodynamic properties of residues that effect ligation of the F1-ATPase metal cofactor

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The relationship between ATP hydrolysis and the rotation of the F1-ATPase 'gamma' subunit was examined. Arrhenius analyses were used to compare the thermodynamic properties site-directed mutants to wild-type F1-ATPase. Residues were selected for rotation that were known to alter the ligands of the metal cofactor for the enzyme or were at an interface with between the 'beta' and 'gamma' subunits at positions that had direct connection with the metal cofactor binding site. These results provide insight into the role of the metal cofactor in the mechanism of coupling of ATP hydrolysis to 'gamma' subunit rotation.

Keywords: F1-ATPase; Metal ligand

A.13. Topological and functional study of subunit *h* of the F1-Fo ATP synthase of *Saccharomyces cerevisae*

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The subunit *h* of the F1-Fo ATP synthase, encoded by the *ATP14* gene, has been discovered and purified in 1996 [1]. As ATP synthesis activity of the complex is abolished in the Δ*atp14* strain, subunit *h* is essential for either the coupling or the assembly of F1 and Fo sectors. Moreover, most of these cells (>90%) present a "Pet" phenotype. The disruption of the *ATP14* gene causes instability and finally a loss of mitochondrial genome. As subunits 6, 8 and 9 are encoded by this genome, this instability forbids the structural and functional analysis of the F1–Fo ATP synthase in a Δ*atp14* strain.

At the beginning of this work, the only environmental data available about subunit *h* in the F1–Fo ATP synthase complex described a proximity between the 98th residue of subunit 4 and an unspecified region of subunit *h* [2]. Using site-directed mutagenesis and molecular cross-linking, we build a model of the topological environment of the subunit *h*. Subunits *d*, *f*, 4 and α are its principal neighbours in the complex.

The study of a Δ*atp14* strain in which the mitochondrial genome is artificially maintained suggests an implication of subunits *h* and *f* in the coupling of F1 and Fo sectors. Our first data show an accumulation of subunits 4, *d*, OSCP, 6 and 9 in the mitochondria and the presence of a functional F1 sector.

Keywords: Mitochondrion; ATP synthase; Topology; Cross-linking; Assembly

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A.14. ATP-induced energy-dependent inhibition of photophosphorylation

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Pre-illumination of DTT-unmodulated broken chloroplasts in the presence of ATP results in a decreased photophosphorylation rate. The decrease is time- and ATP concentration-dependent and reaches its maximum after 3 min illumination with half maximal ATP concentration of about 2 μM. Pre-illumination of chloroplasts induces neither notable activation of ATP hydrolysis nor increased permeability of the thylakoid membrane. The inhibiting effect of ATP pre-illumination is reversed by short dark incubation. These results suggest that illumination of unmodulated broken chloroplasts in the presence of ATP inhibits ATP synthase activity of CF_oCF_1.

Keywords: ATP synthase; Photophosphorylation; Hysteretic inhibition; Chloroplast

A.15. Interconversion between different states of the yeast mitochondrial F_0F_1-ATPase triggered by the natural inhibitor protein (IF1) in the presence of the protonmotive force

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The effect of protonmotive force (pmf) and nucleotides on the interaction between the F_0F_1-ATPase of coupled yeast submitochondrial particles and IF1 was studied. Addition of IF1 during coupled ATP hydrolysis in the presence of pmf nearly completely inhibited the activity. However, subsequent addition of an uncoupler caused burst of ATPase activity up to 100% of overall uncoupled ATPase rate, followed by its IF1-dependent decay. Similar uncoupler-induced stimulation of IF1-inhibited hydrolysis was observed when ITP or GTP were used as ATPase substrates.

The results suggest that inhibition induced by IF1 in the presence of pmf and nucleotides consists of two steps: (1) slow binding of IF1 to NTP-loaded enzyme accompanied by inhibition; (2) fast NDP-dependent conversion of the enzyme to a latent form, unable to hydrolyse ATP, accompanied by IF1 release. Finally, upon an uncoupler addition, the latent form of the enzyme is rapidly converted to an active IF1-sensitive ATPase.

A quite unexpected finding is that in the presence of pmf, IF1 is able to inhibit F_0F_1-ATPase without remaining bound on the enzyme. This process of creating a latent form of the enzyme recalls the case of the energy-dependent transformation of the bovine heart (Mg)ADP-inhibited mitochondrial F_0F_1-ATPase [1].

Keywords: ATP synthase; F_0F_1-ATPase; Protonmotive force; IF1; Yeast mitochondria; Unidirectional inhibition

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A.16. Variability of subunit *c* stoichiometry in isolated F_o complexes

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F_o complexes from different organisms comprise individual numbers of *c* subunits, ranging from 10 to 14. To elucidate whether these different *c* stoichiometries are fixed or possibly due to dynamic variations, isolated F_o complexes from *Escherichia coli* were investigated with respect to both functional and structural aspects. Wild type as well as mutant F_o, the latter comprising a significantly reduced amount of subunit *c*, was used for functional reconstitution, cross-linking, and electron microscopic analysis. Rates of passive proton translocation strongly correlated with the amount of subunit *c* present, with the mutant F_o complexes showing significantly lower rates, which could be restored upon addition of purified subunit *c*. Electron microscopic analysis of freeze fractured specimen of reconstituted F_o complexes revealed that compared to wild type, the isolated F_o complexes of the mutant strain comprise a significantly reduced diameter according to a lower subunit *c* stoichiometry, which could also be restored by the addition of subunit *c* during reconstitution.

These data argue in favor of a variable subunit *c* stoichiometry at least in vitro. In contrast, Cys−Cys cross-linking experiments in membrane vesicles using bisubstituted *c* subunits led to the formation of c_{10} oligomers in either case, favoring a fixed stoichiometry in vivo.

Keywords: ATP synthase; F_o, Subunit *c*; Stoichiometry

A.17. Rotation of the 'gamma' subunit in photosynthetic F1-ATPases is driven by both decoupled CaATPase and coupled MgATPase

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A highly active photosynthetic F1-'alpha3beta3gamma'-complex was assembled from mixtures of *Rhodospirillum rubrum* RF1-'alpha' and 'beta' subunits and the spinach chloroplast CF1-'gamma' subunit [1]. This hybrid complex exhibited higher CaATPase than MgATPase activities, which were regulated by its CF1-'gamma'-specific thiol modulation [1] and inhibited by the CF1'epsilon' subunit [2]. An actin filament connected to the hybrid F1-'gamma' subunit revealed clear rotational movement upon induction of either MgATPase or CaATPase activities. Since we have earlier shown that in membrane-bound RF0F1, as in CF0F1, only the MgATPase activity is coupled to proton translocation [3], the clear induction of our hybrid F1-'gamma' rotation by its proton-decoupled CaATPase as well as the fully coupled MgATPase activity, was rather surprising. It could, however, be explained by our recent work with various RF0F1-'beta'-Thr-159 mutants, which revealed clear differences in ligands for binding of either calcium or magnesium to the RF0F1 catalytic sites [4]. The resulting different conformational states of these sites when occupied by calcium as compared to magnesium, lead to long range effects that still enable the decoupled CaATPase induced F1-'gamma' rotational movements, but block their transmission to the F0–C subunits.

Keywords: F1-ATPase; Rotational catalysis; Proton translocation; Coupling

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A.18. Structure of the V-ATPase and the A_3B_3EG-subcomplex from *M. sexta*

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The V-ATPase from the tobacco hornworm *M. sexta* consists of a membrane-embedded sector V_0, which contains the ion channel, a central connecting stalk, and an extrinsic complex V_1 ($A_3B_3CDEFG_yH_z$), in which ATP hydrolysis takes place [1]. Electron microscopy and small-angle X-ray scattering data have shown that the major, nucleotide-binding subunits A and B of the V_1-ATPase are hexagonally arranged, and alternate around a central cavity in which probably the E subunit is located (reviewed in Ref. [1]). The hexameric barrel is attached to the V_0 part by at least one stalk. A recent three-dimensional structure of V_1 without subunit C [2] at a resolution of 1.8 nm confirm these features and show the stalk part extending from, and therefore partly composed of the V_1 subunits D–H. In the studies presented, the complete V_1-ATPase ($A_3B_3CDEFG_yH$) has been isolated and its structure has been determined by 3D electron microscopy. In addition, a stable A_3B_3EG-subcomplex, with high ATPase-activity has been isolated and visualized by electron microscopy. This is the first demonstration of an active core-complex of the V_1-ATPase from *M. sexta*.

Keywords: V_1-ATPase; Electron microscopy; *M. sexta*

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A.19. Interaction of the two inhibitory peptides IF1 and STF1 with yeast ATP synthase

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The interaction of the regulatory peptides IF1 and STF1 (= "9 kDa protein") with yeast F_0F_1 was studied in intact mitochondria, in deenergized submitochondrial particles (SMP) and in alamethicin-treated mitochondria from wild-type cells of from mutants depleted in these peptides. Contrary to that was previously reported [1], STF1 added to SMP at high concentration fully inhibited ATP hydrolysis, like IF1. Its binding to MF_0MF_1 (pH 6.0, MgATP present) was almost 10 times slower than IF1 binding. In deenergized intact mitochondria (pH 7.3), ATPase activity was more than 70% inhibited by endogenous IF1 alone, and 90% inhibited by endogenous IF1 alone. In SMP or alamethicin-treated mitochondria, both peptides were released (pH 7.3, no MgATP), but this process was 10 times faster with STF1 than with IF1. No evidence was found for an interaction between IF1 and STF1. MgATP favoured IF1 and STF1 binding, and the protonmotive force favoured their release. Finally, STF1 was found to play no role in intact mitochondria. These data contradicts previously proposed effects of STF1, like the stabilization of the IF1–F_0F_1 complex [2] or the replacement of IF1 on its binding site in the presence of the protonmotive force [3], and questions the role of STF1 in vivo.

Keywords: ATP synthase; F_0F_1-ATPase; IF1; 9 kDa protein; Inhibitor protein; Yeast mitochondria

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A.20. Defective torque generation in the βSer-174 to Phe mutant F1 and its suppression by second mutations

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We established systems for observing continuous rotation of fluorescently labeled actin filament connected to *Escherichia coli* F1 subunit in a single molecule during ATP hydrolysis. These progresses enabled us to analyze rotational catalysis, taking advantage of the wealth of information from the genetic and biochemical approaches of *E. coli* enzyme. We examined γ subunit rotation in a mutant F1 having βSer-174 to Phe substitution (10% ATPase activity of the wild-type). The rotary torque produced by the mutant enzyme was significantly reduced (∼ 20 pN nm) compared to that of the wild-type (∼ 40 pN nm). This defective rotation was restored by the introduction of second site mutation (βGly-149 to Ala, βIle-163 to Ala or βIle-166 to Ala). From these results, we concluded that the region of βGly-149 to βSer-174 (also including catalytic residues) plays an important role in the coupling between ATP hydrolysis and mechanical work in F1.

Keywords: F1-ATPase; Rotation; Mutation

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A.21. Interactions between peripheral stalk subunits

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Interactions between peripheral stalk subunits OSCP, F_6, b and d have been studied by solution nmr and by binding studies. Their predicted structures are predominantly α-helical. We have examined they might interact with each other and with the F_1 domain and propose a structural model.

A.22. Role of NtpI subunit in Na^+ translocation by *Enterococcus hirae* V-ATPase

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The 76-kDa NtpI subunit constitutes the membrane-embedded V0 moiety of *Enterococcus hirae* V-type Na^+-ATPase with 16-kDa NtpK hexamer containing Na^+-binding sites. In this study, we investigated the role of an arginine residue, which is highly conserved among the corresponding subunits of bacterial V-ATPases, at position 573 of NtpI. Substitution of Glu, Leu, or Gln for Arg-573 abolished sodium transport and sodium-stimulated ATP hydrolysis of the enzyme. The conservative replacement of Arg by Lys lowered both activities about one fifth of those of the wild-type enzyme. We have previously reported on ATP-dependent negative cooperativity for Na^+ coupling of this enzyme [J. Biol. Chem. 276 (2001) 48337]. The negative cooperativity for Na^+ dependence of ATPase activity was weakened by the mutation Arg-573Lys; the Hill coefficients for the wild type and mutant enzymes at a saturated ATP concentration were 0.22 ± 0.03 and 0.40 ± 0.05, respectively. The Hill coefficients of both enzymes at limited ATP concentrations approached 1. These results indicate that NtpI Arg-573 is indispensable for sodium translocation and for the cooperative features of *E. hirae* V-ATPase.

Keywords: Vacuolar-ATPase; Sodium transport

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A.23. Structure of the OSCP subunit of bovine ATP synthase

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The N- and C-terminal domains of the bovine OSCP subunit have been studied by solution nmr. The poster will describe their structures and discuss how they interact with other subunits in the complex.

A.24. Allotopic expression of yeast mitochondrially encoded ATP synthase subunits 8 and 6

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Yeast mitochondrial ATP synthase (mtATPase) contains three integral membrane protein subunits, 6, 8 and 9, encoded in the mitochondrial genome. To facilitate the study of these proteins, we devised the strategy of allotopic expression [1], which involves recoding of mitochondrial genes for nuclear expression. This approach has been particularly successful for subunit 8 (Y8) such that questions of structure–function, topology and proximity to other subunits of the complex have been addressed by expression of Y8 variants combined with cysteine-scanning mutagenesis and cross-linking studies. More recently, we have directed our attention to achieving the allotopic expression of subunit 6 (Y6). A mitochondrial genome segment from a wild-type yeast strain encompassing the coding sequence for Y6 was recovered by PCR. We then used site-directed mutagenesis to recode four codons in order to overcome codon dictionary differences between the mitochondrion and nucleus/cytosol. The codon-adjusted Y6 gene cassette sequence was then fused to DNA specifying a mitochondrial targeting sequence and cloned into a yeast expression vector enabling expression in yeast cells expressing only a truncated nonfunctional form of native Y6. Here we report on the outcome of these experiments and on our most recent experiments involving allotopic expression of Y8.

Keywords: Yeast; Mitochondria; ATP synthase; Subunit 6; Subunit 8; Allotopic expression

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A.25. H^+-ATP-synthase of *Rhodobacter capsulatus*: proton transfer through the membrane F_O part of the enzyme

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The protonic F_OF_1-ATP synthase is built from two parts: the catalytic F_1 and the membrane-embedded, proton-transferring F_O. We studied the properties of the F_O part in chromatophores vesicles from phototrophic bacterium *Rhodobacter capsulatus*, which were depleted from F_1 by sonication/EDTA treatment [1,2]. Protons were driven through F_O by the flash-induced protonmotive force. The extent and the rate of proton transfer were revealed from the difference between the decay kinetics of the flash-induced electrochromic bandshifts of intrinsic carotenoids measured (at 523 nm) with or without specific inhibitors of F_O as in Ref. [2]. When pH was decreased, the extent of proton transfer through F_O dropped to 1/3 of the initial value with an apparent pK of 7.0 ± 0.2. Then, it remained constant up to pH 5.5. In D_2O, the respective pK value was 7.8 ± 0.1. The rate of proton transfer was the highest at pH 8.0 and decreased both at higher and at lower pH. In D_2O, the proton transfer rates were two times slower than in H_2O. We estimated the activation energy of proton transfer through F_O at different pH values. Activation energy was the lowest at pH = 8.0 (approximately 45 kJ/mol) and increased both at higher and lower pH.

Keywords: ATP-synthase; Proton transfer; *Rhodobacter capsulatus*

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A.26. Involvement of the cytoplasmic loop L6-7 in the entry mechanism for transport of Ca^{2+} through the sarcoplasmic reticulum Ca^{2+}-ATPase

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We previously found that mutants of conserved aspartate residues of sarcoplasmic reticulum Ca^{2+}-ATPase in the cytosolic loop, connecting transmembrane segments M6 and M7 (L6-7 loop), exhibit a strongly reduced sensitivity towards Ca^{2+} activation of the transport process. In this study, yeast membranes, expressing wild-type and mutant Ca^{2+}-ATPases, were reacted with Cr.ATP and tested for their ability to occlude $^{45}Ca^{2+}$ by HPLC analysis, after cation/resin and $C_{12}E_8$ treatment. We found that the D813A/D818A mutant that displays markedly low calcium affinity was capable of Ca^{2+}-occlusion to the same extent as wild-type ATPase. Using NMR and mass spectrometry, we have analysed the conformational properties of the synthetic L6-7 loop and demonstrated the formation of specific 1:1 cation complexes of the peptide with calcium and lanthanum. All three aspartate D813/D815/D818 were required to coordinate the trivalent lanthanide ion. Overall, these observations suggest a dual function of the loop: in addition to mediating contact between the intramembranous Ca^{2+} binding sites and the cytosolic phosphorylation site [J. Biol. Chem. 276 (2001) 15232], the L6-7 loop, in a preceding step, participates in the formation of an entrance port for Ca^{2+}, before high affinity binding inside the membrane.

Keywords: Ca^{2+}-ATPase; Loop NMR structure

Reference

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A.27. Interaction of the inhibitor protein IF1 with F0F1ATPsynthase from ox heart under different pH and aggregation states

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The inhibitor protein IF1 reversibly binds to the F1 sector of F0F1ATPsynthase by inhibiting the enzyme in response to decrease of pH and/or transmembrane potential, conditions that occur under energy deficiency, like ischemia. Kinetic studies of IF1 binding to the isolated F1 from ox heart at different pH have been performed using the resonant mirror biosensor. The results demonstrate that pH decreases increase K_d by influencing $k_{(ass)}$ only.

At low pH, ox heart IF1 occurs in solution as a dimer, which can bind two F1 molecules simultaneously [1]. Nevertheless, evidence has been provided that full inhibition can be achieved without obligatory dimerization of IF1–F1 [2]. In this study, the IF1 interaction with the monomer (Vmon) and dimer (Vdim) of F0F1ATPsynthase in membrane have been also investigated. Bovine heart mitochondria have been solubilized with Triton and the different enzyme aggregation states have been separated by native electrophoresis. The catalytic activity and the IF1 content have been evaluated by histochemical staining and immunoblotting, respectively. The results indicate that both Vmon and Vdim bind IF1. However, only in Vmon the IF1 interaction was related to the inhibition, while Vdim was inactive even when no IF1 was bound.

MURST cofin2000, CNR.

Keywords: F0F1ATPsynthase; Inhibitor protein; Dimerization

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A.28. Structural organization of the V-type Na^+-ATPase of the thermophile *C. fervidus*

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The Na^+ pumping V-ATPase of the anaerobic thermophile *C. fervidus* is one of a few described bacterial V-ATPases. The overall structure of the V-ATPases is similar to that of the well-characterized F-ATPases. Both consist of an extramembranous catalytic domain, called the headpiece V1 or F1, which is linked by means of a stalk region to a membrane-bound ion translocating domain called V0 or F0. Because of the structural similarity, it is believed that the energy coupling mechanism between ATP hydrolysis and ion translocation in V-ATPases is similar as in F-ATPase: rotational catalysis. In spite of the overall structural similarity of V-ATPases and F-ATPases, significant differences are observed especially in the stalk region. Most prominently, electron microscopy images of V-ATPases reveal a more complex stator structure involving two or three peripheral stalks that seem to contact the central stalk. Furthermore, the length of the central stalk is considerably longer in V-ATPases than in F-ATPases. The differences in the stalk regions of V-ATPases and F-ATPases are reflected in the subunit composition of the complexes. We show that the central stalk of the V-ATPase of *C. fervidus* consists of two different subunits.

Keywords: V-ATPase; Stator structure; Central stalk

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A.29. Molecular architecture of a biological turbine

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ATP is the universal carrier of free energy in all cells and is mostly generated by F_1F_0 ATP-synthases. These enzymes work as efficient turbine engines, which are driven by an electrochemical Na^+ or H^+ gradient. The protein complex is composed of the catalytic active site carrying F_1 moiety and the membrane embedded F_0, which is responsible for ion translocation and torque generation [1]. A high resolution structure is available from the soluble F_1 [2] but detailed structural information of F_0 is missing [3]. We present the structure of the stable subunit c ring from *Ilyobacter tartaricus*, which was determined from 4 Å resolution data of 2-D crystals. The 89-residue subunits consist of two helices linked by a hairpin loop. The helices form two concentric rings. Whereas the inner ring shows an extremely tight packing, the helices of the outer ring are more separated. One outer helix contacts the groove formed by two inner helices. The Na^+ binding site is located in the middle of the membrane, the binding site residues are localized on two outer and one inner helix. Cavities between the two outer and the inner helices could provide Na^+ access channels.

Keywords: F_1F_0 ATP synthase; c-Oligomer; Na^+ translocation; Electron crystallography

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A.30. Anisotropy of proton fluxes at the membrane/water interface and its role in ATP synthesis by *Rhodobacter capsulatus* chromatophores

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We checked whether the entropic component of the proton-motive force corresponds to the difference in proton activities in two membrane-adjoining bulk water phases (ΔpH^B) or at two opposite membrane surfaces (ΔpH^S).

Using the previously described experimental set-up [1], we found out that the flash-induced ATP synthesis in chromatophore vesicles from the phototrophic bacterium *Rhodobacter capsulatus* was driven by ΔpH^S together with $\Delta\psi$ but not by ΔpH^B. We estimated the value of ΔpH^S after a single saturating flash as $\geq 1.0 \pm 0.1$ pH unit (at $\Delta pH^B = 0$). By solving the Poisson–Boltzmann equation, we showed that the low dielectric permittivity of the surface water, which was experimentally established in Ref. [2], results in a potential barrier for cations (of ~ 0.2 eV) peaking 1 nm away from the membrane/water interface. The retardation of proton equilibration by this barrier could result in $|\Delta pH^S| > |\Delta pH^B|$ by 1–2 pH units at steady state. We modelled the kinetics of proton escape from the surface into the bulk by solving the Smoluchowski equation and quantitatively reproduced the experimentally observed anisotropy of proton displacement at the membrane surface [3,4], with the longitudinal and perpendicular proton mobility differing by a factor of 10^4.

Keywords: Proton transfer; ATP synthesis; Coupling mechanism

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A.31. Pre-steady state kinetic analysis of rotational catalysis in the F_1-ATPase

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Because catalysis of ATP hydrolysis involving rotation of the gamma subunit requires participation of all three catalytic sites, analysis of the partial reactions of rotational catalysis is being carried out in pre-steady state conditions in the millisecond time domain. Using a Kintek rapid quench flow device, 23 to 430 μm [gamma^{32}P]ATP·Mg is rapidly added to *Escherichia coli* F1 complex with 4 mol of bound nucleotide (1 catalytic plus three noncatalytic sites) in the presence of 0.9 mM free Mg^{2+} at pH 7.5, 25 °C, and the reaction stopped at various times by addition of acid. The amount of radioactive ATP hydrolyzed is determined. A lag phase of up to 0.2 s occurs before entry into steady state hydrolysis rates. In contrast, when the presence of free Mg^{2+} is dropped to 0.05 mM, a burst of hydrolysis up to 1 mol ATP per mole of F1 enzyme is followed by a 0.2 s lag then entry into steady state. The length of the lag phase appears to depend upon the concentration of free Mg^{2+}, and may reflect the release of bound ADP.

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Keywords: F1-ATPase; Rotational catalysis; Pre-steady-state kinetics

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A.32. Subunit rotation of ATP synthase embedded in membranes: *a* or β subunit rotation relative to the *c* subunit ring

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ATP synthase FoF1 ($\alpha_3\beta_3\gamma\delta\varepsilon ab_2c_{10-12}$) couples an electrochemical proton gradient and a chemical reaction through the rotation of its subunit assembly. In this study, we engineered FoF1 to examine the rotation of the catalytic F1 β or membrane sector Fo *a* subunit when the Fo *c* subunit ring was immobilized; a biotin-tag was introduced onto the β or *a* subunit, and a His-tag onto the *c* subunit ring. Membrane fragments were obtained from *E. coli* cells carrying the recombinant plasmid for the engineered FoF1, and immobilized on a glass surface. An actin filament connected to the β or *a* subunit rotated anticlockwise upon the addition of ATP, and generated essentially the same torque as one connected to the *c* ring of FoF1 immobilized through a His-tag linked to the α or β subunit. These results established that the $\gamma\varepsilon c_{10-12}$ and $\alpha_3\beta_3\delta ab_2$ complexes are mechanical units of the membrane-embedded enzyme involved in rotational catalysis.

Keywords: ATP synthase; Rotation; Membrane

A.33. ATP synthase assembly proteins—expression of *ATP11* and *ATP12* genes in mammalian tissues

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Biosynthesis and structure of mammalian and yeast F_oF_1-ATPases are closely related, although several differences exist in the location of their subunit genes, some of the subunits and also in supramolecullar structures of the enzyme. In yeast, three proteins have been found that are involved in assembly of the F_1-catalytic part from individual subunits: the Atp11p, Atp12p and Fmc1p. In agreement with Wang et al., we have identified mammalian genes with high similarity to the yeast *ATP11* and *ATP12* [1]. In order to investigate expression of these genes in different tissues, we have analysed *ATP11* and *ATP12* mRNA levels, in comparison to mRNA levels of F_1-ATPase subunits, by Northern blots and real-time PCR. The same approach has been used for analysis of *ATP11* and *ATP12* transcripts in fibroblasts from patients with ATPase deficiency of the nuclear origin [2], whose phenotype resembles yeast Δatp11 or Δatp12 mutants. Our analysis revealed low *ATP11* and *ATP12* mRNA levels, in comparison to transcripts of F_1-ATPase subunits, and comparable levels of *ATP11* and *ATP12* transcripts in control and patient fibroblasts. These results indicate that the ATPase defect is not caused by aberrant transcription of *ATP11* or *ATP12* genes.

Keywords: Mammalian ATPase; Assembly factor; Expression

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A.34. Structural comparison of three ABC-transporters in detergents or reconstituted in membranes

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YvcC, a multidrug transporter from *Bacillus subtilis*, is a member of the ATP-binding cassette superfamily, highly homologous to each half of human multidrug-resistance P-glycoprotein and to several other bacterial half-ABC transporters [1–2]. Controlled detergent removal from YvcC-lipid micelles allowed the production of ring-shaped particles, well suited for single particle analysis by cryo-electron microscopy [3]. A computed volume has been determined to 2.5-nm resolution. The repetitive unit in the ring-shaped particles was consistent with a homodimeric organization of YvcC. Each subunit was composed of three domains: a 5-nm height transmembrane region, a stalk of about 4 nm in height and a cytoplasmic lobe (NBD) of about 5–6 nm in diameter. The 3D reconstruction of the YvcC homodimer well compared with the very recent X-ray crystallographic data on the MsbA homodimer from Escherichia coli [4]. In addition, our 3D reconstruction revealed an asymmetric organization of the two NBDs sites within the homodimer, as well as a dimeric interaction between two homodimers. We have also performed image analysis of detergent-solubilized Pgp and the lipid-reconstituted Pdr5. Comparison of the two structures with the $(TMD\text{-}NBD)_4$ structure of YvcC strongly suggested that the full ABC transporters, Pgp and Pdr5, had a $(TMD_2\text{-}NBD_2)_2$ organization.

Keywords: ABC transporter; 3D structure; Cryo-electron microscopy; Single particle analysis; PgP; YvcC; Pdr5

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A.35. ATP synthase from *Saccharomyces cerevisiae*: location of the OSCP subunit in the peripheral stalk region

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A biotinylation signal has been fused to the C terminus of the oligomycin sensitivity conferral protein (OSCP) of the ATP synthase complex from *Saccharomyces cerevisiae*. The signal is biotinylated in vivo and the biotinylated complex binds avidin in vitro. By electron microscopy of negatively stained particles of the ATP synthase:avidin complex, the bound avidin has been localised close to the F_1 domain. The images were subjected to multireference alignment and classification. Because of the presence of a flexible linker between the OSCP and the biotinylation signal, the class averages differ in the position of the avidin relative to the F_1 domain. These positions lie on an arc, and its centre indicates the position of the C terminus of the OSCP on the surface of the F_1 domain almost 10 nm from the point distal from F_o. The labelling technique has also allowed a reliable 2-D projection map to be developed for the intact ATP synthase from *S. cerevisiae*. The map reveals a marked asymmetry in the F_o part of the complex that can be attributed to subunits in the F_o domain.

Keywords: ATP synthase; Peripheral stalk; OSCP subunit; Electron microscopy; Biotin; Avidin

A.36. Photoaffinity labeling of ATP synthase by mono- and bifunctional 3′-biotinylated 8-azidoadenine nucleotides

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In order to characterize nucleotide binding sites of ATP synthases, we have synthesized various mono- and bifunctional photoactivatable ATP analogs [1]. The six nucleotide binding sites—three catalytic and three noncatalytic—of ATP synthases are located on the F1 complex of the enzyme alternately at the interfaces between the major subunits alpha and beta as demonstrated by photoaffinity labeling and photoaffinity cross-linking [2].

The introduction of an additional biotin residue, yielding 3′-biotinyl-8-azido-ATP [3], is advantageous for an easy detection of labeled proteins. Irradiation of the F1-ATPase from the thermophilic bacterium PS3 in the presence of 3′-biotinyl-8-azido-ATP resulted in the nucleotide-specific inactivation of the enzyme as well as in the nucleotide-dependent labeling of alpha and/or beta subunits.

Dimerization of 3′-biotinyl-8-azido-ADP resulted in the formation of the bifunctional diadenine dinucleotide 3′-dibiotinyl-8-diazido-AP4A. Irradiation of TF1 in the presence of 3′-dibiotinyl-8-diazido-AP4A yielded the nucleotide-specific inactivation of TF1 and the nucleotide-dependent formation of alpha–beta cross-links.

All these results demonstrate the suitability of the biotinylated azidonucleotides for photoaffinity labeling and photoaffinity cross-linking of ATP synthase complexes.

Keywords: ATP synthase; Photoaffinity labeling; Nucleotide binding site

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A.37. The mechanism of inhibition and reactivation of chloroplast and thermophilic bacterium F_1-ATPases by tentoxin

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Inhibition and reactivation of chloroplast ATP-synthase by fungal cyclotetrapeptide tentoxin was investigated by photolabeling, binding and kinetic studies. Photolabeling with a tentoxin derivative provided the first direct evidence of tentoxin binding to the alpha subunit of chloroplast F_1-ATPase (CF_1), and tentoxin was placed at the alpha–beta interface [1]. Thermophilic bacterium F_1-ATPase (TF_1) proved to be also tentoxin-sensitive at temperatures ranging from 50–60 °C (but not below). Enzyme turnover increased tentoxin binding rate to its inhibitory site in TF_1, but not in epsilon-free CF_1 [2]. We propose that tentoxin preferentially binds to an ADP-loaded catalytic interface and mechanically blocks its conversion into an ATP-loaded interface. Using selected synthetic analogues, we showed that tentoxin needs to be in the boat-up conformation to be inhibitory, while boat-up and chair-left conformers both overactivate the enzyme. Toxin tight binding to inhibitory site of CF_1 proved to modulate the affinity of reactivatory site. ^{14}C binding experiments revealed that in reactivated F_1-ATPase, the two filled tentoxin-binding sites do not exchange their role during catalytic turnover, indicating an impairment between nucleotide occupancy and shape of tentoxin-binding pocket. This provides a mechanical interpretation of F_1-ATPase inhibition by tentoxin and a clue for understanding the reactivation process [3].

Keywords: ATP synthase; F_0F_1-ATPase; Tentoxin; Cyclopeptide; Photolabeling; Rotatory mechanism

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A.38. Structure of the F_6 subunit of bovine ATP synthase

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The F_6 subunit of the bovine ATP synthase has been studied by solution nmr. The poster will describe its structure and discuss its interactions with other peripheral stalk subunits.

A.39. In the absence of the first membrane-spanning segment of subunit *4* (*b*), the yeast ATP synthase is functional but does not dimerize or oligomerize

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The subunit 4 (*b*) is the main component of the stator of the yeast ATP synthase. Its N-terminal portion is anchored in the inner mitochondrial membrane by two hydrophobic segments. We investigated the role of the first membrane-spanning segment, which is absent in prokaryotic and chloroplastic enzymes, by shortened versions in the N-terminal domain. The deletion of the hydrophilic region of this N-terminal portion showed a similar phenotype to the wild type. In the absence of the first membrane-spanning segment, a strong decrease in the amount of subunit *g* was found. The mutant ATP synthase did not dimerize or oligomerize, as shown by BN PAGE experiments, and mitochondria displayed anomalous morphologies with onion-like structures. This phenotype is similar to that of the null mutant in the *ATP20* gene that encodes subunit *g*, a component involved in the dimerization/oligomerization of ATP synthase [1]. Our data indicate that the first membrane-spanning segment of the mitochondrial *b*-subunit is not essential for the function of the enzyme since its removal did not directly alter the oxidative phosphorylation. It is proposed that the unique membrane-spanning segment of subunit *g* and the first membrane-spanning segment of subunit *4* interact, as shown by cross-linking experiments [2].

Keywords: Yeast; Mitochondrion; ATP synthase dimerization; Subunit *b*; Subunit *g*

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A.40. The H^+/ATP coupling ratio measured in CF_0F_1 proteoliposomes

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According to the chemiosmotic theory, the equilibrium state of the H^+-translocating ATP synthase is described by

$$\log \frac{[\mathrm{ATP}]}{[\mathrm{ADP}][\mathrm{Pi}]} = -\frac{\Delta G_\mathrm{p}^{\mathrm{o}\prime}}{2.303\ RT} + n(\Delta \mathrm{pH}_\mathrm{eq} + F\Delta\varphi_\mathrm{eq}) \quad (1)$$

where $\Delta G_\mathrm{p}^{\mathrm{o}\prime}$ is the standard Gibbs energy of the ATP synthesis reaction, $\Delta \mathrm{pH}_\mathrm{eq}$ and $\Delta\varphi_\mathrm{eq}$ are the transmembrane pH and electric potential differences at equilibrium and n is the H^+/ATP stoichiometry.

This relationship implies that, by having constant $\Delta\varphi_\mathrm{eq}$ and a set of experimentally determined couples [$\Delta \mathrm{pH}_\mathrm{eq}$, log ([ATP]/[ADP]/[Pi])], one can determine the H^+/ATP ratio n and the standard free energy $\Delta G_\mathrm{p}^{\mathrm{o}\prime}$.

We have isolated the ATP synthase of chloroplasts and reconstituted it into liposomes. The rates of ATP synthesis and hydrolysis following an acid–base transition have been taken. For each ΔG_p value, the transition from hydrolysis to synthesis direction has been observed by measuring the net catalytic rate as a function of different ΔpH values. The ΔpH at which the net catalytic rate was zero has been interpolated and considered as the ΔpH of thermodynamic equilibrium for the corresponding ΔG_p.

The plot of the different ΔG_p values as a function of the corresponding $\Delta \mathrm{pH}_\mathrm{eq}$ yielded a value of $\Delta G_\mathrm{p}^{\mathrm{o}\prime} = 36.5 \pm 1.8$ kJ/mol at pH 8.45, in agreement with literature data, and a value of $n = 3.9 \pm 0.2$.

Keywords: ATP synthase; H^+/ATP stoichiometry; Standard Gibbs energy; Chloroplast; Acid–base transition

A.41. Two ATP synthases can be linked through subunits i in the inner mitochondrial membrane of *Saccharomyces cerevisiae*

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Cross-linking experiments showed that the supernumerary subunit i is close to the interface between two ATP synthases. These data were used to demonstrate the presence of ATP synthase dimers in the inner mitochondrial membrane of Saccharomyces cerevisiae. A cysteine residue was introduced into the inter-membrane space located C-terminal part of subunit i. Cross-linking experiments revealed a dimerization of subunit i. This cross-linking occurred only with the dimeric form of the enzyme after incubating intact mitochondria with a bis-maleimide reagent, thus indicating an inter-ATP synthase cross-linking, whereas the monomeric form of the enzyme exhibited only an intra-ATP synthase cross-linking with subunit 6, another component of the membranous domain of the ATP synthase.

Keywords: Yeast; Mitochondrion; ATP synthase dimers; Subunit i; Subunit 6; Cross-linking

A.42. Membrane topography of the coupling ion binding site in Na^+-translocating F_1F_0 ATP synthase

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A carbodiimide with a photoactivatable diazirine substituent was synthesized and incubated with the Na^+-translocating F_1F_0 ATP synthase from both *Propionigenium modestum* and *Ilyobacter tartaricus*. This caused severe inhibition of ATP hydrolysis activity in the absence of Na^+ ions but not in its presence, indicating the specific reaction with the Na^+-binding cE65 residue. Photocrosslinking was investigated with the substituted ATP synthases in reconstituted 1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphocholine (POPC)-containing proteo-liposomes. A subunit c/POPC conjugate was found in the illuminated samples but no a–c crosslinks were observed, not even after ATP-induced rotation of the c-ring. Our substituted diazirine moiety on cE65 was therefore in close contact with phospholipid but does not contact subunit a. $Na^+_{in}/^{22}Na^+_{out}$ exchange activity of the ATP synthase was not affected by modifying the cE65 sites with the carbodiimide, but upon photoinduced crosslinking, this activity was abolished. Crosslinking the rotor to lipids apparently arrested rotational mobility required for moving Na^+ ions back and forth across the membrane. The site of crosslinking was analyzed by digestions of the substituted POPC using phospolipases C and A_2, and by mass spectroscopy. The substitutions were found exclusively at the fatty acid side chains, which indicates that cE65 is located within the core of the membrane.

Keywords: ATP synthase; DCCD; Subunit c; Photo crosslinking; Phospholipid; Reconstitution

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A.43. Multiple subunit isoforms of mouse vacuolar H^+-ATPase

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Mammalian endomembrane organelles including Golgi apparatus, lysosomes, endosomes, and secretory vesicles have luminal acidic pH established by vacuolar type H^+-ATPase (V-ATPase). The V-ATPase is also localized to the plasma membrane of highly differentiated cells such as osteoclasts and kidney epithelial cells. Thus, an obvious question is whether V-ATPases at various locations have exactly same subunits or utilize different isoforms. We found multiple isoforms of mouse Vo subunit *a* (*a*1, *a*2, *a*3 and *a*4) [1,2], whereas *c* and *c*″ subunits are encoded by single genes [3,4]. In undifferentiated cells, *a*1 is predominantly localized to secretory vesicles, and *a*2 is associated with Golgi, whereas *a*3 is a late endosomal/lysosomal resident. *a*4 is specifically expressed in renal intercalated cells. As well as the Vo, the membrane periperal V1 sector also exhibits structural divergence. V-ATPase with a novel *E*1 isoform is expressed specifically in the acrosomal membrane of sperm, whereas the ubiquitously expressed *E*2 is the component in other tissues [5]. Brain expresses a unique *G*2 isoform as well as a ubiquitous isoform *G*1. Thus, the divergent physiological functions of V-ATPase in various acidic compartments are established, in part, by utilizing distinctive subunit isoforms.

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A.44. Molecular mechanism of ATP synthesis by ATP synthase

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We propose a molecular mechanism for ATP synthesis drawing on properties of catalytic sites including the transition state [1–3], on X-ray structure [4], and on our hypothesis for ATP-driven subunit rotation [3].

Major steps are: (A) Initial gamma rotation (proton-gradient-driven) inserts alphaArg-376 (“arginine finger”) into the catalytic site, promoting “energy-linked” phosphate binding. This prohibits ATP but allows ADP binding. (B) Rotation compresses the alpha/beta catalytic interface, forcing phosphate toward ADP. The pentacovalent transition state forms, with ADP–O to P bond length 2.0–2.2 Å. Further compression to 1.55 Å forms ATP, while residue betaGlu-181 sequesters product water. (C) Rotation removes alphaArg-376, preventing ATP hydrolysis. (D) Rotation drastically lowers ATP affinity, releasing ATP.

During each 120° rotation step, enzyme steps A–D occur contemporaneously at different sites. A occurs at one site, B/C at a second, D at the third. Sites are transiently unoccupied between steps D and A [5].

All steps require energy input, *including* chemical synthesis, contradicting conventional wisdom based on ^{18}O exchange and “unisite” reactions. Scrutiny [1] indicates neither reaction occurs in enzyme molecules undergoing rapid, multiple turnovers during ATP synthesis. Likely, they are manifestations of an “idling” mode designed to conserve ATP when no work is being performed.

Keywords: ATP synthase; ATP synthesis; Molecular mechanism

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A.45. Quantitative determination of binding affinity of delta subunit in *Escherichia coli F-ATPase*

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To study the stator function in ATP synthase, a fluorometric assay has been devised for quantitative determination of binding affinity of δ subunit to *Escherichia coli* F_1-ATPase. The signal used is that of the natural tryptophan at residue δ28, which is enhanced by 50% upon binding of δ subunit to $\alpha_3\beta_3\gamma\varepsilon$ complex. K_d for δ binding is 1.4 nM, which is energetically equivalent (50.2 kJ/mol) to that required to resist the rotor strain. Only one binding site for δ was detected. The δW28L mutation increased K_d to 4.6 nM, equivalent to a loss of 2.9 kJ/mol binding energy. While this was insufficient to cause detectable functional impairment, it did facilitate preparation of δ-depleted F_1. The αG29D mutation reduced K_d to 26 nM, equivalent to loss of 7.2 kJ/mol binding energy. This mutation did cause serious functional impairment, referable to interruption of binding of δ to F_1. Results with the two mutants illuminate how finely balanced is the stator resistance function. δ′ fragment, consisting of residues δ1–134, bound with the same K_d as intact δ, showing that, at least in absence of F_o subunits, the C-terminal domain of δ contributes zero binding energy.

Keywords: ATP synthase; F_1-ATPase; Stator stalk; Delta subunit; Delta binding affinity

A.46. Relation of *Escherichia coli* membrane proton conductivity with membrane and redox potential

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Escherichia coli membrane proton conductivity measured by acid-pulses method was shown to depend on the growth pH and the other parameters. It was lower for bacteria under fermentation of glucose and nitrate/nitrite respiration that that for cells performing oxygen respiration. Proton conductivity was increased with lowering growth pH. Relation of proton conductivity change with cell membrane and redox potential values was determined. Energy-dependent H^+ efflux from bacteria and the F0F1-ATPase activity in membrane vesicles had different characteristics for cells grown in anaerobic and aerobic conditions: H^+ efflux was higher and *N*,*N*′-dicyclohexylcarbodiimide (DCCD)-inhibited ATPase activity became K^+-dependent under fermentation of glucose. When growth pH was lowering DCCD-sensitive ATPase activity markedly decreased, and ATPase activity not inhibited by DCCD was induced. These results might be discussed suggesting relation of bacterial membrane proton conductivity with energy-dependent H^+ efflux and ATPase activity.

Keywords: *Escherichia coli*; pH; Fermentation and respiration; Proton conductivity; Membrane potential; Redox potential; ATPase activity

A.47. Functional association of *Escherichia coli* membrane transport systems: external formate stimulates the F0F1-ATPase activity at slightly alkaline pH

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The *Escherichia coli* H^+-translocating F0F1-ATPase under fermentation at slightly alkaline pH (pH ~ 7.5) is proposed to operate in functional association with the K^+ uptake TrkA system and formate hydrogen lyase constituted with hydrogenase 4 (Hyd-4) [1]. As one of experimental evidences, K^+-stimulated F0F1-ATPase activity has been shown in right-side-out membrane vesicles and the F0F1-ATPase preparations, and was observed in in-side-out vesicles. Moreover, formate can additionally increase this activity. K^+- and formate-stimulating activity appeared in the parent strains but was negatively affected in trkG or trkH mutants with an atpB-deletion as well as in hyf mutants.

In-side-out membrane vesicles from *E. coli* grown in the presence of 30 mM formate at pH 7.5 demonstrated formate-stimulated *N*,*N*′-dicyclohexylcarbodiimide (DCCD)- or sodium acid-sensitive ATPase activity. This could be dependent on formate concentration in the assay medium and was absent in atp and hyc mutants. Increase in hydrogenase 3 (Hyd-3) but not in Hyd-4 activity has been shown previously [2]. Thus, association of the F0F1-ATPase with formate hydrogen lyase constituted with Hyd-3 is also probable when bacteria were grown in the presence of formate, and so external formate may be a factor regulating operation of the F0F1-ATPase in different manners.

Keywords: *Escherichia coli*; Membrane vesicle; ATPase activity; Hydrogenase; Formate

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A.48. Electron Paramagnetic Resonance Studies of Drug Transport by P-glycoprotein

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Multidrug resistance mediated by human P-glycoprotein poses a serious problem to medical therapy. Membrane permeability of transported drugs and consequent lack of an experimentally defined drug position have made resolution of the transport mechanism difficult. To overcome these obstacles, we devised a novel EPR spin labeled verapamil for use as a transport substrate. Transport studies were performed utilizing pure human P-glycoprotein expressed in *Saccharomyces cerevisiae* and reconstituted into defined proteoliposomes [1]. Spin labeled verapamil had apparent turnover number, K_m and K_i values of 5.8 s^{-1}, 4 μM and 210 μM respectively at pH 7.4 and 37° C. The apparent affinities were $\sim$10 fold higher than for unlabeled verapamil. Spin labeled verapmil stimulated ATPase activity $\sim$5 fold, was relatively hydrophilic and had a very low flip-flop rate, making it an ideal transport substrate. Measurements of the mobility of spin labeled verapamil were used to resolve the location of the drug in proteoliposome suspensions. Steady state gradients of spin labeled verapamil within the range of K_i/K_m ratios were observed. Measurements of transport velocity as a function of drug load established that the conformational change leading to drug transport was directly coupled to the ATP hydrolysis transition state.
Supported by PHS grant GM52502.

Keywords: P-glycoprotein, transport mechanism, EPR, energy coupling.

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Session B: Biogenesis

B.1. Quality control of membrane proteins in mitochondria

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Impaired proteolysis of membrane proteins has been recognized as the basis of many human disorders. A number of membrane-embedded proteases were identified, which are capable of clipping membrane-spanning segments or completely degrading membrane proteins. We are studying the proteolytic system of the inner membrane of mitochondria that consists of several highly conserved components: Firstly, the *m*-and *i-AAA proteases* with catalytic sites at opposite membrane surfaces. These ATP-dependent peptidases form a membrane-integrated quality control system and exert crucial regulatory functions during mitochondrial biogenesis. Yeast cells lacking both proteases are inviable, whereas inactivation of one of these proteases causes neurodegeneration in humans. Secondly, the *prohibitin complex* that presumably exerts chaperone activity and modulates the degradation of membrane proteins by the *m*-AAA protease. Prohibitins affect cellular longevity in yeast and mammals, which might reflect effects of a disturbed stability of membrane proteins on mitochondrial metabolism. Thirdly, the *ABC-transporter Mdl1* that exports peptides generated by the *m*-AAA protease in the matrix space allowing their subsequent release from mitochondria. Fourthly, a novel class of membrane-embedded peptidases that have overlapping activities with the *m*-AAA protease. Recent experiments on the turnover of a polytopic membrane protein by AAA proteases and this novel peptidase will be discussed.

B.2. Characterization of the assembly pathway of human mitochondrial complex I

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The mitochondrial respiratory chain consists of four different enzyme complexes (I–IV). The largest and most complicated one is the NADH/ubiquinone oxidoreductase (complex I). In human, this complex is composed of 7 mitochondrial-encoded subunits (ND subunits) and probably at least 36 nuclear-encoded subunits [1]. It has been proved that several mutations in complex I genes can be associated with reduction of the complex I activity. These mutations are responsible for various mitochondrial cytopathies and severe diseases. Our aim is to study the assembly pathway for complex I subunits in normal mitochondria and to find out how this assembly is disturbed by pathological mutations.

To this end, we are studying several lines of cultured human cells that were given by Dr. G. Hofhaus (University of Düsseldorf, Germany) and Pr G. Attardi (Caltech, Pasadena, CA, USA): 143 B Rho° cells (cells completely depleted of mitochondrial DNA), C4T cells (ND4-mutated cells) and CF9 cells (ND5-mutated cells) [2]. The parental cell lines (143 B human osteosarcoma cells) are used as a control of normal assembly process.

We have characterized these cells by several methods such as Western blot, measurement of the respiratory chain complex activities, cells fractionation and subcomplex immunoprecipitation to analyse the structure of the partially assembled complex I as well as their subcellular localisation. This work will help to understand the different key steps of the biogenesis of the human mitochondrial complex I.

Keywords: NADH/ubiquinone oxidoreductase; Mitochondrial cytopathy; Biogenesis; Complex I; Subcomplex

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B.3. In vivo measurements of the energetic of tat protein transport into and across thylakoid membranes: translocation mutants of the Rieske protein

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Proteins translocated by the TAT pathway have usually a twin arginine motive in the amino-terminus before a hydrophobic stretch that confers translocation specificity. TAT protein transport in vitro does not require ATP hydrolysis, but relies only on a *trans*-thylakoid ΔpH. In this work, we studied in vivo the TAT translocation of three proteins: 16 and 23 kDa components of PSII oxygen-evolving complex and Rieske subunit of cytochrome $b_6 f$ complex.

We compared the wild-type and three mutants of *Chlamydomonas reinhardtii*: FUD50 that lacks ATPsynthase–ATPase CF0–CF1 and is therefore devoided of ΔpH in the dark [1] and two site-directed [2] new Rieske protein mutants, K12R (wild-type KR motive changed in canonical RR motive) and R13K (motive removed into KK).

Measurements of photosynthetic activity and protein accumulation rates indicate that ΔpH removal in vivo, induced either by the absence of the ATPase, or by addition of specific ionophores has no consequences on the efficiency of protein association to the thylakoid membrane. Instead, a marked decrease was observed upon disruption of the Rieske TAT import sequence. This confirms that TAT machinery operates in vivo in *Chlamydomonas reinhardtii*, while suggesting that its energetic requirements are profoundly modified with respect to in vitro conditions.

Keywords: Translocation; Thylakoid; ΔpH; Rieske protein; Oxygen-evolving complex

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B.4. Domain structure of the dual targeted pea glutathione reductase signal peptide

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Import of nuclear encoded proteins into mitochondria and chloroplasts is generally organelle specific, which depends on the N-terminal targeting peptide. Yet, a group of proteins known as dual targeted proteins have a targeting peptide capable of leading the mature protein to both organelles. We investigated the domain structure of the dual targeted pea glutathione reductase targeting peptide using N-terminal truncations. A mutant starting with the second methionine residue of the targeting peptide directed import into both organelles, showing that dual import was not controlled by the nature of the N terminus. The removal of 16 N-terminal amino acids stimulated mitochondrial import without significantly affecting chloroplast import and the removal of the first 30 N-terminal residues strongly inhibited import efficiency into both organelles, especially mitochondria. Furthermore, N-terminal signal peptide truncations greatly stimulated the mitochondrial processing activity measured with the isolated processing peptidase. These results imply a domain structure for dual targeting peptides and indicate the existence of regulatory domains therein.

B.5. Role of positively charged transmembrane segments in the insertion and assembly of mitochondrial inner-membrane proteins

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The biogenesis of membrane oligomeric complexes is an intricate process that requires the insertion and assembly of transmembrane (TM) domains into the lipid bilayer. The Oxa1p family plays a key role in this process in organelles and bacteria. Hell et al. have recently proposed that Oxa1p could act as part of a general membrane insertion machinery for mitochondrial respiratory complex subunits [1]. We have previously shown that mutations in the TM domain of Cyt1p can partially compensate for the absence of Oxa1p [2]. We demonstrate that a single amino acid substitution in the TM domain of Qcr9p can bypass Oxa1p in yeast [3]. The mutations we have isolated in Cyt1p or Qcr9p introduce positively charged amino acids and we show that the mutant TM domain of Cyt1p mediates the restoration of complex assembly. We propose that the positive charges introduced in Cyt1p and Qcr9p TM domains promote interactions with negatively charged TM domains of other respiratory complex subunits, allowing the co-insertion of both domains into the membrane, in the absence of Oxa1p. This model argues in favor of a role of Oxa1p in the insertion and the lateral exit of less hydrophobic TM domains from the translocation site into the lipid bilayer.

Keywords: Oxa1; Respiratory complex; Membrane insertion; Mitochondria; Yeast

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B.6. Biosynthesis and acquisition of the molybdenum cofactor in *Escherichia coli*

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Despite the critical role of metalloproteins in processes as fundamental as cellular respiration, little is known about the molecular mechanisms of metalloprotein biosynthesis and assembly. For instance, the molybdenum cofactor (Moco) is an essential component of a diverse group of enzymes involved in the global carbon, nitrogen, and sulfur cycles [1]. Genes involved in molybdenum cofactor biosynthesis (*mo.* genes) have been identified in eubacteria, archaea, and eukarya. Some aspects of the biosynthesis process, e.g. the formation of the molybdopterin unit, are shared by all molybdoenzymes whereas the subsequent addition of a nucleotide is specific to some prokaryotic molybdoenzymes. Additionally, a specific chaperone-type protein is required for insertion of the appropriate Moco in a manner that must be coordinated with both Moco biosynthesis and apoenzyme folding pathways [2–4].

Here, we report results on the interaction existing in vivo between the *mo.* gene products involved in the final stages of Moco biosynthesis in *E. coli*. Effects of various *mo.* mutations on these interactions have been assessed and allowed the identification of protein complexes for which formation is dependent upon binding of a Moco intermediate. A comprehensive model is presented for the protein interaction network existing during the course of Moco biosynthesis in *E. coli*.

Keywords: Metalloprotein maturation; Molybdenum cofactor; Chaperone; Biogenesis nitrate reductase

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B.7. Identification of a novel mitochondrial metalloprotease that degrades targeting presequences

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Nuclear encoded mitochondrial precursor proteins contain an N-terminal extension called the presequence that carries targeting information for import into the organellar matrix. The presequences represent a group of amphiphilic, positively charged, membrane-interacting peptides with a propensity to form alpha-helices. Presequences are cleaved off after import by the mitochondrial processing peptidase, MPP. Previous studies revealed that the presequence of the ATP synthase F1beta subunit undergoes rapid degradation catalysed by a matrix located protease(s). In contrast, the mature portion of the precursor was not degraded [1]. We have developed a three-step chromatographic procedure for isolation of a protease involved in degradation of mitochondrial presequences. The proteolytic activity was measured by immunological detection of the F1beta presequence degradation and cleavage of specific synthetic fluorescent peptides (Pep Tag Protease assay). Two-dimensional gel electrophoresis of the isolated fraction followed by ESI MS/MS and database searches allowed identification in *A. thaliana* database of a novel mitochondrial metalloprotease. The identified metalloprotease contains an inverted zinc binding motif and belongs to the pitrilysin family.

Keyword: Mitochondria presequences degradation

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B.8. Cytochrome-*c* oxidase subassemblies in patients with cytochrome-*c* oxidase deficiency

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Although significant progress has been made concerning the structure and catalysis of cytochrome-*c* oxidase, the assembly of the 13-subunit mammalian enzyme has remained relatively unexplored. Patients with cytochrome-*c* oxidase deficiency generally show decreased subunit steady-state levels with a residual subunit pattern that is characteristic for the mutation causing the deficiency. Therefore, we hypothesised that any failure to incorporate one of the subunits or prosthetic groups leads to rapid proteolytic degradation of the remaining subunits, unless they have progressed sufficiently in their assembly pathway to form stable assembly intermediates.

To dissect the assembly of human cytochrome-*c* oxidase, we are comparing control fibroblast cultures with cultures from patients with cytochrome-*c* oxidase deficiency due to mutations in their nuclear SURF1, COX10 or SCO1 genes. The protein products of these genes are all critical for assembly of the holo-enzyme, but they are not constituents of the final complex. Western blot analysis of mitochondrial fractions resolved on native gels revealed the presence and subunit composition of distinct subassemblies in both patients and controls. Identification of these subassemblies has improved temporal positioning of activities within the assembly pathway of cytochrome-*c* oxidase and has increased our understanding of the function of the three assembly factors.

Keywords: Cytochrome-*c* oxidase; Assembly; Enzyme deficiency; SURF1; COX10; SCO1

Session C: Emerging Structure

C.1. Identification of structural attributes specific to proton pumps

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In a search for the structural attributes specific to proton pumps, we compared the X-ray structures of membrane energy-converting proteins either involved or not involved in proton pumping. As proton pumps, we took cytochrome bc_1 complexes, fumarate-reductase, various cytochrome *c* oxidases and bacteriorhodopsin. As ''nonpumping'' proteins, we took the light-harvesting complexes and the photochemical reaction centers of phototrophic bacteria.

The average distance between acid groups at the *p*-surface of proton pumps was found to be ~ 7 Å, in correspondence with the optimal distance providing the fastest proton transfer along both phospholipid monolayers [1,2] and anionic polymers [3]. In the nonpumping proteins, the acidic net is less dense.

In proton pumps, the distribution of charged residues from two sides of the hydrophobic barrier was found to by asymmetric: from the *n*-side the hydrophobic layer was found to be flanked by a band of positively charged amino acid residues. According to our calculations, the cumulative electrostatic effect of this positively charged belt facilitates the proton transfer towards the *p*-side of the membrane and blocks the proton escape in the wrong direction. In the nonpumping membrane, proteins this positively charged band is absent.

Keywords: Proton pump; Proton transfer; Cytochrome-bc_1 complex; Cytochrome *c* oxidase

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C.2. Functional roles of conserved domains in the diverse hydrophilic carboxy-terminal halves of yeast Na^+/H^+ antiporters (Nha1p)

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We have cloned and determined the nucleotide sequences of genes encoding the Na^+/H^+ antiporter (Nha1p) from *Candida tropicalis* (C.t.), *Hansenula anomala* (H.a.) (also named *Pichia anomala*), and *Aspergillus nidulans* (A.n.). In the deduced primary sequences, highly conserved hydrophobic regions, and rather diverse hydrophilic regions were found. We found six conserved regions (C1–C6) within the hydrophilic regions. We constructed various truncated forms of the C-terminal half of S.c. and C.t., expressed them in S.c., and analyzed the effect of truncation. Deletion of the C4–C6 region caused cell growth to be more resistant to high salinity than the wild type, suggesting an inhibitory function of these domains. However, complete loss of C1–C6 caused a severe growth defect under conditions of high salinity, suggesting a defect in the antiporter activity. The C2–C6 form containing only C1, restored the retarded cell growth at high salinity more than the control vector alone, but to a value lower than the wild type. These results suggested an essential role for C1 and an activating role of the C2–C3 region in the functional expression of Nha1. We also surveyed interacting partners of the conserved domains and found a candidate new protein.

Keywords: Na^+/H^+ antiporter; Yeast; Salinity resistance

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C.3. Characterisation of respiratory chain supercomplexes of *Paracoccus denitrificans*

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Paracoccus denitrificans is a Gram-negative soil bacterium that contains NADH–ubiquinone–oxidoreductase, cytochrome bc_1 complex and cytochrome *c* oxidase, as well as further respiratory chain enzymes like succinate dehydrogenase and different terminal oxidases [1]. Complex I of *P. denitrificans* is a detergent-sensitive enzyme that could not be isolated so far using the detergent laurylmaltoside. Complexes III and IV were purified by Berry and Trumpower [2] as assemblies of the two complexes with variable.

We found that digitonin is a milder detergent and isolated supercomplexes containing monomeric complex I, tetrameric complex III and four copies of complex IV. Complex IV probably exists as two complex IV dimers since we could find smaller supercomplexes containing tetrameric complex III with two or four copies of complex IV, which seem to be dissociation products of the large supercomplex.

The supercomplexes from *P. denitrificans* are structurally similar to those of mammalian mitochondria as described by Schägger and Pfeiffer [3], although the stoichiometry of the individual complexes is different.

In contrast to mammalian supercomplexes, the supercomplexes from *P. denitrificans* seems to represent a complete respirasome with stably bound cytochrome *c* oxidase.

Keywords: Supercomplex; Bacteria; Assembly; Respiratory chain; Respirasome

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C.4. Site-directed sulfhydryl labeling of the oxaloacetate decarboxylase Na^+ pump of *Klebsiella pneumoniae*: helix VIII comprises a portion of the sodium ion channel

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Helix VIII of the β-subunit of the oxaloacetate decarboxylase of *Klebsiella pneumoniae* contains the functionally important residues N373, G377, S382 and R389 [1–4]. Using a functional oxaloacetate decarboxylase mutant devoid of Cys residues in the β-subunit, each amino acid residue in helix VIII was replaced individually with Cys. Structural and dynamic features of this region were studied by using site-directed sulfhydryl modification of 20 single-Cys replacement mutants with methanethiosulfonate reagents in the absence or presence of sodium ions. Cys residues introduced in the lower part of helix VIII were accessible to the water-soluble compounds MTSEA, MTSET and MTSES and therefore believed to be exposed to water on the cytoplasmic side of the membrane. Based on the pattern of accessibility of residues 376–386 located at the upper part of helix VIII towards hydrophilic MTS compounds, we suggest that this part of the transmembrane segment is α-helical. In particular, a water-accessible face comprising G377, G380/V381, A383 and M386 may be part of a portion of a sodium-ion channel. The distinct results on accessibility towards the different MTS reagents obtained in the presence or absence of Na^+ ions may suggest a conformational change upon binding of Na^+ in this region.

Abbreviations: MTSEA; MTSET; MTSES

Keywords: Oxaloacetate decarboxylase; Methanthiosulfonate; Sodium ion pump; *Klebsiella pneumoniae*

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Session D: Oxidases

D.1. NO reductase as an O_2 reductase: the reaction of the fully reduced NO reductase with O_2

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The NO reductase from *P. denitrificans* is an integral membrane protein that belongs to the superfamily of heme–copper oxidases [1]. 'Traditional' heme–copper oxidases have a binuclear site consisting of a heme and a copper ion where oxygen is bound and reduced to water. The NO reductase has an iron instead of a copper at its catalytic center, something that is thought to be important for the reduction of NO. The NO reductase is also capable of oxygen reduction, albeit at a slow rate. The reaction between fully reduced NO reductase and O_2 was studied using the flow-flash technique in combination with time-resolved optical spectroscopy in the μs–s range. The results from this study will be presented and compared to the oxygen reaction for the 'conventional' oxidases.

Keywords: NO; O_2; Electron transfer; Proton transfer

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D.2. Probing the active site structure and mechanism of the plant alternative oxidase

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Mitochondria of higher plants are characterised by a cyanide-resistant alternative oxidase that is present in addition to the conventional cytochrome *c* oxidase. Based on primary structural homology between the alternative oxidase and well-characterised di-iron carboxylate enzymes, a structural model has previously been developed in which the active site of the oxidase is predicted to comprise a non-haem binuclear iron centre. This centre is proposed to be coordinated within a four-helical bundle that is peripherally associated with the matrix side of the mitochondrial inner-membrane [1]. Site-directed mutagenesis experiments have recently yielded some evidence for this structure as well as clues regarding the alternative oxidase reaction [2]. We are currently investigating the steady-state respiratory characteristics of two recently engineered mutant proteins (expressed in *Schizosaccharomyces pombe* mitochondria) in which a highly conserved tryptophan residue of potential catalytic importance has been substituted by either phenylalanine or tyrosine. Furthermore, we have partially purified the alternative oxidase from *Arum maculatum* to enable spectroscopic as well as transient and steady-state kinetic experiments to gain further insight in the active site structure and mechanism of the enzyme. Preliminary results of these studies will be presented and discussed in relation to our present ideas on alternative oxidase catalysis [3].

Keywords: Plant alternative oxidase; Ubiquinol–oxygen oxidoreductase; Di-iron carboxylate protein; Active site structure; Reaction mechanism

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D.3. Are there specific electron transfer pathways in cytochrome *c* oxidase?

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Cytochrome *c* oxidase catalyses the four electron reduction of dioxygen to water. The electrons are delivered to the enzyme one at a time by cytochrome *c* and are transferred via Cu_A and heme *a* to the binuclear center, consisting of heme a_3 and Cu_B, where oxygen reduction takes place. There are two main theories of how electron transfer (eT) in biological systems occurs. In one theory, the rate of electron tunnelling depends on the edge-to-edge distance between the donor and acceptor, and can occur through a variety of different routes [1], whereas the other theory favours electron transfer through specific pathways, which would allow structural control of the eT rates [2]. Key amino acids in suggested electron transfer pathways of cytochrome *c* oxidase [3,4] were mutated, and the eT rates between the different redox centres were investigated following CO-photolysis from the mixed-valence enzyme. The rates of electron transfer between Cu_A, heme *a* and heme a_3 were not affected by these mutations. These results do not support the existence of specific eT pathways in cytochrome *c* oxidase.

Keywords: Electron transfer; Cytochrome *c* oxidase

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D.4. Proton pumping on cytochrome *c* oxidase re-reduction

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Cytochrome *c* oxidase belongs to the family of terminal oxidases and is part of the respiratory chains of mitochondria and several aerobic bacteria. It catalyzes the process of oxygen reduction to water, and conserves energy as proton-motive force.

In a single turn-over the oxidase goes through several intermediates starting from the fully oxidized (O) to the fully reduced (R) state. The reaction with oxygen returns R back to the fully oxidized state, but this differs from the initial O-state by the values of midpoint potentials of the redox centers. The newly formed fully oxidized state is metastable, and decays into the relaxed O-state. Reduction of the metastable O-state, but not of relaxed O, is linked to proton pumping.

Keywords: Cytochrome *c* oxidase; Proton pumping; Re-reduction

D.5. FTIR spectroscopic evidence for the presence of acidic residues in the vicinity of the quinone and the oxygen binding site in cytochrome *bd* oxidase from *E. coli*

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Cytochrome *bd* oxidase is a widely distributed bacterial oxidase with unknown structure. Redox-induced FTIR difference spectra reveal the presence of several acidic residues in the active sites of cytochrome *bd* oxidase from *E. coli* [1]. These acidic residues may be involved in quinone binding, in proton uptake/release processes from/to the quinone as well as to the oxygen reducing site. Comparing the difference spectra for preparations of the enzyme isolated from cells grown at variable oxygen levels in which the quinone content of the membrane is altered, signals at 1738 and 1595 cm^{-1} have been shown to be involved in quinone binding. CO photolysis on the fully reduced form of the enzyme at low temperatures specifically perturbs the vicinity of the heme *b*–*d* binuclear center [2]. Several bands at 1760–1710 cm^{-1} can be observed, indicating that more than three protonated aspartic or glutamic acid residues are present in the direct vicinity of the binuclear center and reorganize upon CO dissociation. We suggest this residues to participate in proton translocation for oxygen reduction.

Keywords: Cytochrome *bd* oxidase; FT-IR spectroscopy; *E. coli*; CO photoperturbation

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D.6. Reduction of heme a_3 in cytochrome *c* oxidase is coupled to protonation of a heme a_3-ligated hydroxide molecule

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Protonation coupled to the internal reduction of the binuclear centre in cytochrome *c* oxidase can be studied using the reversed process, in the so-called back-flow experiment. Photo-dissociation of CO from the mixed-valence state of the enzyme results in internal electron transfer from heme a_3 to heme *a*. This oxidation of heme a_3 is coupled, at high pH, to the deprotonation of a group close to the binuclear centre and the release of a proton through the K-pathway.

We measured the kinetic difference absorption spectrum of the reaction associated with the proton release and it shows that a ligand binds to heme a_3 when it gets oxidised. We also studied the pH dependence on the kinetics with which CO recombines to the mixed-valence state and recorded an EPR-spectrum of this state. Results from both measurements indicate that the oxidation of heme a_3 results in deprotonation of a water molecule at the binuclear centre and that the formed hydroxide then ligates to heme a_3.

Keywords: Cytochrome *c* oxidase; Heme a_3; Redox-linked protonation; EPR-spectra

D.7. Exploring the role of tyrosine residues in the reaction of the plant alternative oxidase with ubiquinone

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The site-directed mutagenesis and subsequent functional expression of the plant alternative oxidase in the fission yeast *Schizosaccharomyces pombe* has provided a powerful tool in assessing amino acid residues of potential structural and/or catalytic significance. Recently, we have mutated two highly conserved tyrosines (Y253 and Y299, *S. guttatum* numbering), putatively involved in quinol binding, to phenylalanines. Respiratory analysis with isolated yeast mitochondria reveals that Y299F exhibits only ~ 45% of the wild type activity, whereas Y253F remains fully active. Y253F activity is marginally more sensitive to salicyl hydroxamic acid compared to the wild type enzyme (I_{50} ~ 45 and 83 μM, respectively). However, the sensitivity with respect to octyl gallate is comparable for both proteins (I_{50} ~ 0.11 μM). Simultaneous measurements of oxygen uptake rates and Q-reduction levels reveal that the kinetic behaviour with respect to the Q-redox poise of the Y253F mutant is very similar to that of the wild type. In contrast, the partial activity of Y299F would suggest that this enzyme does differ kinetically from the wild type. Currently, we are investigating the extent to which mutation of Y299-F has affected alternative oxidase activity to phenolic antagonists as well as alternative oxidase activity with respect to the Q-redox poise.

Keywords: Plant alternative oxidase; Tyrosine; Site-directed mutagenesis; Quinone pool; Redox poise; Fission yeast; Mitochondrial respiration

D.8. Interaction of ligands with cytochrome *a* in fully oxidized bovine cytochrome *c* oxidase

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Mammalian cytochrome *c* oxidase utilizes four redox centers in electron transfer (ET) from ferrocytochrome *c* to dioxygen. Cu_A and cytochrome *a* are electron acceptors and electrons from these sites are delivered to the binuclear center of cytochrome a_3 and Cu_B, where oxygen is reduced to water.

Ligand binding studies mostly focus on the binuclear center because of its catalytic significance. However, there are reports that cytochrome *a* can also interact with ligands [1–3]. In this work, the interaction between cytochrome *a* and external ligands was characterized by EPR spectroscopy. All ligands tested can be divided into two groups. One group consists of halides (F^-, Cl^-, Br^- and I^-) and induce a 24 G an up-field shift of $g = 3$ signal of cytochrome *a*. Nitrogen-containing anions (CN^-, NO_2^-, N_3^-, NO_3^-) are in the second group and shift the $g = 3$ signal down-field. At present, the number of binding sites was not established conclusively but chloride binding suggests a single interacting site. The possible catalytic significance of the binding site(s) may be in the regulation of ET or in involvement of hydroxide/water movement during enzyme function.

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Keywords: Cytochrome *c* oxidase; Cytochrome *a*; EPR

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D.9. Cytochrome *c* oxidase—proton pumping during single turnover

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Single-turnover proton pumping by cytochrome *c* oxidase from *R. sphaeriodes* incorporated into vesicles has been studied using the flow-flash technique. Monitoring the proton release with a pH-sensitive dye, phenol red, shows proton pumping during the F to O phase with a rate of 1.5 ms. However, pumping during the P to F transition could not be revealed [1] while the results from electrical measurements of charge translocation across the membrane indicate equal proton pumping in the P to F and F to O transitions [2]. The discrepancy between these results may be due to the fact that protons are bound to the lipid surface before release to the bulk [3]. To test this hypothesis, we did experiments in D_2O where the F to O transition is slowed by a factor of ~ 7. In D_2O, a biphasic proton release with similar amplitudes was seen, which indicates that proton pumping occurs to the same extent in the P to F and F to O transitions. Moreover, the fast release of protons is only visible if the F to O transition is slowed. Further experiments with mutant enzymes are on the way.

Keywords: Cytochrome *c* oxidase; Proton pumping; Vesicle

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D.10. The formation of intermediate E in cytochrome *c* oxidase causes structural changes at Cu_B and promotes the reaction with NO

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Oxidized Cu_B in cytochrome oxidase is likely liganded to a OH^-, which can be replaced by Cl^-. By studying by stopped-flow spectrophotometry, the redox-linked protonation of the soluble beef heart enzyme (using phenol red as the pH indicator), we find that bound Cl^- significantly decreases (by $\sim 0.9\ H^+/aa_3$) the apparent H^+ uptake stoicheiometry, by eliminating the net H^+ uptake linked to Cu_B reduction. We propose that upon formation of intermediate E, Cu_B is reduced and the anionic ligand of this metal (OH^- or Cl^-) picks-up a H^+ and diffuses into the bulk (as H_2O or HCl), where HCl dissociation explains the lower apparent stoicheiometry.

Nitric oxide is a very efficient inhibitor of cytochrome oxidase in turnover. By investigating the reduction kinetics of the mutants K354M and D124N of the *Paracoccus denitrificans* aa_3 in the absence/presence of NO, we provide evidence that this efficient inhibition is due to the ability of NO to react with the single-electron reduced active site of intermediate E. Such a conclusion is fully consistent with previous computer simulations. These results also suggest that the H^+-conducting K-pathway, but not the D-pathway, controls the kinetics of formation of this intermediate.

Keywords: Proton and electron transfer; Respiration; Nitric oxide

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D.11. $Q_H^{\bullet -}$ quinone binding pocket in quinol oxidases studied by EPR spectroscopy

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Ubiquinol oxidase (*bo*$_3$) from *E. coli* contains the so-called high-affinity, redox-active ubiquinone (Q_H). Its location and binding has yet to be accurately determined by X-ray crystallographic studies; however, based on mutagenesis studies, a putative binding-site has been proposed [1].

Nitrogen hyperfine couplings to the bound $Q_H^{\bullet -}$ have been investigated using three Pulse-Electron Spin-Echo Envelope Modulation (ESEEM), Hyperfine Sub-level Correlation (HYSCORE) and Double Nuclear Coherence Transfer (DONUT)-HYSCORE spectroscopies. Spectra revealed the interaction of the unpaired electron of $Q_H^{\bullet -}$ with a single nitrogen nucleus from the protein surrounding, which has been assigned to a protein-backbone nitrogen [2]. Time-domain simulations allowed a detailed description of the structural arrangement of this nitrogen to be determined.

The $Q_H^{\bullet -}$ binding site was further characterised using multi-frequency (35 and 94 GHz) electron paramagnetic resonance (EPR) spectroscopy. Experiments were performed on the native protein and on samples with selectively ^{13}C-labelled quinone derivatives. Evidence is provided for a strongly asymmetrical (or even one-sided) hydrogen bonding of $Q_H^{\bullet -}$ within its protein-binding pocket. These results are compared with the corresponding information available on other protein binding sites and model systems and are discussed with regard to the location and function of Q_H.

Keywords: Quinone; EPR spectroscopy; ^{13}C-labelling; Quinol oxidase

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D.12. FTIR difference spectra of cytochrome *c* oxidase reveal a coupling of histidine in the reduction cycle

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The catalytic steps and pathways of proton pumping in the heme–Cu terminal oxidases present several fascinating mechanistic questions. In the work presented here, we have isolated cytochrome *c* oxidase from *R. sphaeroides* and bovine heart, and used this for a series of electrochemically mediated FTIR difference spectroscopy measurements to examine the involvement of histidine in the catalytic cycle. Through both global ^{15}N-labeling and specific ^{15}N-histidine incorporation in the bacterial oxidase, a characteristic histidine vibration at 1104 cm^{-1} is found. This mode is ^{15}N-sensitive, showing a 10 cm^{-1} downshift, and is also present in the mammalian oxidase enzyme. We will present measurements of simultaneous IR and UV–Vis optical spectra where we correlate the appearance of the histidine mode with the reduction status of hemes *a*/a_3. The spectroscopic finding of a dynamic histidine residue provides an indication for a functional role of histidine in the O_2 reduction chemistry and associated proton pumping sequence.

Keywords: Cytochrome *c* oxidase; FTIR; Histidine

D.13. ATR-FTIR studies of catalytic intermediates of bovine cytochrome *c* oxidase

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Specific structural changes in protein, cofactors, and ligands of the catalytic intermediates of bovine cytochrome *c* oxidase were studied by perfusion-induced attenuated total reflection Fourier transform infrared (ATR-FTIR) spectroscopy in the 700–3000 cm^{-1} range. The IR absorption changes of the enzyme film, which was attached to an ATR prism, were measured under different aqueous environments. CO/O_2 perfusion at pH 9 allowed measurement of the transition from the fully oxidized (O) state to the peroxy (P_M) state. P_M formation was confirmed by simultaneous optical measurement of visible absorption band changes. Features in the FTIR difference spectra can be assigned to changes of heme a_3 and to changes in surrounding protein including, possibly, formation of a tyrosyl radical. The results will be interpreted in the light of crystallographic data and by comparison with available vibrational information on related proteins and model compounds.

Keywords: ATR; Cytochrome *c* oxidase; FTIR

D.14. Computational study of one and two electron reduced Complex IV

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The binuclear centre of cytochrome *c* oxidase is studied by means of computer simulations. The electronic and atomic structure of an approximately 250 atom model-system of the enzyme, embedded in either water or vacuum, is discussed. Quantum chemical calculations are used in the study of the oxidised, as well as the one and two electron reduced centre [1,2]. Furthermore, the ligation of CuB by H_2O, OH^- or Cl^- is investigated and the probabilities of such ligations are estimated.

Keywords: Cytochrome *c* oxidase; Quantum mechanical calculation

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D.15. Structural dynamics of cytochrome *c* oxidase

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Cytochrome *c* oxidase (COX) is a terminal enzyme of the respiratory chain. It catalyses the reduction of molecular oxygen to water and maintains a proton gradient across the membrane, used to drive ATP synthesis. In spite of extensive research, the structure–function relation of COX is not fully understood. The structure of COX from *Rhodobacter sphaeroides* has been solved to a resolution of 2.3/2.8 Å [1]. We also solved the structure of the inactive EQ286 mutant enzyme (E286 is a key residue in one of the proton pathways), which shows that the proton conducting hydrogen bond network is broken in the mutant enzyme. We are now in the process of refining the structure of the ED286 mutant enzyme, with a 50% reduced pumping activity. Together, these structures will add new information about the role of glutamate-286.

My main focus is the structural dynamics of COX, investigated using a combination of spectroscopy and X-ray crystallography. We have preliminary X-ray data on the DN132 mutant enzyme (D132 is located at the entry point of the same proton pathway), in which the oxygen reaction cannot proceed beyond the so-called F-state. We will use the DN132 mutant enzyme to trap this kinetic intermediate in the crystal.

Keywords: Cytochrome aa_3 oxidase; *Rhodobacter sphaeroides*; Redox protein; X-ray crystallography; Structural dynamics

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D.16. Coupling of electron uptake with propionyl group protonation at the low-spin heme B of cytochrome bo_3

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We have studied redox-dependent changes of the heme–copper ubiquinol oxidase cytochrome bo_3 of *Escherichia coli*, using Fourier transform infrared spectroscopy (FT-IR). Electron transfer is induced by a auto-photoreduction technique. This causes the complete reduction of the enzyme by means of UV irradiation at low (noninvasive) intensity. After in vivo labeling of the enzyme with 1-^{13}C-aminolaevulinate, carboxyl groups of the heme propionate side chains could be identified due to band shifting. The absorbance difference signals could be assigned specifically to the low-spin and the high-spin heme groups by comparison with FT-IR spectra of purified cytochrome bb_3, which was obtained after deletion of the *cyoE* gene encoding farnesyltransferase. One of the carbonyl groups of the low-spin heme B was protonated during the electron transfer. Using photoreduction spectra with cytochrome bo_3 equilibrated at the mixed valence state [1], we could allot the protonation event specifically to the electron transfer step on the heme B redox center itself.

Keywords: Cytochrome bo_3; FT-IR; Heme reduction; Heme protonation; Isotopic labelling

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D.17. Electron transfer between *Paracoccus denitrificans* cytochrome c_{552} and cytochrome *c* oxidase Cu_A soluble domains

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The electron transfer process between the soluble Cu_A domain of cytochrome *c* oxidase [1] and soluble domain of c_{552} [2] has been studied in the forward (physiological) and reverse electron transfer directions by stopped-flow spectroscopy as a function of ionic strength (I). The time-dependent reaction profiles in either direction show a monoexponential behaviour under most conditions with apparent second-order rates of 3.4×10^6 and 17.7×10^6 M^{-1} s^{-1} (errors within 10%) at $I = 15$ mM for the forward and reverse directions, respectively. The equilibrium constant for the physiological direction is thus about 0.2 under these conditions and does not appear to vary significantly with ionic strength up to 200 mM. The bimolecular rates in both directions decrease according to the Brönsted law as the ionic strength is increased with slopes (the $z_A z_B$ product) of about -5, indicating that two to three effective charges of opposite sign on each protein interface interact in the bimolecular electron transfer reaction. The temperature dependence of both the forward and reverse ET reactions was also studied, yielding activation energies of 44.3 and 24.7 kJ/mol. Although these findings show that the cytochrome c_{552}–Cu_A ET process is kinetically and thermodynamically unfavourable, the initial encounter between cytochrome *c* oxidase and its substrate is coupled to the highly exergonic subsequent reaction of water formation.

Keywords: *Paracoccus denitrificans*; Cu_A domain; Cytochrome c_{552}; Stopped-flow

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D.18. Pulse radiolysis study on one-electron reduction processes in subunit I mutants of cytochrome *bo*-type ubiquinol oxidase from *Escherichia coli*

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Cytochrome *bo*-type ubiquinol oxidase from *Escherichia coli* belongs to the heme–copper terminal oxidases and serves as a redox-driven proton pump of the aerobic respiratory chain. To understand the molecular mechanism of proton pumping, we have been carrying out site-directed mutagenesis studies on subunit I [1–3] where dioxygen reduction and proton translocation take place.

We applied pulse radiolysis technique, one of the powerful methods for studying intramolecular one-electron transfer processes [4,5], to subunit I mutants lacking the Cu_B center or having defects either in D- or K-channel for proton translocation. Upon pulse radiolysis of the wild-type enzyme in the presence of *N*-methylnicotinamide as an electron mediator, we observed the generation of ubisemiquinone anion radical with a broad peak at 440 nm at the Q_H site and subsequent electron transfer to hemes *b* and *o* with the first-order rate constant of 1.5×10^{-3} s^{-1} [5]. In His-333Ala, a biphasic reduction of the hemes with the rate constants of 1.1×10^{-5} and 8.9×10^{-2} s^{-1} was observed, indicating the perturbation in heme-to-heme electron transfer. Experiments with the channel mutants suggest that both D- and K-channels can be operative upon one-electron reduction of the oxidized enzyme.

Keywords: Quinol oxidase; Pulse radiolysis; Electron transfer; Channel; Site-directed mutagenesis

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D.19. Mapping the K-pathway of heme–copper oxidases

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Heme–copper oxidases are molecular energy transducers. Energy input is from a redox reaction: $4e^- + 4H^+ + O_2 \rightarrow 2H_2O$, and energy output is in the form of an electrochemical proton membrane potential ($\Delta\mu_{H^+}$). The enzyme functions both as a consumer of protons and as a proton pump. Thus, protons must be taken up, and also ejected, against the gradient while additional protons are taken up, against the gradient, to make water. For this reason, the structures that facilitate proton movement in the enzyme are of some interest. Studies by mutation and by X-ray crystallography have revealed two pathways for proton uptake in the enzyme. These are known as the "K" and "D" pathways because they are marked by a lysine and an aspartate, respectively. The K-pathway is believed to function primarily during reduction of the unliganded oxygen-reduction site, while the D-pathway is believed to function during the reduction of O_2 to H_2O, carrying protons for pumping as well as protons that will go to make water. Mutants have been made and characterized, which further define the K-pathway and confirm the rate-limiting character of this pathway for reduction of the oxygen-reduction site and hence net enzyme turnover.

Keywords: Cytochrome oxidase; *Rhodobacter sphaeroides*; Proton channel

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D.20. Modification of nuclear-coded subunits of oxidized bovine heart cytochrome *c* oxidase by hydrogen peroxide

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Bovine cytochrome *c* oxidase (CcO) is known to react with hydrogen peroxide and generate two intermediates, the 607- and 580-nm forms [1]. However, detergent-solubilized CcO also reacts with hydrogen peroxide, resulting in a time- and concentration-dependent inactivation. Electron transport activity decreases by 80% when the enzyme is incubated with 1 mM hydrogen peroxide for 30 min at pH 7.4 and room temperature. The amounts of subunits VIa and VII(b + c) decrease by about 70% and 30% in the hydrogen peroxide-treated enzyme as detected by reversed-phase HPLC (RP-HPLC). All 10 nuclear-coded subunits of intact CcO are detected by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF); the measured molecular masses are in excellent agreement with values calculated from the known sequences. MALDI-TOF analysis of hydrogen peroxide-treated CcO confirms the RP-HPLC data: (i) decreased subunit VIa content and (ii) modification of subunit VIIc with increased mass of +16 Da. We conclude that hydrogen peroxide does not simply react at the binuclear center of CcO, but also causes irreversible structural changes, i.e., dissociation of subunit VIa and oxidation of an amino acid in subunit VIIc (most likely a tyrosine [2]).

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Keywords: Cytochrome *c* oxidase; Hydrogen peroxide; Nuclear-encoded subunit; Modification

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D.21. Alteration of the thermodynamic properties of the D-pathway in cytochrome *c* oxidase results in uncoupling of proton pumping from the oxygen reduction activity

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The D-pathway of cytochrome *c* oxidase is used for the uptake of both substrate protons and pumped protons during the oxidative phase of the catalytic cycle. It has been shown that insertion of acidic amino-acid residues in the D-pathway results in uncoupling of proton pumping from the oxygen reduction activity, i.e. the mutant enzymes reduce oxygen to water but do not pump protons.

In this study, time-resolved optical absorption spectroscopy was used to investigate the reaction between the reduced enzyme and oxygen. We have compared the pH dependence of the reaction in the wild-type (WT) enzyme with that in different D-pathway mutants of cytochrome *c* oxidase from *Rhodobacter sphaeroides*. Our data show that the apparent pK_a values of the observed rates for the P to F and F to O transitions are altered in the nonpumping mutants compared to the WT enzyme. We propose that the observed pH dependence of these rates reflects the apparent pK_a value of a conserved glutamate residue, E(I-286), in the upper part of the D-pathway. The results reveal insights into the coupling between the thermodynamic properties of the D-pathway and the proton pumping machinery in cytochrome *c* oxidase.

Keywords: Cytochrome *c* oxidase; Kinetics; Optical absorption spectroscopy; Proton pumping

D.22. Molecular dynamics simulation of cytochrome *c* oxidase in a dimyristoyl-phosphatidylcholine bilayer membrane

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Cytochrome *c* oxidase couples the one-electron oxidation of cytochrome *c* to the four-electron reduction of molecular oxygen and links these electron transfer to proton translocation across the inner mitochondrial membrane, or the bacterial cytoplasmic membrane [1].

The subject of the present work is to study the structure and dynamics of the protein–membrane–solvent system by means of molecular dynamics simulations on the bases of the atomic structure [2] and the kinetics of proton movement coupled to electron transfer and conformational fluctuations associated with these processes.

The model consists of I and II subunits of the cytochrome *c* oxidase from *Paracoccus denitrificans* (two subunits of 549 and 252 amino acids), 181 DMPC (90 and 91 in the top and bottom layers, respectively), 72,686 water molecules in a bulk solution. The total number of atoms in the model more then 100,000 [3,4]. Energy minimization, membrane modelling are performing using of the biomolecular simulation program CHARMM c27b2 [5].

Here, we present preliminary results about the system set-up (positioning if internal waters, protonation states) and the equilibration of the system.

Keywords: Cytochrome *c* oxidase; Molecular dynamics simulation; Energy minimization

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D.23. NO binding and ultrafast dynamics in the active site of reduced heme-copper oxidases

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Nitric oxide (NO) is involved in regulation of respiration through interaction with the active site of cytochrome c oxidase (CcO) [1]. We study the dynamics of NO in this site in its fully reduced form by femtosecond flash photolysis. These are complicated, as more than one NO can be accommodated in or near the active site, even at very low NO concentrations [2]. By extending our measurements to the nanosecond timescale, here we show that two distinct phase of recombination of NO with heme a3 can be observed: a slow phase (> 4 ns) which is the only phase observed at low (up to stoichiometric) NO concentrations (1 NO/enzyme), and a faster one (~ 250 ps) with an increasingly higher relative amplitude at higher concentrations (2 NO/enzyme). Interestingly, in CcO ba3 from Thermus thermophilus the kinetics are NO-concentration independent and consist of the slow phase only. This protein is known to have a considerable NO reductase activity [3], which prevents the steady-state simultaneous presence of 2 NO molecules in the reduced active site. Additional techniques are presently explored to test various models of NO binding and dynamics in the binuclear center.

Keywords: Cytochrome oxidase, NO, femtosecond spectroscopy

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D.24. Investigation of protonable residues in the d-channel of *Rhodothermus marinus* caa_3 terminal oxidase

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The thermohalophilic bacterium *Rhodothermus marinus* has a caa_3 terminal oxidase, which does not have the glutamate residue present in mitochondrial-like oxidases [1–3]. Homology modelling analysis showed that the side chain of a tyrosine residue is in the spatial space of the glutamyl side chain. This observation led to the proposal that this tyrosinyl and a consecutive serine residue were the functional substitutes of that glutamate residue. In order to check whether this tyrosinyl (or other residues) could be protonable, pK_a calculations using continuum electrostatic methods were performed on this structure, and compared with control calculations done in the structure of *Paracoccus denitrificans* terminal oxidase. The results obtained will be discussed in the framework of intraprotein proton pathways in haem–copper oxidases.

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D.25. Purification and characterization of the alternative cytochrome *c* oxidase from *Rhodobacter sphaeroides*

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Rhodobacter sphaeroides has a branched respiratory chain with two cytochrome *c* oxidases, cytochromes aa_3 and cbb_3, of which the cbb_3-type terminal oxidase is expressed under microaerobic conditions. Cytochrome cbb_3 belongs to the family of heme–copper oxidases since it has a similar binuclear metal center where the oxygen reduction chemistry occurs [1]. It also contains the common low-spin heme moiety, but instead of Cu_A there are three heme *c* binding sites [1,2].

An efficient method to purify a highly active and homogeneous cytochrome cbb_3 enzyme has been developed for structural and spectroscopic studies. The *cco*NOQP gene cluster of *R. sphaeroides* was overexpressed in *Paracoccus denitrificans* (Δ*cco*NO) to obtain a large amount of starting material and a histidine-tag has been added to facilitate purification.

Spectroscopic studies show that full reduction of the enzyme is hard to achieve and that the occupancy of Cu_B–CO after flash-photolysis of the CO-bound enzyme is much higher than in the other heme–copper oxidases, both of which complicate the investigation of this enzyme when the same methods as has been used with the classical cytochrome *c* oxidase are applied.

Keywords: Cytochrome cbb_3; Cytochrome *c* oxidase

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D.26. Electron transfer in mixed valence cytochrome oxidase

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An essential functional property of cytochrome oxidase is electron transfer from the low spin heme (heme *a*) to the binuclear center formed by heme a_3 and Cu_B. One approach for the determination of the dynamics of this process is to form the mixed valence CO-bound enzyme (MV-CO) in which heme *a* and Cu_A are oxidized and heme a_3 and Cu_B are reduced and then photodissociate the CO. This will initiate a kinetic electron transfer process and will terminate in an equilibrium distribution based on the midpoint potential of the four redox centers. Recently, Verkhovsky et al. [1] reported that there is a very fast tunneling phase ($k>10^8\ s^{-1}$) followed by a slower phase ($k=3\times10^5\ s^{-1}$), the total of which yields a new distribution in which $\sim$ 60% of the electron density from heme a_3 is transferred to heme *a*. In contrast, prior studies reported only one phase (the slower one) with a $\sim$ 25% transfer. To resolve this issue, resonance Raman spectroscopy was used to follow the reaction until it reached a stationary state. The data could be deconvoluted into four species yielding a quantitative determination of the electron distribution in the two-electron reduced enzyme.

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D.27. Gene destruction and energetics of the two respiratory pathways of the amino-acid producing bacterium *Corynebacterium glutamicum*

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The high G + C gram-positive bacterium *Corynebacterium glutamicum* has two electron-transferring pathways in the respiratory chain. One consists of a new type of cytochrome *bc*-type quinol–cytochrome *c* oxidoreductase [1] and cytochrome *aa*3-type cytochrome *c* oxidase [2], while the other cytochrome *bd*-type quinol oxidase [3]. The energy coupling efficiency of the former pathway is expected higher than that of the latter. To investigate the roles of these two pathways and to see if it is possible to improve the cellular energetics, each of the genes for the two terminal oxidases was destructed. The growth rate of the *bd*-less mutant cells was halved, but the cell yield at the stationary phase was higher than that of the wild-type cells. The *aa*3-less mutant showed both a much less growth rate and cell yield than the wild cells. The H^+/O ratios of the wild, the *bd*-less and the *aa*3-less mutant cells were about 4, 5.5 and 2, respectively. The rates of dioxygen consumption of the membrane preparations are reduced even in the *aa*3-less mutant. The inhibitory effects of cyanide on the rate are consistent with those expected from the combination of oxidases. These findings suggest that it is possible under certain conditions to improve the cellular energetics and consequently the growth yield by gene manipulation of the respiratory enzyme complexes.

Keywords: Hydrophobic diheme cytochrome *c*; Cytochrome oxidase; Respiratory chain; High G + C gram-positive bacterium; *Corynebacterium glutamicum*

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D.28. The start of the water exit pathway in bacterial cytochrome *c* oxidase

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Cytochrome *c* oxidase (CcO) is the terminal enzyme in the electron transport chain, responsible for reduction of dioxygen to water with the simultaneous translocation of protons across the membrane. Entry pathways for protons have been identified, but exit pathways for protons and water remain unclear. A nonredox active Mg is located immediately above the active site in a buried region of organized water that could be part of an exit route. Our previous studies with Mn-substituted CcO, using a rapid-freeze-quench/ESEEM analysis, have shown that the protons of the three water ligands of the metal can be exchanged with deuterium from bulk solvent on a catalytically relevant time scale [1]. Additional studies using ^{17}O-labelled water also demonstrate rapid water exchange from bulk. By turning the enzyme over in the presence of ^{17}O-labelled O_2 and monitoring the appearance of ^{17}O-labelled water at the Mn, we have shown the product water exit route passes the Mn on its way to the bulk.

Keywords: Water transport; Cytochrome *c* oxidase; Cytochrome *aa*3; Oxygen reduction

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D.29. Asp51Asn mutation of bovine heart cytochrome *c* oxidase subunit I

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HeLa cells were transfected with a mammalian expression vector carrying bovine cytochrome *c* oxidase subunit I gene in which codons specific to mitochondria were changed to the universal one. The gene was also modified to express subunit I with the mitochonrial targeting signal and a histidine-tag at N- and C-termini, respectively. Western blot analysis using anti-histidine tag antibody demonstrated that the transfected HeLa cells expressed the bovine wild type or Asp51Asn subunit I in the mitochondria, but the mock-treated cells did not. Mitoplasts containing the bovine wild type subunit I showed a cyanide-sensitive ferrocytochrome *c* oxidation at the rate almost identical to that of mitoplasts from the mock treated cells in the presence of valinomycin and inhibitors for complexes I, III, and V. The mitoplasts showed a clear initial acidification, followed by alkalinization. FCCP completely abolished the acidification. The proton/electron ratios during the initial linear acidification were about 0.8. Mitoplasts containing bovine Asp51Asn subunit I showed no initial acidification, but oxidized ferrocytochrome *c* at the rate 30% faster than those containing the wild type subunit I. These results indicate that Asp-51 is essential to the proton pumping.

Keywords: Bovine cytochrome *c* oxidase; Site-directed mutagenesis; Proton pumping

D.30. Interactions between the cytochrome pathway and the alternative oxidase in isolated *Acanthamoeba castellanii* mitochondria

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The steady-state activity of the two quinol-oxidizing pathways of *Acanthamoeba castellanii* mitochondria, the phosphorylating cytochrome pathway (i.e. the benzohydroxamate(BHAM)-resistant respiration in state 3) and the alternative oxidase (i.e. the (KCN)-resistant respiration), is shown to be fixed by Ubiquinone (Q) pool redox state independently of the reducing substrate (succinate or exogenous nicotinamide adenine dinucleotide (NADH)), indicating that the active Q pool is homogenous. For both pathways, activity increases with the Q reduction level (up to 80%). However, the cytochrome pathway respiration partially inhibited (about 50%) by myxothiazol decreases when the Q reduction level increases above 80%. The decrease can be explained by the Q cycle mechanism of complex III. It is also shown that BHAM has an influence on the relationship between the rate of ADP phosphorylation and the Q reduction level when alternative oxidase is active, and that KCN has an influence on the relationship between the alternative oxidase activity and the Q reduction level. These unexpected effects of BHAM and KCN observed at a given Q reduction level are likely due to the functional connections between the two pathways activities or to protein–protein interactions.

This work was supported by the Polish K.B.N. and the Belgian F.N.R.S.

Keywords: Alternative oxidase; Cytochrome pathway; Oxidative phosphorylation

D.31. Simulation of water in cytochrome oxidase: dynamics of water chains and H_2O exit channels

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By computer simulation, we investigate the distribution and dynamics of water molecules within cytochrome *c* oxidase (CcO). In particular, we are interested in dynamics of water molecules that are produced at the binuclear center. We specifically address the following questions:
How many water molecules are in CcO, how are they distributed, what are their dynamics?
How stable are water (HB) networks inside CcO (D and K channels)?
How does a newly formed water molecule exit from the catalytic site?

The answers to these questions have considerable implications for CcO electron transfer and redox coupled proton transfer mechanism. We find:
There are two (quasi-) stable chains of water molecules, formed from water produced by the enzyme, which connect Glu-242 (end of D-channel), catalytic site, and a propionate of heme *a*3.
There are water molecules between the edges of heme *a* and heme *a*3.
These may facilitate electron transfer between the hemes and explain recent puzzling data on fast *a*/*a*3 ET.
Two channels for water exit from the catalytic site were identified.
The exit via one of the channel is only open when His-291 is dissociated from CuB. (His-291 dissociation is part of Wikström's His cycle model).

Keywords: Cytochrome *c* oxidase; Water dynamics; Electron transfer; Proton transfer; Pumping mechanism

D.32. The physiological significance of the cyanide-binding slow and fast forms of bovine cytochrome *c* oxidase

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It has been accepted that the cyanide-binding slow and fast forms occur in the purified bovine cytochrome *c* oxidase [1]. We have previously reported that the slow form of the oxidase is cased by the binding of ADP to the oxidase [2]. In the present study, we found that the fast and slow forms of the oxidase show the difference also in the reaction with carbon monoxide; the cyanide-binding fast and slow forms react with carbon monoxide at fast and slow rates, respectively. This suggests that the microstructure in vicinity to heme a_3–Cu_B binuclear center of the oxidase is different between the two forms. Furthermore, the slow form of the oxidase showed the proton pumping activity, while the fast form did not show it. In ESR spectrum, the slow form showed a more intense signal at $g = 2.01$. If this is attributable to a change of state of Cu_B., this suggests that, at least, one His liganding to Cu_B may dissociate by the binding of ADP to the oxidase in the slow form. As the slow form showed the proton pumping activity, the His liganded to Cu_B seems to be responsible for the activity. This result is in good agreement with the finding reported by Ralle et al. [3]. It seems rational in vivo that when ATP is consumed and ADP increases, ADP binds to cytochrome *c* oxidase to make the oxidase change to the cyanide-binding slow form, and the oxidase acquires the proton pumping activity.

We wish to thank Prof. Yoko Nagata for her interest in this study.

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D.33. Electron injection experiments on the D124N mutant of *Paracoccus denitrificans* cytochrome *c* oxidase

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In this study, we have measured membrane potential generation due to electron injection into an oxidized D-channel mutant enzyme of *Paracoccus denitrificans* cytochrome *c* oxidase. In the mutant enzyme, the aspartic acid 124, situated in the beginning of the D-channel, is mutated to asparagine. On injection of the first electron, we observe a fast phase of membrane potential generation with the same time constant of 11 μs as measured for wild type *P. denitrificans* cytochrome *c* oxidase [1,2]. As multiple electrons are injected, a subsequent slower electrometric phase starts to appear. The time constant of this slower phase is similar but the amplitude is much smaller than in the wild type enzyme where this phase is due to proton uptake into the binuclear center upon its reduction by a second electron. However, in the case of the mutant enzyme, this phase of electrometric potential generation cannot be connected with uptake of a proton through the D-channel because it is blocked by the mutation. We suggest that in this mutant enzyme, the slow electrometric phase may be due to proton transfer from E278 to the binuclear site.

Keywords: Proton transfer; Cytochrome *c* oxidase; *Paracoccus denitrificans*

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D.34. Electrometric properties of a metastable form of oxidized cytochrome *c* oxidase

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The full catalytic cycle of cytochrome *c* oxidase consists of oxidative and reductive parts. Using electrometric and single turnover proton pumping measurements, it was recently demonstrated that if the reaction is started with the fully reduced enzyme *plus* O_2, both the oxidative and reductive parts of the cycle contribute to proton pumping to the same extent—two protons are pumped in each [1]. It was suggested that the oxidative part of the reaction ends with a metastable, high-energy intermediate that has different properties from the normal oxidized enzyme. Here, we present electrometric data on the kinetics of enzyme reduction after an oxidative phase, with very small or very large concentrations of reductant. If there is no reductant in the medium (or only a tiny amount), the time constant of relaxation of the electrometric signal is 300 ms after pulsing reduced enzyme with O_2. With a large amount of reductant present (sufficient to fully reduce the enzyme within 300 ms), the relaxation time becomes 1.5 s, which is comparable to the intrinsic time constant of signal dissipation across the measuring membrane. The results indicate that immediately after oxidation, the metastable oxidized enzyme is in an ''open'' state in which the generated potential dissipates with a time constant of 300 ms.

Keywords: Cytochrome *c* oxidase; Electron transfer; Proton pumping

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D.35. Effects of various wavelengths in the near infrared range on cytochrome *c* oxidase in primary neurons functionally inactivated by tetrodotoxin

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Near-infrared light (NIR) has documented benefits in promoting wound healing [1] but the mechanism of its action is poorly understood. Our working hypothesis is that it activates cellular cytochrome *c* oxidase, a major photoacceptor in the NIR range [2,3]. Primary neuronal cultures were a model system because neurons are highly oxidative and their cytochrome *c* oxidase levels are regulated by neuronal activity [4]. Functional impulse blockade with tetrodotoxin (TTX) induces a down-regulation of this enzyme reversible by light-emitting diode array (LED) treatment at 670 nm [5]. To determine the optimal wavelength for activation in neurons, GaAlAs LEDs at 830, 880, 770, and 728 nm were tested in cultured neurons functionally inactivated by TTX at a power intensity of 50 mW/cm^2 and energy density of 4 J/cm^2 when applied for 73–105 s. Sister cultures were exposed to LED once a day for the last 5 of 6 days in the presence of 0.4 μM TTX. Results indicate that 830 nm was most beneficial in reversing the detrimental effect of TTX, followed by 880 and 770 nm, whereas 728 nm was the least effective. These findings indicate that all of the effective wavelengths correspond to the absorption spectrum of copper centers in cytochrome *c* oxidase.

Keywords: Near-infrared light; Light-emitting diode; Cytochrome *c* oxidase; Primary neuronal culture; Tetrodotoxin

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D.36. Heme a as the driving element of proton pumping of bovine heart cytochrome *c* oxidase

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Refined X-ray structures of bovine heart cytochrome *c* oxidase at 1.8–1.9 Å resolution showed a redox-coupled conformational change in the hydroxyfarnesylethyl group of heme a giving a large capacity change in the water channel placed from the matrix side to the formyl group of heme a. The refined structures show a hydrogen-bond network including the formyl group, Arg-38, a fixed water, Tyr-371, another fixed water, a peptide bond, Asp-51 in tandem and a large hydrophilicity increase in the environment of Asp-51 carboxyl group upon reduction. The latter is consistent with the infrared results showing deprotonation of Asp-51, induced solely by heme a reduction. No detectable X-ray structural change in the Arg-38-formyl group system despite a large electron density increase in the formyl group upon heme a reduction suggests protonation of Arg-38 upon the reduction. These structural results suggest an active proton transfer from Arg-38 to Asp-51 driven by heme a oxidation. Upon heme a reduction, the deprotonated Arg-38 extracts a proton from a water in the water channel leaving a hydroxide ion. The ion is squeezed off to the matrix space by the water channel capacity decrease upon heme a oxidation.

Keywords: Cytochrome *c* oxidase; Proton pumping; X-ray structure; Infrared spectroscopy

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D.37. Heme–heme and heme–ligand interactions in a binuclear cytochrome *bd* oxygen-reducing site studied by nanosecond laser absorption spectroscopy

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Cytochrome *bd* is a terminal quinol oxidase of bacterial respiratory chains containing three hemes: *b*-558, *b*-595 and *d* [1]. The role of heme *b*-595 remains obscure. We studied interaction of *Escherichia coli* membrane-bound cytochrome *bd* with CO in fully reduced (R) and mixed valence (MV) states by nanosecond absorption spectroscopy with different excitation wavelengths. Wild type (WT) and E445A mutant (lacking heme *b*-595) were compared. Photolyzed CO recombines with WT-R in a simple monoexponential process ($t_{1/2} = 8$ μs) giving a typical W-shaped difference spectrum. Recombination of CO with WT-MV shows three kinetic phases with half-times of 10 ns, 7–10 and 100–200 μs. The 10-ns phase, absent in WT-R, reflects geminate recombination of CO with part of WT-MV on ns-time-scale (cf. Refs. [2,3]). In the case of E445A, we similarly observed a nanosecond geminate recombination. However, in contrast to WT, such a recombination of CO with part of heme *d* in E445A was not confined to E445A-MV, but was observed as well in E445A-R. The data support electronic interactions between (and hence close location of) high-spin protoporphyrin *b*-595 and chlorin *d* in oxygen-reducing-site.

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Keywords: Cytochrome *bd*; Terminal oxidase; Respiratory chain; Ligand binding; Carbon monoxide; Electronic interaction; Geminate recombination; Nanosecond laser absorption spectroscopy; *Escherichia coli*

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D.38. Time-resolved studies of intraprotein proton transfer in the D-channel mutants of *Rhodobacter sphaeroides* cytochrome *c* oxidase

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Time-resolved electrogenic reactions of cytochrome *c* oxidase (COX) from *R. sphaeroides* coupled to transfer of the 4th electron have been studied using photoreduction of the ferryl form of the enzyme by Ru(Bpy)32+. Three mutants in the protonic D-channel have been compared: E286D (upper part of the D-channel), D132N (entrance of the D-channel) and E286D/D132N double mutant. Apart from the rapid electronic electrogenic phase (15 mks) observed in all forms of COX studied, there are two cyanide-sensitive electrogenic protonic phases characteristic of the wild-type COX: intermediate (0.4 ms) and slow (1.5 ms). In E286D, only the latter is resolved. In contrast, the D132N substitution removes specifically the slow phase, whereas the intermediate (0.4 ms) phase is only partly decreased in amplitude. Double substitution E286D/D132N eliminates the slow protonic phase, as in D132N, and this allows to observe in the double mutant the intermediate phase of the same amplitude as in D132N that is, however, decelerated four- to five-fold relative to WT or D132N. The deceleration explains the apparent absence of this phase in the E286D mutant, as it simply merges with the slow phase. The data indicate E286 to be specifically involved in the initial electrogenic proton movement (intermediate electrogenic phase) in the F-to-O transition.

Keywords: Cytochrome *c* oxidase; Proton transport; Time-resolved electrogenic reaction; D-channel mutant

D.39. Mechanism of superoxide generation by cytochrome *c* oxidase

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Cytochrome *c* oxidase is a key respiratory enzyme of eukaryotes and many bacteria that catalyses $4e^-$ reduction of O_2 to H_2O. During turnover, the enzyme passes a number of intermediate states in which partially reduced O_2 remains bound in the catalytic center. It was shown that P- and F-intermediate formation, which corresponds to transfer of the 2nd and 3rd electron to O_2, could be observed and studied under steady-state conditions simply by adding H_2O_2 to oxidized COX. A pseudo-catalase cycle has been proposed to explain the reaction of oxidized COX with H_2O_2, where H_2O_2 acts both as a ligand to the binuclear center and as an electron donor reducing P to F and F to Ox, generating superoxide radicals as a product [1].

Superoxide generation by bovine cytochrome *c* oxidase (COX) in the presence of excess hydrogen peroxide was detected earlier in our group using a classical spin-trap DMPO [2]. However, the spin-trapping technique is not suitable for quantitative measurements. Another conventional assay for O_2^- generation is based on measuring superoxide dismutase sensitive reduction of the Nitro Blue Tetrazolium (NBT) to brightly colored formazan [3]. We have shown that oxidized bovine COX catalyzes superoxide dismutase sensitive reduction of NBT to monoformazan in the presence of excess H_2O_2 and this reaction has been used for further characterization of the process. The O_2^- generation rate has been found to be proportional to concentrations of COX and H_2O_2 and strongly pH-dependent with the maximal rates observed around pH 6.5 (ca. 15 min^{-1} at 8 mM H_2O_2 and 25 °C). As the final product of NBT reduction is water insoluble, the rates obtained have been confirmed using a new sulfonated tetrazolium salt WST-1, which produces a highly water soluble formazan dye [4].

In a different approach, H_2O_2-sensitive electrode constructed as described in Ref. [5] has been used to monitor the rate of H_2O_2 degradation by bovine COX in the pseudocatalase cycle. The rate of H_2O_2 consumption has been shown to be KCN sensitive and to depend linearly on COX concentrations. The O_2^- release by the enzyme may indicate a possibility of endogenous tightly bound O_2^- generation in the binuclear center as a part of the catalytic mechanism.

Keywords: Cytochrome oxidase; Superoxide; NBT; WST-1; Hydrogen peroxide electrode

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D.40. Ca-binding characteristics of D477A mutant of cytochrome *c* oxidase from *Paracoccus denitrificans*

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Crystallographic data reveal a Ca-binding site in mammalian [1] and bacterial [2] cytochrome *c* oxidases (COX). In the bacterial enzyme, calcium is tightly bound [2] whereas the mammalian COX binds the cation reversibly and the binding induces a small red shift of heme *a* absorption spectrum [3].

Replacement of residue D477 in coordination sphere of Ca in COX from *Paracoccus denitrificans* leads to the appearance of the Ca-induced spectral changes similar to those observed in mammalian enzyme. Although affinity of D477A mutant for Ca^{2+} is close to that of bovine heart COX [4] (ca. 1 μM), the association and dissociation of calcium proceed much slower ($k_{on} = 6.8 \times 10^{3}\ M^{-1}\ s^{-1}$, $k_{off} = 6.8 \times 10^{-3}\ s^{-1}$). The dissociation rate of bound Ca^{2+} strongly depends on temperature ($E_{act} = 23$ kcal/mol).

In D477A mutant, calcium competes with sodium ions, as in bovine COX. In the bovine enzyme, dependence of K_d for Ca on Na concentration is parabolic [4] (1 Ca competes with 2 Na). In D477A, the plot yields a straight line indicating that calcium ion competes with 1 Na. The result indicates that aspartate-442 in bovine COX (homologous to D477 in *P. denitrificans*) may be one of the two Na binding sites, lost in the D477A mutant COX from *P. denitrificans*.

Keywords: Cytochrome *c* oxidase; Ca-binding site

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D.41. The *cbo*-type cytochrome oxidase from the obligate methylotroph *Methylobacillus flagellatus* KT: substrate specificity

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A novel representative of the haem–copper oxidase superfamily the cytochrome *cbo* from the obligate methylotroph *Methylobacillus flagellatus* KT is a four-subunit complex with subunits of apparent molecular masses of 57(I), 35(II), 30(III) and 40(IV) kDa. Subunit II is a cytochrome *c* homodimer, subunit III is a dihaem cytochrome *c* [1]. Substrate specificity of the purified by preparative gel electrophoresis oxidase was studied. The *cbo* oxidase revealed respiratory activity towards ascorbate, presumably due to the presence of high-spin *c*-type cytochrome, with pH optimum at 8.3. The pH optimum for ascorbate/TMPD oxidation was at pH 8.3 either due to the input of ascorbate oxidase activity: ascorbate/TMPD minus ascorbate respiration revealed a broad pH optimum at 7.0–7.6 for TMPD oxidation. The cytochrome *cbo* oxidized reduced by ascorbate TMPD ($K_M = 0.86$ mM, $V_{max} = 1.1$ μmol O_2/min mg protein) and horse heart cytochrome *c* ($K_M = 0.09$ mM, $V_{max} = 0.9$ μmol O_2/min mg protein). Ascorbate/TMPD oxidation was sensitive to cyanide ($K_i = 5$ uM). Kinetic constants were measured at pH 7.0. It was demonstrated that the physiological electron donor to the oxidase is the soluble cytochrome c_H from *M. flagellatus* KT.

Keywords: *Cytochrome oxidase*; *Cytochrome c*; *Methylobacillus*

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D.42. Energy coupling at the terminal part of the respiratory chain of extremely alkaliphilic *Thioalkalivibrio*

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Proton transport at the terminal part of the respiratory chain in extremely alkaliphilic halotolerant bacterial strain *Thioalkalivibrio versutus* was studied under near optimum growth conditions (pH 9–9.5). Under these conditions, the bacterial cells generated electric potential the negative charge being inside the cells. When the terminal part of the respiratory chain functioned, it was found that: (a) in the presence of K^+ and valinomycin, the cell suspension produced no acidification of the outer space in contrast to other known bacteria, (b) in the presence of uncoupler CCCP but in the absence of valinomycin, the anaerobic cell suspension responded on O_2 injection by reversible alkalinization of the medium. Cyanide prevented this alkalinization. According to difference spectra the cell membranes contained cytochromes *c* and (*b* + *o*) part of which reacted with CO. The membrane respiratory activity at the terminal part of the respiratory chain was optimal at pH 9.5 and specifically depended on sodium ions ($C_{1/2}$ = 10 mM). The data obtained suggest the presence of Na^+-pump at the terminal part of the respiratory chain of the studied strain that could pump Na^+ ions out of the cells.

D.43. Removal of bound cardiolipin destabilizes the quaternary structure of bovine heart cytochrome *c* oxidase

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The quaternary stability of bovine heart cytochrome *c* oxidase (CcO), either cardiolipin-containing or CL-free, was monitored by measuring individual subunit dissociation as a function of urea concentration. When 4–6 CL are bound per CcO monomer, the order of subunit dissociation is: VIa and VIb < VIIa < III < Vb. Complete removal of CL by PLA2 digestion destabilizes the quaternary structure of CcO. Subunits VIa and VIb are released, even without urea [1], while subunits VIIa, III and Vb are dissociated at lower urea concentrations (1.5–2.0 M lower) compared to the CL-containing enzyme. The electron transport activity of CcO is unaffected by the removal of subunits VIa and VIb. However, dissociation of subunit VIIa from either CL-containing, or CL-free CcO causes a coincident and complete loss of electron transport activity. CL removal has almost no effect on secondary structure but does perturb the cytochrome *a* environment. These results support our previous findings that functionally important CL binds in close proximity to subunit VIIa and that removal of CL from this site perturbs cytochrome *a*, decreasing the rate of electron transfer from cytochrome *a* to a_3.

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Keywords: Cytochrome *c* oxidase; Bovine heart; Cardiolipin; Subunit interaction; Urea denaturation; Quaternary stability

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Session E: Bacterial Redox Proteins

E.1. Characterization of the succinate-quinone-reductase of the thermoacidophilic archaeon *Thermoplasma acidophilum*

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Succinate-quinone-reductases play an important role in aerobic organisms as complex II in the respiratory chain catalyzing the oxidation from succinate to fumarate. The electrons are funnelled to the membrane quinone pool via the enzyme bound cofactors, which consist of three different iron–sulfur clusters, one FAD-molecule and, in some cases, of up to two additional *b*-type cytochromes [1]. The SQR from the thermoacidophilic euryarchaeon *Thermoplasma acidophilum*, growing at 59 °C and pH 1, is highly expressed in membranes thus allowing for the characterization of its iron–sulfur centers in the most integral, the membrane bound state [2]. For further characterization, we purified the enzyme in the presence of *N*-dodecyl-maltoside (DM) by three different chromatographic steps, yielding a purification factor of about 40, based on catalytic activity. The purified enzyme consisted of four different subunits with relative molecular masses of 63.5, 27.5, 14.8 and 13.9 kDa for subunits SDH A, SDH B, SDH C and SDH D, respectively, as already predicted from the recently published genome project of this archaeon [3]. Interestingly, however, we were able to detect the resonances of all three iron–sulfur clusters individually in the partially purified state, thus allowing for the first time the direct biophysical characterization of the canonical iron–sulfur centers.

Keywords: Archaea; Iron–sulfur center; EPR; Thermoacidophile

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E.2. *Escherichia coli* nitrate reductase A (NarGHI): identification of quinol binding sites

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Under anaerobic conditions and in the presence of nitrate, *Escherichia coli* synthesises an electron transfer chain comprising a terminal membrane bound nitrate reductase (NarGHI). NarI, a *b*-type cytochrome, is the site of quinol binding and anchors NarGH to the membrane. In order to define the residues involved in quinol binding and oxidation, mutants have been generated and their quinol binding and oxidation capability have been tested using quinol analogue substrates (menadiol, duroquinol) and inhibitors (HOQNO, stigmatellin) in combination with optical, fluorescence and EPR spectroscopies. From these studies, a putative quinol binding site has been identified. Residues located near the periplasmic end of the transmembrane helix III and residues from the loop (IV−V) might be involved in the formation of this site. Moreover, the heme b_L ligand His187 might be involved in this quinol binding site as HOQNO binding is sensitive to the absence of heme b_L. This is reminiscent to the quinone binding in formate dehydrogenase-N where the N-oxide group of HOQNO accepts a hydrogen bond from His-169, a heme *b* ligand. All these results are in accordance with the location of a quinol binding site towards the periplasmic side of NarI in close association to heme b_L.

Keywords: Respiratory chain; Anaerobiosis; *E. coli* nitrate reductase; Quinone binding site

E.3. Defining the Q_P-site of *Escherichia coli* fumarate reductase (FrdABCD) by fluorescence quench titrations and site-directed mutagenesis

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We have used fluorescence quench titrations and EPR spectroscopy to study the effects of site-directed mutants of FrdB, FrdC, and FrdD on the proximal menaquinol binding site (Q_P) of *Escherichia coli* fumarate reductase (FrdABCD) [1]. Fluorescence quench titrations with the menaquinol analog 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide (HOQNO) indicate that the Q_P site is defined by residues from FrdB, FrdC, and FrdD. In fluorescence quench titrations, wild-type FrdABCD binds HOQNO with a K_d of 5 nM, and the following mutations significantly increase this value: FrdB-T205H ($K_d = 16$ nM), FrdB-V207C ($K_d = 28$ nM). FrdC-E29L ($K_d = 26$ nM); FrdC-W86R ($K_d > 1000$ nM); and FrdD-H80K ($K_d = 27$ nM). Where binding was detected, data were fit to a monophasic binding equation, indicating that no additional high-affinity binding sites exist in FrdABCD. With the exception of the FrdB-V207C and FrdC-W86R mutants, HOQNO also has an effect on the EPR line shape of the [3Fe−4S] cluster. In the case of the FrdB-V207C mutant, the [3Fe−4S] cluster is converted to a [4Fe−4S] cluster [2], and in this case, HOQNO has an effect on the EPR spectrum of the fully reduced mutant enzyme. In addition to its effect on the affinity for HOQNO, the FrdB-T205H mutant elicits a $\Delta E_{m,7}$ of +82 mV on the [3Fe−4S] cluster. We conclude that residues from FrdB, FrdC, and FrdD play important roles in defining the Q_P site.

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E.4. Quinone binding sites in succinate dehydrogenase and fumarate reductase from *Escherichia coli*

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Succinate–ubiquinone oxidoreductase (SQR) and menaquinol–fumarate oxidoreductase (QFR) are structurally and functionally related membrane-bound electron transport enzyme complexes [1]. Two binding sites for quinone species have been shown in the crystal structure of the *Escherichia coli* QFR, which agrees with site-directed mutagenesis studies and labeling with azido quinone molecules [2,3]. The site proximal to the [3Fe–4S] cluster of QFR has been termed Qp and this site also helps stabilize an anionic semiquinone. Mutation of an acidic residue at the proximal quinone binding site increases the stability constant for the semiquinone by more than four-orders of magnitude [4]. A basic amino acid residue is also part of this quinone binding site and mutagenesis of this amino acid affects the ability of the enzyme to reduce quinone or oxidize quinol differentially as described in this communication. A similar site in *E. coli* SQR has also been shown by site-directed mutagenesis and EPR analysis to affect the catalytic activity of SQR and also effects the redox properties of the heme moiety of SQR [5]. Comparison of the quinone binding sites in SQR and QFR provides insights into the functional differences between these two evolutionarily related enzyme complexes.

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E.5. Estimation of the photo-induced and/or permanent electric potential across the membranes of *Rhodopseudomonas viridis*

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In photosynthetic reaction centers, the photo-induced electron transfers between the electron donors and acceptors generates a transmembrane electric field, which is used to produce ATP. As a consequence, the physicochemical parameters of these electron transfer reactions are expected to depend on the magnitude of the transmembrane electric field. It has been shown that in *Rhodpseudomonas viridis*, the reduction kinetics of the photo-oxidized chlorophyll dimer P^+ by a nearby cytochrome, which accounts for about 15% of the total photo-induced electric field [1], is indeed dependent on the membranes permeability [2]. We show here that both the intrinsic rate and equilibrium constant of this reaction may be changed by varying either the amplitude of the photo-induced electric field or the permanent electrostatic potential difference resulting from the cell metabolism. We present a method that makes use of this dependence to quantify both the photo-induced and permanent electric field.

Keywords: Electron transfer; Electrochemical difference potential; Photosynthesis

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E.6. Role of the endogenous ubiquinone as electron acceptor of the periplasmic PQQ-deshydrogenases of *Acetobacter diazotrophicus PAL5*

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Acetobacter diazotrophicus is an obligatory aerobe that fixes nitrogen; it belongs to the well-known group of the acetic acid bacteria that is recognized by its ability of to carry out oxidative fermentation of alcohols, aldehydes and sugar alcohols. Oxidation reactions are performed by membrane-bound deshydrogenases that directly feed electrons to the respiratory system. Primary dehydrogenases are located in the outer face of the cytoplasmic membrane with its active sites oriented to the periplasmic space. The purified primary dehydrogenases so far are either quinoproteins having a pirroquinoline quinone (PQQ) or flavoproteins having a covalently bound FAD as prosthetic groups.

In the case of *A. diazotrophicus*, we demonstrated [1] the presence of very active membrane-bound PQQ-deshydrogenases for glucose, acetaldehyde and ethanol that were up-regulated under culture conditions where diazotrophic activity was instrumental for growth. In our scheme of the respiratory system, we proposed that the endogenous ubiquinone was the common electron acceptor of the above three primary dehydrogenases, in analogy with other acetic acid bacteria [2]. Here we present evidence indicating that the endogenous ubiquinone is not required for the respiration of membranes with glucose, ethanol and acetaldehyde; on the other hand, ubiquinone is instrumental for the respiration with NADH and succinate.

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Keywords: PQQ-dehydrogenase; Ubiquinone; Bacterial respiration; *Acetobacter diazotrophicus*

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E.7. Investigation of the topology and menaquinol binding of DMSO reductase in *Escherichia coli*

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DMSO reductase (DmsABC) from *Escherichia coli* is a trimeric, oxidant specific menaquinol oxidase. The DMSO reducing DmsA subunit binds a molybdo-bis(molybdopterin guanine dinucleotide) co-factor. DmsB binds four [4Fe–4S] clusters and transfers electrons from menaquinol to DmsA. DmsC is a menaquinol binding transmembrane anchor subunit. DmsA and DmsB form a heterodimer that is attached to DmsC [1].

The site of menaquinol binding in DmsC is not fully identified. Mutation of the conserved His-65 located on the periplasmic side of the membrane affects the menaquinol oxidation [1]. To identify other residues that define the menaquinol binding site, we have done site-directed mutagenesis of conserved residues in DmsC. The mutants have been analysed for their capability to support anaerobic growth, enzyme activity and quinol binding.

Most biochemical data indicate a cytoplasmic location of the catalytic DmsAB-dimer even though DmsA contains a periplasmic targeting signal [3,4]. We have addressed the location of the DmsAB dimer by using in frame sequence tag insertions in DmsC. We have also used cysteine-scanning mutagenesis to address the location of DmsAB and the topology of DmsC. Several mutants have been analysed for the effect on growth on Glycerol/DMSO media and enzyme activity.

Keywords: Oxidoreductase; Menaquinol binding; Topology

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E.8. Electrochemical and FTIR spectroscopic investigations of the quinol:fumarate reductase from *Wolinella succinogenes*

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Electrochemically induced FTIR difference spectroscopy [1] has been used to investigate the dihaem quinol:fumarate reductase (QFR) from *Wolinella succinogenes*. The structure of QFR was recently solved at 2.2 Å resolution [2]. The difference spectra were taken between 1800 and 1000 cm^{-1} and the obtained difference bands represent transitions between oxidative and reductive potentials. The applied reductive and oxidative potentials were chosen such that all involved protein cofactors were either fully reduced or oxidised. Analysis of the difference spectra indicates structural reorganisations of the protein backbone upon redox reaction. The difference spectra exhibit spectral contributions in the range of stretching vibrations of protonated carboxyl groups. Possible origin of those spectral features are protonated aspartic or glutamic acid residues or heme propionates. Via site-directed mutagenesis and H/D isotope exchange, it was possible to identify an individual acidic amino acid residue to be partially responsible for the derivative-shaped spectral feature above 1700 cm^{-1}. The signal pattern is affected by H/D exchange and shows a downshift of several wave numbers. Spectra of WT QFR have been recorded between pH 5.5 and 8.8 showing a strong pH dependency of the carboxylic acid bands above 1700 cm^{-1}, indicating pK_a changes of the involved protonated carboxylic groups.

Keywords: Complex II; Dihaem cytochrome *b*; Electron transfer; Proton transfer; UV/VIS spectroscopy

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E.9. Purification, crystallisation and preliminary crystallographic studies of succinate:ubiquinone oxidoreductase from *Escherichia coli*

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Succinate:ubiquinone oxidoreductase (SQR), also referred to as succinate dehydrogenase or Complex II, is a four-subunit membrane-bound respiratory enzyme. SQR catalyses the oxidation of succinate to fumarate and the reduction of ubiquinone during aerobic respiration. This reaction directly connects the TCA cycle and the electron transport chain (ETC); however, SQR does not directly contribute to the proton gradient generated by the ETC like other respiratory proteins. Electron transfer is facilitated in SQR by five prosthetic groups; one flavin–adenine dinucleotide (FAD), three iron–sulfur clusters and one heme b_{556}. Here we present preliminary crystallisation and X-ray diffraction results for SQR from *Escherichia coli*. Crystals have been obtained and a data set, to 4 Å resolution, has been collected. The crystals belong to the hexagonal space group $P6_3$ with unit-cell dimensions of $a = b = 123.8$ Å and $c = 214.6$ Å. Using this data, 88.1% completeness and 0.106 R_{merge}, molecular replacement was possible. The solution, which uses the soluble domain of *E. coli* QFR as a search model, shows sensible molecular packing and suggests that *E. coli* SQR is a crystallographic trimer rather than a dimer as observed for the *E. coli* QFR.

Keywords: Succinate dehydrogenase; SQR; Membrane protein; X-ray crystallography

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E.10. Proton motive force generation through the redox loop: structure of formate dehydrogenase-N

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In anaerobic environments, the respiration of nitrate constitutes a major respiratory pathway, utilising electrons from formate oxidation and nitrate reduction to generate ATP for cellular processes. The enzymes involved in the pathway are formate dehydrogenase-N (Fdn-N) and nitrate reductase (Nar). These belong to the large molybdopterin oxidoreductase family, which contain a molybdenum atom and two molybdopterin guanine dinucleotides as cofactors in the active site. Both Fdn-N and Nar are composed of three separate subunits, a soluble alpha subunit, a membrane-anchored beta subunit, and an integral membrane gamma subunit containing two haem *b* groups. In order to increase our understanding of this important respiratory pathway, we have crystallized and successfully analyzed the structure of the Fdn-N complex. The structure has been refined to 1.6 Å resolution with R_{cryst} of 0.177 and R_{free} of 0.195, revealing an electron transfer pathway, a quinone binding site and a putative proton pathway. The structure explains how this respiratory system generates PMF through the redox loop mechanism.

Keywords: Formate dehydrogenase; Redox loop; Proton motive force

E.11. Structure and mechanism of quinol:fumarate reductase, a respiratory membrane protein complex from *Wolinella succinogenes*

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We have determined at 2.2 Å resolution the structure of *Wolinella succinogenes* quinol:fumarate reductase (QFR) by X-ray crystallography [1]. Based on the structure of the three protein subunits A, B, and C and the arrangement of the six prosthetic groups (a covalently bound FAD, three iron–sulfur clusters, and two haem *b* groups), we propose a pathway of electron transfer from the quinol-oxidising dihaem cytochrome *b* in the membrane to the site of fumarate reduction in the hydrophilic subunit A. Based on crystallographic analysis of three different crystal forms of the enzyme and the results from site-directed mutagenesis, we have derived a mechanism of fumarate reduction and succinate oxidation [2], which is generally applicable throughout the superfamily of succinate:quinone oxidoreductases. By combining the results from site-directed mutagenesis, functional and electrochemical characterisation, and X-ray crystallography, we have identified a residue that is essential for menaquinol oxidation [3]. The location of this residue in the structure indicates that the coupling of the oxidation of menaquinol to the reduction of fumarate by *W. succinogenes* QFR should be associated with the generation of a transmembrane electrochemical potential. The latter could not be confirmed [4]. A hypothesis is presented which reconciles these apparently conflicting experimental observations [5].

Keywords: Proton transfer; Electron transfer; X-ray crystal structure

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E.12. Positive and negative regulation of cytochrome *bd* expression in *Bacillus subtilis*

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Cytochrome *bd* is a terminal oxidase that is widespread among prokaryotic organisms. The *cydABCD* genes encoding cytochrome *bd* in *Bacillus subtilis* are transcribed as a polycistronic message from one identified promoter. The operon is expressed maximally when cells are growing in the aerobic stationary phase at conditions of low oxygen tension.

In this report, we dissect the promoter to define essential regulatory regions using various *cyd–lacZ* transcriptional fusions. Inspection of the promoter region revealed a 16-bp perfect palindromic sequence 98 bp upstream of the AUG translation initiation codon for CydA. This sequence was found to be required for the regulated expression of the *cydABCD* operon and it may comprise the operator site for a putative regulatory protein. The carbon catabolite protein A (CcpA), which represses the transcription of various genes encoding proteins involved with the utilization of secondary carbon sources, was found be required for maximal promoter activity. The molecular mechanism of transcriptional activation by CcpA is unknown, but our data indicate that regions upstream of the -35 region are important for this activation. Thus, expression of the *cydABCD* operon appears to be under positive (transcripional activation) and negative control (transcriptional repression).

Keywords: Cytochrome *bd*; Terminal oxidase; Regulation; *Bacillus subtilis*; Ccpa

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E.13. Bifunctional catalase–peroxidases (EC 1.11.1.7) and monofunctional catalases (EC 1.11.1.6) from cyanobacteria

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Cyanobacteria, to be regarded as the ancestors of all higher plants (viz., of the oxygen-evolving chloroplast), are unique oxygen-producing phototrophic prokaryotes which, in both photosynthesis and respiration [1], are potentially threatened by the formation of dangerous reactive oxygen intermediates (ROS) such as hydrogen peroxide or superoxide radical. The typical hydrogen peroxide-scavenging enzyme of cyanobacteria is a bifunctional catalase-peroxidase [2,3]. Among >20 species screened, only *Nostoc* spp., *Oscillatoria* spp., and *Chlorogloeopsis fritschii* gave unequivocal evidence for the presence of a monofunctional (“classical”) catalase. The enzyme from the latter species was isolated and purified to homogeneity. Molecular, spectral and kinetic (inhibition) characteristics will be amply discussed and shown to be clearly distinctive of either type of catalase. A gene coding for a monofunctional catalase may, indeed, be present in *Nostoc punctiforme* (http://www.jgi.doe.gov/JGI_microbial/html/).

Keywords: Respiration; Photosynthesis; Reactive oxygen species; Evolution; Catalase

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E.14. Modulation of the relative efficiency of the two electron transfer branches in Photosystem 1

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Photosynthetic reaction centres (RCs) allow plants and certain bacteria to convert light into chemical energy. All known RCs have symmetric structures: two similar integral membrane subunits form a dimeric core and bind the cofactors through which electrons are shuttled across the membrane. This symmetric arrangement gives rise to two similar branches of cofactors, down which light-driven electron transfer (ET) could proceed. The first two members of each branch are chlorins, the third is a quinone. It is known that the initial ET occurs almost exclusively along one of the two branches in the well-characterized type 2 RCs, although the origins of this asymmetry are still debated. Photosystem I (PS1) is the best-characterized representative of the type 1 RCs. Recent data suggests that ET proceeds down both cofactor branches of PS1 [1–3]. In this framework, both phylloquinones are reduced and the time constants of their reoxydation differ by one order of magnitude. We have used this kinetic signatures to assess the relative efficiency of the two ET branches in PS 1. Site-directed mutations were introduced at different positions along the two ET chains upstream the phylloquinones. The consequences of these mutations on the reduction of both phylloquinone will be presented.

Keywords: Photosynthesis; Electron transfer; Kinetics

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E.15. Delipidation of cytochrome *c* oxidase from *Rhodobacter sphaeroides* decreases stability of its quaternary structure

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Cytochrome *c* oxidase, isolated from *Rhodobacter sphaeroides* (Rbs-CcO), is a three-subunit integral membrane protein. Delipidation of the purified enzyme can be accomplished by phospholipase A2 hydrolysis of the bound phospholipids (PL) in the presence of detergent. The delipidated enzyme remains intact and fully active with all of its PL replaced by detergent except for two tightly bound phosphatidylethanolamines. In contrast to the enzyme prior to delipidation, exposure of the delipidated enzyme to urea (>5 M for 10 min at RT) substantially decreases its electron transport activity. This treatment has almost no effect on either the secondary or tertiary structure. It does, however, disrupt the quaternary structure, i.e., the interactions between subunits, as shown by sedimentation velocity and chromatographic analysis. Because of the strong correlation between the percent of intact enzyme and the remaining electron transport activity, we conclude that removal of PL inactivates Rbs-CcO by destabilizing the interactions between subunits. This result is quite different from that obtained with bovine heart enzyme since exposure of this enzyme to similar concentrations of urea does not disrupt interactions between the three homologous large mitochondrially encoded subunits [1].

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Keywords: Cytochrome *c* oxidase; *Rhodobacter sphaeroides*; Phospholipid; Subunit interaction; Urea denaturation

Reference

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E.16. Structure, stability, and function of cytochromes *c* from thermophilic and mesophilic bacteria

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Gram-negative bacteria are widely spread in different environments, having diverse modes of electron transport chains. In contrast to the diversity, soluble monoheme cytochromes *c* in the chains have remarkable similarity in their structures. Such cytochromes *c* are used for investigating protein stability as well as function in electron transport. We cloned genes coding for highly homologous cytochromes *c* from a thermophile, *Hydrogenobacter thermophilus* and a mesophile, *Pseudomonas aeruginosa*. We then established heterologous gene expression system using *Escherichia coli* as a host organism for these cytochromes *c* with the aim of protein engineering studies. The less stable mesophilic cytochrome *c* became as stable as the thermophilic one by introducing only five amino acid substitutions [1]. The five residues, distributed in three spatially separated regions, were selected and mutated with reference to the corresponding residues in the thermophile through careful structure comparison [2]. Furthermore, strength of heme iron coordination and redox property were influenced by the mutations in the three regions responsible for the overall stability, although these regions were away from the heme binding site. Here, we could provide molecular mechanisms underlying protein stability and redox property in the cytochromes *c* through the mutagenesis study.

Keywords: Cytochrome *c*; Thermophilic bacteria; Protein engineering; Stability

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E.17. Characterization of the respiratory nitrate reductase of *Wolinella succinogenes*

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Bacterial nitrate reductases involved in anaerobic respiration are located either in the membrane with the catalytic site of nitrate reduction oriented to the cytoplasm (Nar), or in the periplasmic space (Nap) [1]. Both enzyme types catalyze nitrate reduction to nitrite but the respective electron transfer pathways from the quinone pool to the catalytic sites are different. *Wolinella succinogenes* can grow by respiratory nitrate ammonification with formate as electron donor [2]. Although its nitrite reductase has been characterized in great detail [3,4], little is known about the nitrate reductase. Here we report the sequence of a putative *nap* operon from *W. succinogenes* (*napAGHBFLD*). Inactivation of *napA* prevented growth of *W. succinogenes* by nitrate respiration. The activity of nitrate reduction by formate as well as that of chlorate reduction by reduced benzyl viologen was abolished in fumarate-grown mutant cells. In contrast to wild-type cells, the mutant did not react with an anti-*Paracoccus pantotrophus*-NapA serum.

It is concluded that *W. succinogenes* contains only one respiratory nitrate reductase which is of the Nap type. Possible functions of the individual *nap* gene products in electron transfer or enzyme biogenesis are discussed.

Keywords: Nitrate reductase; Nitrite reductase; Anaerobic respiration; *Wolinella succinogenes*

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E.18. The oxygen respiratory chain of anaerobic sulfate-reducers

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Desulfovibrio species are sulfate-reducing bacteria described as strict anaerobic organisms since their discovery, more than 100 years ago. Nevertheless, several reports have shown that these bacteria are quite resistant to oxygen [1].

Our studies in the membranes of *Desulfovibrio gigas* showed that this organism contains a respiratory chain able to fully reduce dioxygen to water, with oxygen consumption rates comparable to those of aerobic organisms. In particular, two components of this respiratory chain were purified and characterized: a canonical membrane-bound terminal oxygen reductase of the cytochrome *bd* family [2] and a fumarate reductase [3] that is also capable, like most enzymes from this family, to function efficiently as succinate dehydrogenase.

A careful analysis of the unfinished genome of another sulfate-reducing bacterium, *D. vulgaris* Hildenborough, shows that this organism contains putative genes for most of the aerobic respiratory proteins. Interestingly, putative genes encoding for regulatory proteins that control aerobic/anaerobic gene expression are also present in *D. vulgaris* [4].

Thus, it appears that the presence of a canonical oxygen respiratory chain may be a general feature of sulfate-reducing bacteria, an observation with a significant implication for the understanding of the physiology, ecology, and possible pathogenicity of this important group of microorganisms.

Keywords: Anaerobe; Aerobic respiratory chain; Cytochrome *bd*; *Desulfovibrio*

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E.19. The role of different respiratory chain enzymes of *Azotobacter vinelandii* in the respiratory protection

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It was shown that two NADH:ubiquinone oxidoreductases operate in the *Azotobacter vinelandii* respiratory chain: proton-motive NADHdehydrogenase (NDH-I) and enzyme noncoupled to the energy conservation (NDH-II). The level of these oxidoreductases strongly depends upon $[O_2]$ and $[NH_4^+]$ in the growth medium. The gene encoding NDH-II in *A. vinelandii* was cloned, sequenced and used to construct an NDH-II-deficient mutant strain. Compared to the wild type, this strain showed a marked decrease in the respiratory activity. It was unable to grow diazotrophically at high aeration, while fully capable of growth at low aeration or in the presence of NH_4^+. These results suggest the role of NDH-II as a vital component of the respiratory protection mechanism in *A. vinelandii.*

A part of gene encoding cbb_3-type oxidase was cloned from *A. vinelandii* and a mutant *cco*N::Tc strain was constructed. In contrast to the wild type, this strain is fully unable to oxidize cytochromes c_4 and c_5. Thus, it is shown that the *A. vinelandii* respiratory chain contains cbb_3-type cytochrome oxidase. It was also shown that activity of this enzyme is not necessary for diazotrophic growth of *A. vinelandii* at high $[O_2]$.

Based on these results, the scheme of *A. vinelandii* respiratory chain under different environmental conditions was proposed.

Keywords: Respiratory protection; NADH:quinone oxidoreductase; NDH I; NDH II; Energy coupling; Terminal oxidase; *Azotobacter vinelandii*

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E.20. Thermal stability of cytochrome *d*–cyanide complex in *Salmonella typhimurium*

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Kinetics of cytochrome *d*-cyanide complex [1–3] formation at various temperatures (25–80 °C) was investigated in *Salmonella typhimurium* at pH 7.0. K_s (4 mM) [4] remained temperature-independent up to 50 °C, then increased to 71 mM at 60 °C and to 298 mM at 75 °C. V_{max} increased from 20 pmol min^{-1} mg^{-1} at 25 °C to 43 pmol min^{-1} mg^{-1} at 50 °C, then decreased to 12 pmol min^{-1} mg^{-1} at 75 °C. Arrhenius plot indicated 53 °C as optimum temperature for complex formation and 7.1 kJ mol^{-1} as activation energy. Pseudo-first-order rate constant (2×10^{-3} s^{-1} at 25 °C to 5×10^{-3} at 50 °C) dropped to 7×10^{-5} s^{-1} at 70 °C and to 3×10^{-5} s^{-1} at 75 °C. Second-order rate constant (465 M^{-1} s^{-1} at 25 °C to 1160 M^{-1} s^{-1} at 50 °C) dropped to 23 M^{-1} s^{-1} at 70 °C and to 15 M^{-1} s^{-1} at 75 °C. Gibbs free energy of denaturation ($\Delta G_D = -RT \ln (Y - Y_S/Y_D - Y)$) was 12 kJ mol^{-1} at 25 °C, 10 kJ mol^{-1} at 60 °C, dropped to 1.3 kJ mol^{-1} at 75 °C and to infinite negative value at 80 °C. Data showed that cytochrome *d*–cyanide was stably formed at temperatures up to 50 °C; thereafter, the affinity of cyanide for cytochrome *d* dropped and complex formation became undetectable at 80 °C.

Keywords: Cytochrome *d*; Cyanide; Temperature; *Salmonella typhimurium*

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E.21. *Enterococcus hirae* growth and solute transport can be controlled by redox potential and proton motive force

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The aim of this work is to study correlation between the changes of redox potential (E_h) and proton motive force (PMF) in *Enterococcus hirae* during growth under fermentation. Such a study is done to determine the prevailing conditions when *E. hirae* accumulates potassium to a high gradient between the cytoplasm and the medium in order to stabilize PMF at alkaline (pH 8.5) media. Two redox compounds: dithiothreitol (DTT) and potassium ferricyanide, which could change medium's redox potential, were tested. Positive means ($+220$ mV) of E_h created by 1 mM ferricyanide did not affect the growth; meanwhile, the negative E_h (-300 mV) stimulated growth accompanied with the strong acidification of the medium. Despite the noticeable drop in E_h, anaerobic fermentative growth of *E. hirae* was strongly suppressed in the presence of 0.1 mM uncoupler (carbonilcyanide-*m*-chlorphenilhydrazone, CCCP). Elimination of PMF by CCCP also resulted in the suppression of the ATP-dependent and PMF-regulated $2H^+/K^+$-exchange in *E. hirae*. DTT completely restored the growth and ion-exchanging pump operation under fermentation but not under respiration assuming the fact that this effect is only due to a decrease of E_h. The results demonstrate that the PMF can modify pH homeostasis by changing E_h.

Keywords: *Enterococcus hirae*; Fermentation; Redox potential; Proton–potassium exchange

E.22. Q_B position in the photosynthetic reaction center depends on pH: a theoretical analysis of the proton uptake upon Q_B reduction

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Electrostatics-based calculations have been performed to compute the proton uptake upon Q_B reduction in the reaction center of *Rhodobacter sphaeroides* as a function of pH. Two conformations of the reaction center, previously obtained by X-ray crystallography[1], were considered. In these structures Q_B is positioned either proximal or distal to the non-heme iron. These two positions have been attributed to the reduced and the oxidized forms of Q_B, respectively. We calculated the proton uptake expected from four different models. In one of these models, Q_B is allowed to conformationally equilibrate between the two positions independent of its oxidation state. This equilibrium can vary with pH. In the other models, the distribution of Q_B between the proximal and the distal position was pH-independent with Q_B either occupying the distal or the proximal position or been distributed between the two positions with a fixed ratio. Only the model including the pH-dependent conformational equilibrium reproduces the experimentally-measured pH-dependent proton uptake[2] and the crystallographically-observed conformational equilibrium at pH 8. From this model, we find that Q_B occupies only the distal position at low pH and only the proximal position at high pH, independent of its oxidation state. Between pH 6.5 and 9.0 both positions are partially occupied and the reduced quinone has a higher occupancy in the proximal position than the oxidized quinone. The analysis leads to the conclusion that the conformational equilibrium of Q_B depends on both the redox state of Q_B and the pH of the solution.

Keywords: Conformational Transition , Continuum Electrostatics, Photosynthesis, Proton Uptake, Protonation States

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Session F: Others

F.1. Oxidatively damaged proteins and senescence in *S. cerevisiae*

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Electron transport chains (ETC) using oxygen as a final electron acceptors are risky devices. They leak electrons that can partially reduce diatomic oxygen to superoxide consequently generating internal oxidative stress. Enzymes capable of efficiently scavenging reactive oxygen species (ROS) are present in all biological kingdoms, thereby implying an ancient necessity of protection against free radicals. Under normal conditions, the cell can balance ROS production and protective activities, in so doing, maintaining redox homeostasis. However, oxidative damage increases with age in almost all organisms tested so far and overactive protection systems often lead to longer lifespan [2]. In our group, we study protein oxidation and its implication to aging in *Saccharomyces cerevisiae* by measuring carbonyl groups levels by immunodetection. We showed that protein oxidation correlates with respiratory state more than respiratory rate thereby challenging the "rate of living hypothesis" [3]. This implies that the oxygen consumption rate (i.e. the electron flow through the ETC) is mechanistically not relevant to ROS production. In contrast, the degree of coupling between respiration and ADP phosphorylation (i.e. the respiratory state) directly correlates with the level self-inflicted oxidative damage [1]. In vivo, the oxidative phosphorylation apparatus can switch from a phosphorylating to a nonphosphorylating state under some nutrient starvation condition [1]. This leads to a sudden increase of protein oxidation. Interestingly, preliminary results suggest that a constitutive nonphosphorylating respiratory state is observed in cells transformed with the oncogenic allele of RAS. However, the relevance of such a respiratory state to normal replicative age is not established.

In order to investigate the effect of protein oxidation management on replicative age, we designed a method allowing us to visualize oxidized proteins in situ. We observe that yeast can deal with oxidative damage at the time of cytokinesis through a segregation mechanism. This discovery reveals a new protection mechanism against oxidative damage.

Keywords: Protein carbonylation; Replicative aging; Imaging; Cytokinesis

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F.2. Stimulation of menaquinone-dependent electron transfer in the respiratory chain of *Bacillus subtilis* by membrane energization

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At pH ≤ 7, respiration of *Bacillus subtilis* cells as well as sphaeroplasts shuts down almost completely upon addition of an uncoupler *m*-chlorocarbonyl cyanide phenylhydrazone (CCCP) and K^+-valinomycin. For endogenous respiration, $K_{50\%}$ of CCCP in the presence of K^+-valinomycin is below 100 nM. Either CCCP or valinomycin alone are much less efficient than combination of the two. The inhibitory effect is easily reversible and depends specifically on H^+ and K^+ concentration in the medium. Similar inhibition is observed with respect to the reduction of artificial electron acceptors that intercept reducing equivalents at the level of menaquinol. A loss of the electron transfer activities is also observed upon disruption of the bacterial cells during isolation of the membranes; the residual activities are not further inhibited by the uncoupler and ionophores. We conclude that the menaquinone-dependent electron transfer in *B. subtilis* respiratory chain is facilitated, thermodynamically or kinetically, by membrane energization. Requirement for energized state of the membrane is not a specific feature of succinate oxidation, as proposed in the literature [1–3], since it is observed also in the mutant of *B. subtilis* lacking succinate:quinone reductase as well as for the substrates other than succinate.

Keywords: Menaquinone; Proton motive force; Succinate:quinone reductase; Uncoupling; *Bacillus subtilis*

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F.3. Metabolic and genetic alterations of mitochondrial oxidative phosphorylation in aging

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Several works supported that mitochondrial oxidative phosphorylation (OXPHOS) is altered in skeletal muscle during the human aging process. Activities of mitochondrial respiratory chain enzymes decreased [1], with a progressive accumulation of random mtDNA mutations induced by radical oxygen species and lack of mtDNA protection [2]. Nuclear OXPHOS gene regulation seems to play an important role to compensate this mitochondrial impairment [3]. However, nutritional state and physical activities have to be considered as factors that could modulate such observations [4].

In order to understand the real effect of aging on mitochondria, we are carrying out molecular and biochemical analysis on either human muscle biopsies from healthy subjects or cell cultures originated from these biopsies. The mtDNA content and its random mutation rate, deletions and point mutations, are evaluated by real-time quantitative PCR. This methodology was previously finalized by the quantification of mtDNA depletion in patients with mitochondrial disorders [5]. Respiratory chain enzymatic activities are simultaneously measured, and we showed a decrease of cytochrome *c* oxidase activity in aging.

Finally, the study of the nuclear response to aging is undertaken by the quantification of nuclear and mitochondrial gene expression in both muscle biopsies and cultured myoblasts by quantitative RT-PCR and microarray analysis.

Keywords: Mitochondria; Aging; Skeletal muscle; Real-time quantitative PCR; Enzymatic activity

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F.4. Factors affecting the binding of lipophilic anions to biological and artificial membranes

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Lipophilic ions are a class of charged molecules with the unique property that they are soluble both in hydrophilic and hydrophobic solvents. Best-known examples of lipophilic ions are the indicators of membrane voltage, e.g. tetraphenylphosphonium, tetraphenylboron, phenyldicarbaundecaborane (PCB) and their analogues. In the early studies of transport of lipophilic ions across lipid bilayers, it was observed that lipophilic anions (LPA) bind several orders of magnitude more strongly and translocate several orders of magnitude more rapidly than structurally similar cations. The extensive interactions of LPAs with planar lipid bilayers and liposomes have been studied intensively (see Ref. [1]), but it is not clear yet why intact microbial cells bind very low amount of LPAs [2].

We studied the binding of LPAs to bacterial and yeast cells, to *Escherichia coli*-derived membrane vesicles as well as to pure phospholipid vesicles and membrane-containing bacteriophage particles. The inactivation of the cells by heat or phenol treatments led to six to eight times increase in the binding of PCB. A simultaneous inhibition of cellular oxidative phosphorylation and glycolysis also led to a considerable increase in PCB-binding. The results with polycationic compounds and bacterial viruses suggest that the lower binding of LPAs to intact cells is due to the difficulty in partitioning into the interior of phospholipid bilayers. Screening of the cell surface charge by high concentrations of salts affected the binding of LPAs rather weakly, but the amount of LPAs bound reached the level of heat-inactivated cells when pH of the medium was decreased to 3 and lower. Polycationic antibiotic Polymyxin B increases the PCB-binding some nine times to intact *E. coli* cells. It also increases the binding to heat-inactivated cells (about 15%) and to envelope-containing virus PRD1 (up to 60%). We demonstrated that the plasma membrane of microbial cells, in addition to the outer membrane of Gram-negative bacteria, creates an effective barrier for LPAs. We conclude that a barrier to lipophilic compounds in intact microbial cells includes several stages and is regulated by intrinsic membrane as well as metabolism-dependent factors.

Keywords: Lipophilic anion; Membrane permeability; Bacterial envelope

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F.5. Effects of the treatment by nucleoside analogs of reverse transcriptase (NRTIs) on mitochondrial function of adipose superficial and deep tissues of rats

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The use and development of tritherapy treatment (combination of protease inhibitors and NRTIs) led to a decrease in mortality and morbidity associated to HIV infection. Unfortunately, different secondary effects obliterated the efficiency of this treatment. Among them, HIV associated lipodystrophy (LD) is one of the most important [1]. Nowadays, it is usually considered that NRTIs could have a role in LD development [2], via effects that have been ascribed to the induction of mitochondrial dysfunction of adipose tissue [3].

The aim of this work was to test whether NRTIs cause mitochondrial metabolism modifications in adipose superficial (inguinal) and deep (epidydimal) tissues from rats treated with AZT compared with control rats. For that, we treated Wistar male rats for different times (1 and 4 weeks) with 70 mg AZT/kg/day.

We found that the specific activity of respiratory complexes (complexes II and IV) was significantly lower in adipose superficial tissue of AZT treated rats as compared to control rats. In contrast, no significant difference was observed for deep adipose tissues, thus suggesting a higher sensitivity of superficial adipose tissue towards NRTIs.

Since the synthesis of some respiratory complexes requires the expression of mitochondrial genome, mtDNA deletions and depletions analysis are under investigation.

Keywords: NRTI; Lipodystrophy; Adipose tissue; Respiratory chain

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F.6. On mitochondrial processes in apoptotic photosensitised cells

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Mitochondria and bioenergetic status of the cell have been shown to be the important factors in apoptosis. We have investigated a relation between cell death and bioenergetic parameters in murine hepatoma MH22 cells in vitro. Cell death was induced with either staurosporin targeting proteinkinase C, or doxorubicin targeting nucleus, or mTHPC-sensitised photodamage targeting cell membranes including mitochondria. Changes of mitochondrial membrane potential were followed by staining with fluorescent probes JC-1 or DiOC6. Cytochrome *c* leakage was detected by Western blotting or cytoimmunofluorescent microscopy. Intracellular ATP pool was assayed using luciferin–luciferase. The mode of cell death was revealed by activity of caspase-3 and changes of nucleus morphology.

Apoptosis was detected after the treatment with any of the applied cell death inductor as well as the leakage of cytochrome *c* did. However, the significant change of mitochondrial membrane potential and ATP pool were recorded only following the mTHPC-sensitised photooxidation. The investigation of cyclosporin A effect on cytochrome *c* leakage from the mTHPC-photosensitised cells revealed the complex response to cell death induction.

Keywords: Apoptosis; Photosensitization; ATP; Cytochrome *c*

F.7. Towards the systematic identification of mitochondrial proteins by LC-MS/MS

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In eukaryotic cells, mitochondria is the key organelle for cellular respiration. Mitochondrial proteome analysis is difficult to perform by the classical proteomic approach involving two-dimensional gel electrophoresis (2DE) as this organelle contains a large number of membrane-associated and highly alkaline proteins usually requiring specific treatments to be successfully analyzed. Here, an alternative approach was evaluated and led to the rapid and sensitive identification of around 35% of the yeast mitochondrial proteins. It consists of an SDS-PAGE gel electrophoresis of the total mitochondrial protein in combination with the LC–MS/MS analysis of the digestion products of gel slices. The use of only 40 μg of mitochondrial protein enabled the identification of 179 different gene products divided into similar proportions of membrane and soluble proteins. The distribution of the identified proteins in terms of p*I* and hydrophobicity revealed that the present analytical strategy is largely unbiased as compared to 2DE. The identification of 28 proteins of previously unknown subcellular localization demonstrated the ability of SDS-PAGE–LC–MS/MS to rapidly supplement the knowledge of the mitochondrial proteome.

Keywords: Proteome; Tandem mass spectrometry; Mitochondrion; Yeast; Respiratory complex

F.8. Binding to a lipid layer: a low material consuming approach for structural analysis of membrane proteins

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Difficulties of overexpression of membrane proteins represent a bottleneck for their structural analysis. We have developed strategies based on the specific binding of membrane proteins to a lipid layer spread at the air/water interface, which allowed to decrease to 1–2 μg the amount of proteins per structural experiment. A first approach is the production of 2D crystals that can be analysed by electron crystallography. It involves a binding step of the micellar proteins to the lipid layer, followed by reconstitution of the proteins into a lipid bilayer [1,2]. 2D crystals of different his-tagged proteins have been obtained after specific binding to an Ni-chelating lipid layer (FhuA, TF1FO, MIP, ANC2), and of charged proteins after electrostatic binding to a charged lipid layer (bacteriorhodopsin, ANC2). A second approach consists to bind and to orient single proteins onto the lipid layer. The unique orientation of bound proteins provided rapidly an averaged projection useful per se or a missing projection crucial for a 3D reconstruction by single particles analysis (e.g. ABC transporter, YvcC) [3]. Another application consists to fuse, onto the lipid layer, small 2D crystals obtained by the classical reconstitution in volume, to obtain large 2D crystals useful for high-resolution analysis.

Keywords: Membrane protein; 2D crystal; Single particle analysis; Lipid layer

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F.9. Ultrafast ligand rebinding in the heme domain of the oxygen sensors FixL and Dos: general regulatory implications for heme-based sensors

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Heme-based sensors carry out crucial roles in biological signaling by responding to the physiological messengers NO, CO and O_2. Binding of these gaseous ligands to a heme sensor domain initiates chemistry, eventually resulting in the organism's response to changes in ligand availability. To get insight into the dynamical aspects underlying these features and the ligand specificity of the signal transduction from the heme domain, we used ultrafast spectroscopy to study ligand dynamics in the heme domains of the oxygen sensors FixL [1] from *Bradyrhizobium japonicum* (FixLH) and Dos [2] from *Escherichia coli* (DosH). The transient spectra after ligand photodissociation are distorted compared to the ground state difference spectra. With O_2 as a ligand, this distortion is particularly strong, indicating dramatic differences in the heme environment with respect to the unliganded state. Moreover, heme-O_2 recombination occurs with an efficiency unprecedented for heme proteins, in $\sim$ 5 ps for $\sim$ 90% of the dissociated O_2. For DosH–O_2, where the signal transduction mechanism presumably is quite different, a similarly fast recombination was found with an even higher efficiency. Altogether, these results indicate that the heme pocket in these sensors acts as a ligand-specific trap [3]. The general implications for the functioning of heme-based ligand sensors are discussed in the light of recent studies on heme-based NO [4] and CO [5] sensors.

Keywords: Heme-based ligand sensor; Signal transduction; Dynamics of physiological messenger molecule; Ultrafast spectroscopy; Ligand-specific 'trap'

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F.10. Antioxidant capacity in cybrids harboring the three mtDNA mutations related to complex I subunits and primarily associated with Leber's (LHON)

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MtDNA mutations at position 11778/ND4, 14484/ND5 and 3460/ND1 are associated with LHON. Assessment of respiration in tissues, cells, and cytoplasmic hybrids from patients with LHON reveals reduction in oxygen consumption for all three mutations but such reduction is not coupled with a proportionate defect of complex I activity.

Even subtle dysfunction of complex I have been shown to be coupled with increased ROS production. The aim of our study is to assess the ROS production in cybrids harboring the three main mtDNA primary LHON mutations and correlate it to the intracellular nonenzymatic and enzymatic antioxidant protection systems.

Our results can be summarized as follows:

1) Basal ROS production shows a trend of increase in cybrids endowed with mtDNA mutation.
2) In the same cells, Mn-SOD and GPx activities are higher in respect of that of 143B-TK-parental cells, while the activity of GRx, is not influenced by mtDNA mutations.
3) The high consume of GSH well correlates with the lower level of total GSH in the all cybrids with LHON mutations respect to the parental cells.

In conclusion, our results suggest that mutations in mtDNA result in a modified pattern of antioxidant machinery in an attempt to limit ROS damage produced by defects in OXPHOS activity.

Keywords: LHON; Cybrid; ROS; Antioxidant system; Complex I

F.11. Dysfunction of the respiratory chain complexes cytochrome oxidase and *bc*1: studying disease-related mutations in yeast

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Mitochondrial respiratory chain defects are associated with an increasing group of human diseases. The genetic lesions causing the defect can be of nuclear or mitochondrial origin. More than 100 mutations in the mitochondrial genome have been reported (see for instance http://www.gen.emory.edu/mitomap.html). In this work, we use yeast mutants as models to characterize the effect of disease-related mutations in the mitochondrial genes coding for cytochrome *b* and for cytochrome oxidase subunits 1, 2 and 3. We are currently studying approximately 15 'human' mutations in these genes. The mutations are introduced in yeast mitochondrial genome by the ballistic transformation technique. Their effects on the respiratory competence of the cells, and on the assembly and catalytic activity of the mutant enzymes are then investigated. Some mutations have severe effect on the respiratory function and induce a respiratory deficient phenotype. Those mutants are used to explore ways to restore the respiratory competence.

Keywords: Disease; Cytochrome oxidase; Cytochrome *b*; Yeast

F.12. Paraquat affects mitochondrial bioenergetics by promoting the loss of mitochondrial membrane plasticity

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Paraquat is an herbicide believed to act by generating highly reactive oxygen free radicals accepting electrons from Photosystem I of chloroplasts by exposure to light [1]. This herbicide is very toxic to animals, including humans, with putative toxicity mechanisms associated to microsomal and mitochondrial redox systems [2]. In plants, paraquat also acts on nonchlorophylous tissues and mitochondria are candidate targets of paraquat toxicity, as in animal tissues.

Paraquat depresses mitochondrial $\Delta\psi$, simultaneously with stimulation of state 4 O_2 consumption [3]. However, the protonophoric action suggested by these effects was not confirmed by swelling. Mitochondrial structure observed by electron microscopy after a swelling performed in K-acetate medium with and without paraquat presented a significant difference. In the absence of paraquat, a mitochondrial volume increase and a generalized vanishing of the cristae was observed while in the presence of paraquat it was observed a shrinking, being the cristae still well observed. Paraquat radicals destroyed SMP proteins in rat liver, inducing the formation of high molecular weight bands $\sim$ 100 kDa. These morphological and structural effects clearly point out for a decrease on the protonophoric-dependent swelling as a result of a loss of mitochondrial membrane plasticity induced by paraquat binding or incorporation in the membrane.

Keywords: Paraquat; Mitochondrion; Bioenergetic; Toxicology

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F.13. Kinetic regulation of the mitochondrial glycerol-3-phosphate dehydrogenase by the external NADH dehydrogenase in *Saccharomyces cerevisiae*

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In the yeast *Saccharomyces cerevisiae*, the two most important systems for conveying excess cytosolic NADH to the mitochondrial respiratory chain are external NADH dehydrogenase (Nde1p/Nde2p) and the glycerol-3-phosphate dehydrogenase shuttle. In this last system, NADH is oxidized to NAD+ and dihydroxyacetone-phosphate is reduced to glycerol-3-phosphate by the cytosolic Gpd1p; glycerol-3-phosphate gives electrons to the respiratory chain via mitochondrial glycerol-3-phosphate dehydrogenase (Gut2p). Both Nde1p/Nde2p and Gut2p are located in the inner mitochondrial membrane with catalytic sites facing the intermembranal space.

We showed kinetic interactions between these two enzymes. First, deletion of either one of the external dehydrogenases caused an increase in the efficiency of the remaining enzyme. Second, the activation of NADH dehydrogenase inhibited the Gut2p in such a manner that, at saturating concentration of NADH, glycerol-3-phosphate is not used as respiratory substrate. This effect was not a consequence of a direct action of NADH on Gut2p activity since both NADH dehydrogenase and its substrate were needed for Gut2p inhibition. This kinetic regulation of the activity of an enzyme as a function of the rate of another owning the similar physiological function may be allowed by their association into the same supramolecular complex in the inner membrane.

Keywords: External NADH dehydrogenase; Glycerol-3-phosphate dehydrogenase; Kinetic regulation; Redox; Mitochondria; Yeast; *Saccharomyces cerevisiae*

F.14. Respiratory metabolism in plant cells during starvation and rescue

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In plants, the intracellular pools of sucrose is an important reserve of energy and carbon that helps sustain metabolism when photosynthesis and/or exogenous sugars are limited. Many changes in metabolism and gene expression occur during starvation, which together prolong plant cell survival until rescue occurs, when photosynthesis and/or exogenous sugars return. Cycling of carbon between sucrose and hexoses has been observed in many plants [1–3], and it has been proposed that this process consumes a large proportion of ATP synthesized by oxidative phosphorylation [4]. It is possible that sucrose cycling would greatly exaccerbate the energy crisis in starved cells. We therefore monitored sucrose cycling in cultures of nonphotosynthetic sycamore cells during starvation stress, using NMR spectroscopy. The rates of sucrose synthesis and splitting were quantified in pulse chase experiments using [1-^{13}C]glucose. Label in sucrose and G6P was quantified by ^{13}C-NMR, while the total intracellular amounts of these metabolites were determined by ^{13}C-NMR and ^{31}P-NMR, respectively. These experiments allowed us to determine if changes in metabolism and gene expression during starvation lead to reduced capacity for sucrose cycling.

Keywords: Plant cell bioenergetic; Sugar metabolism in plant; NMR spectroscopy

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F.15. CitS–sodium ion-dependent citrate carrier of *Klebsiella pneumoniae*

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CitS is a membrane protein that can transport citrate in symport with two sodium ions and one proton. The hydrophaty profile predicts 12 hydrophobic domains (TMS) but only 11 of them are spanning the membrane. One of the domain (called Vb) is located in periplasm. The C terminus is in the periplasm and the N terminus is in the cytoplasm.

We studied the properties of the cysteine residues of CitS. The wild-type CitS contains five cysteine residues (CCCCC) and all are located in the C-terminal half. Two of cysteines are in TMS VII and IX, three others in cytoplasmic loops. Mutants have been constructed in which cysteines were mutated into serines in different combinations. These mutants were assayed for sodium ion-motive-force-driven uptake of citrate in right-side-out membrane vesicles. More or less, activity corresponded to the expression levels in the membrane. The expression level of the Cys-less mutant was very low. Treatment of the mutants with NEM revealed that the two cysteines present in the C-terminal cytoplasmic loop were labeled, resulting in reduced activity of the transporter. Inactivation was prevented by the presence of the substrate citrate.

Keywords: Membrane protein; Cysteine labeling

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F.16. Effects of bupivacaine on mitochondrial ATP-sensitive potassium channel

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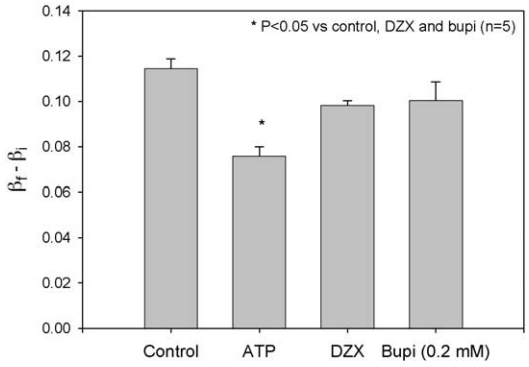
Introduction: Mitochondrial ATP-sensitive potassium channels (mitoK_{ATP}) are involved in cardioprotection and ischemic preconditioning [1]. Volatile anesthetics induce cardioprotection by activating mitoK_{ATP} [2]. The effects of local anesthetics on sarcolemnal K_{ATP} channel have been studied and could in part explained the pro- or anti-arrhythmic properties of these molecules [3]. However, the effects of local anesthetics on the mitoK_{ATP} are not yet known.

Materials and methods: Rat heart mitochondria were isolated by differential centrifugation. K^+ influx into mitochondria through the mitoK_{ATP} was measured using light scattering technique as the changes in mitochondria matrix volume [4]. Results (mean ± S.E.) were expressed as the maximal variation of the reciprocal absorbance ($\beta_f - \beta_i$). Statistical analysis was performed using ANOVA (SPPSS software) with $P < 0.05$ as significant.

Results: K^+ influx through the mitoK_{ATP} was decreased in the presence of adenosine triphosphate (ATP, 2 mM). Diazoxide (DZX, 30 μM) promoted channel opening. Bupivacaine (Bupi, 0.1–0.5 *m*M) acted, like diazoxide, as an agonist of the mitoK_{ATP} (figure).

Discussion: Bupivacaine appears as a mitoK_{ATP} opener. Consequences on the pharmacological properties and toxicity of local anesthetics should be studied.



Keywords: Local anesthetic; Mitochondria; Heart; Cardioprotection; Potassium channel

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F.17. IL-1 and LPS downregulate levels of PPAR gamma in brown adipose tissue

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Brown adipose tissue (BAT) is well known as heat producing organ of rodents and generally all young mammals. There is good experimental evidence that its thermogenic activity plays role also during development of fever and that this activation is mediated exclusively via adrenergic pathway. However, we have already shown that BAT cells highly express receptors for pyrogenic factors (IL-1, LPS) and that after their activation, they are capable of responding by production of IL-6 [1,2]. It is also known that TNFα, another cytokine involved in mediation of febrile response, acts as anti-adipogenic agent via downregulation of PPARγ involved in cell differentiation.

In this study, we have examined effect of chronic treatment of BAT cells in primary culture by IL-1β, IL-6 and LPS on their ability to differentiate into mature adipocytes. We found that treatment by IL-1β and LPS but not IL-6 inhibit differentiation of BAT cells. This seems to be mediated via downregulation of PPARγ levels. Furthermore, we demonstrated that cells after such treatment exhibit higher induction of IL-6 transcript by adrenergic stimulation. These results support the view of BAT such as not only effector but also mediator of febrile response [3].

Keywords: Brown adipose tissue; Pyrogen; PPAR gamma

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F.18. Metabolic regulation of stress response in the cells of sulfate-reducing bacteria

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This work provides experimental data on *Desulfovibrio desulfuricans*' survival in environments with strong respiratory poison carbon monoxide. We have demonstrated that CO in 5% concentration in the gaseous phase causes detectable changes in growth and energy parameters of the cells: prolonged lag-phase, decrease of biomass accumulation, lactate/acetate ratio and economic and energy coefficient. CO influences cytohrome composition, ATP, AMP, NAD(P)H content, inhibits substrate-level phosphorylation and probably serves as electron donor in the reactions of oxidative phosphorylation. This tendency is realized in different ways in dependence of initial metabolic status of the cells, subjected to CO. We suppose that all these changes are of adaptive value and are based on selective inhibition/activation of membrane proteins by CO. Taking into account our previous results [1,2], we propose the working scheme of metabolic regulation on physiological level, aimed to preserve high energy state of the cells in stress conditions.

Keywords: Sulfate-reducing bacteria; Energy metabolism; Carbon monoxide

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F.19. Novel aspects in function of the Pho89 phosphate transporter in *Saccharomyces cerevisiae*

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Phosphate starvation in the yeast *Saccharomyces cerevisiae* leads to an increased expression of the genes, *PHO84* and *PHO89*. They encode plasma membrane high-affinity phosphate transporters and a continued uptake of phosphate under such environmental circumstances is thus ensured [1]. The proton-driven Pho84 protein is primarily active at acidic to neutral conditions while the sodium-coupled Pho89 transporter has its maximal uptake capacity in the alkaline pH range. At standardized phosphate limiting conditions, the activation and inactivation of the Pho89 transport activity occurs early in the growth phase and the Pho84 transport, in contrast, is maximally active at mid-log phase. Furthermore, the phosphate uptake rate catalyzed by the Pho89 transporter is about two magnitudes lower than the incorporation catalyzed by the Pho84 protein [2]. A lower expression level of the *PHO89* is likely, together with other factors, to explain this discrepancy (J.O.L. and B.L.P., unpublished).

To overcome the latter and to enable in vivo studies of the Pho89 protein in a *PHO84*-free environment, we recombined the *PHO89* gene, C-terminally tagged with either a MYC epitope or the green fluorescent protein, into the *PHO84* locus. Our novel findings concerning the bioenergetical and physiological behavior of these strains expressing *PHO89* under control of the *PHO84* promoter will be discussed.

Keywords: Yeast; Phosphate; Transport; PHO84; PHO89

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Session G: Complex I & Transhydrogenase

G.1. NADH:ubiquinone oxidoreductase (Complex I) contains two FMN groups and has two EPR-detectable [4Fe–4S] clusters, N-2a and N-2b, in the TYKY (NuoI) subunit

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Biochemistry textbooks state that Complex I contains one FMN and four to five iron–sulfur clusters. This statement is incorrect for the bovine enzyme as it is based on erroneous protein determinations. A re-investigation showed that Complex I contains two FMN groups, seven NADH-reducible and EPR-detectable Fe–S clusters (one [2Fe–2S] cluster and six [4Fe–4S] clusters) plus one NADH-nonreducible [2Fe–2S] cluster. Two FMN groups were also found in the evolutionary-related NAD^+-reducing [NiFe]-hydrogenase from *Ralstonia eutropha*.

The TYKY subunit in the bovine enzyme (NuoI in the *Rhodobacter capsulatus* enzyme), contains two conserved Cys motifs typical for 2[4Fe–4S] ferredoxins. Otherwise, its amino acid sequence is unique for NAD(P)H:quinone oxidoreductases and certain membrane-bound [NiFe]-hydrogenases [1]. *R. capsulatus* mutants have been constructed in which five out of the eight conserved Cys residues in NuoI were replaced by other residues. Membranes from the C67S and C106S mutants had a diminished or unperturbed Complex I activity, respectively. EPR analysis showed a specific, 50% decrease of the signal attributed to cluster N-2. It is concluded that the NuoI (TYKY) subunit binds two clusters N-2, called N-2a and N-2b.

Keywords: Complex I; Two FMN; Protein determination; Mutant; TYKY; NuoI

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G.2. Cross-linking of transmembrane helices in proton-translocating nicotinamide nucleotide transhydrogenase from *Escherichia coli*. Implications for the structure of the membrane domain

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The proton-translocating nicotinamide nucleotide transhydrogenase from *Escherichia coli* is composed of three domains; domain I and domain III both face the cytosol and contain the NAD(H) and the NADP(H)-binding site, respectively. Domain II is the membrane-spanning domain composed of 13 α-helices [1] and harvests the proton channel. The active form of transhydrogenase is a dimer with a total of 26 transmembrane α-helices.

A cysteine-free construct [2] of transhydrogenase was used to introduce one or two cysteines at a time in domain II. The locations of the introduced cysteines were chosen so that it would be possible to determine the organisation of the 26 helices of the transmembrane domain. All mutants were located in the loops, on both sides of the membrane, that connect the helices. Cross-linking was performed in the presence or absence of an oxidation agent (diamide) or bifunctional maleimide cross-linkers (o-PDM (6 Å), p-PDM (10 Å) and BMH (16 Å)) and the cross-linking products were detected by SDS-PAGE. The results have made it possible to present the first complete model of the organisation of the transmembrane domain in transhydrogenase.

Keywords: Cross-linking; Transmembrane domain; *Escherichia coli*

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G.3. Covalently bound FMN in archaeal type-II NADH dehydrogenases

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The membrane-bound type-II NADH dehydrogenases from the thermoacidophilic archaea *Acidianus ambivalens* and *Sulfolobus metallicus* were isolated and characterised [1,2]. Both enzymes lack iron–sulfur clusters and consist of a single subunit of around 50 kDa and constitute the first examples of this enzyme family having one covalently bound FMN, as determined by fluorescence and ^{31}P NMR spectroscopies [2]. Data on *A. ambivalens* NDH-II point to the presence of an 8α-*N*-histidylflavin covalent bound. This novel feature among type-II NADH dehydrogenases contributes to the higher reduction potentials of these enzymes (68 mV for *A. ambivalens* and 160 mV for *S. metallicus* NDH-II). The affinity towards NADH is indicated by the K_m values (6 μM for *A. ambivalens* and 2 μM for *S. metallicus*) with ubiquinone-2 as electron acceptor. In the presence of phospholipids, the specific activities of these enzymes were determined to be 160 mmol NADH oxidized min^{-1} mg^{-1} for *A. ambivalens* and 192 μmol NADH oxidized min^{-1} mg^{-1} for *S. metallicus* NDH-II. The *A. ambivalens* NDH-II gene was cloned and sequenced, and heterologous expression was carried out in *E. coli*, aiming further studies with the recombinant protein. Analysis of the full sequence of *A. ambivalens* NDH-II shows putative binding sites for both FMN and quinone.

Keywords: Archaea; Type-II NADH dehydrogenase; Covalent FMN

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G.4. Characterization of the flavin radical of Na^+-translocating NADH:ubiquinone oxidoreductase from *Vibrio cholerae*

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Na^+-NQR is the entry point for electrons into the respiratory chain of *Vibrio cholerae*. It oxidizes NADH, reduces ubiquinone and uses the free energy of this redox reaction to translocate sodium. The enzyme is an integral membrane complex of six subunits that accommodates a 2Fe–2S center and several flavins: two covalently bound FMN's in subunits B and C, and one noncovalently attached FAD in subunit F. Na^+-NQR also contains an unusual radical, present in the oxidized and in the reduced enzyme. Here, we present EPR and ENDOR data which demonstrate that this radical is a flavin-semiquinone. In the oxidized enzyme, the radical is in its neutral form, but it is converted to the anionic form upon reduction of the sample. It is likely that the radical resides in a sequestered environment. When the enzyme becomes reduced, the proton on the N(5) position of the isoalloxazine ring shifts to a neighboring proton acceptor. The resulting anionic radical may also require a nearby positive charge for stabilization, suggesting that the flavin radical might be involved in translocation of Na^+ by the enzyme.

Keywords: Na^+-NADH:quinone oxidoreductase; *Vibrio cholerae*; Flavin; Electron transfer; Sodium translocation; EPR; ENDOR

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G.5. Production of superoxide radicals by alternative NADH dehydrogenases of yeast and trypanosome mitochondria

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The production of H_2O_2, the dismutation product of superoxide (O_2^-), by yeast mitochondria (mito) with succinate or malate as substrate was stimulated by antimycin (AA) and inhibited by myxothiazol (myxo) suggesting that the bc_1 complex produces O_2^-. H_2O_2 production with NADH was stimulated by AA but unaffected by myxo. Detergent-treated mito produced O_2^-, determined by the EPR detectable DMPO-adduct, stimulated by AA, blocked by superoxide dismutase, and unaffected by myxo. Mitoplasts produced O_2^- with NADH in the presence of myxo; however, proteinase K digestion of mitoplasts reduced the NADH:UQ reductase activity and O_2^- production. Mild detergent treatment of the proteinase-treated mitoplasts resulted in an increase in NADH:UQ reductase due to the internal NADH dehydrogenase; however, there was little increase in O_2^- production indicating that the external NADH dehydrogenase(s) produce O_2^- in yeast mito. The production of O_2^- by trypanosome mito was stimulated by AA but unaffected by myxo suggesting that the bc_1 complex does not produce O_2^-. With NADH, O_2^- production was inhibited by DPI and SOD suggesting that the rotenone-insensitive NADH dehydrogenase produces O_2^-. The purified alternative NADH dehydrogenase produced O_2^-, which was inhibited by DPI. Possible physiological roles of alternative NADH dehydrogenases in O_2^- production are under investigation.

Keywords: Rotenone-insensitive NADH dehydrogenase; Superoxide; Trypanosome; Mitochondria; Complex I

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G.6. Subunit arrangement in Complex I: an immuno-electron microscopy study

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The NADH-dehydrogenase (Complex I) is the first enzyme in the mitochondrial respiratory chain and translocates protons across the membrane coupled with the transfer of electrons from NADH to ubiquinone. Complex I is a multisubunit complex with a total molecular mass of nearly 1000 kDa and is composed of more than 40 subunits.

We analyzed complex I purified from the strictly aerobic yeast *Yarrowia lipolytica* [1]. The enzyme was studied by single particle electron microscopy and image processing. Several subunits were labeled with monoclonal antibodies.

The 3D reconstruction shows an L-shaped complex formed by a membrane arm (~ 20 nm) and a peripheral arm (~ 12 nm) protruding into the mitochondrial matrix.

Two separate epitopes located on the 49 kDa subunit indicate its extent over about 7 nm within the extrinsic part of the matrix arm. The epitope of the 30 kDa subunit was located close to the outer epitope of the 49 kDa subunit. In complex I of *E. coli*, a minimal model, the two subunits are known to be fused [2].

Two other epitopes in the membrane arm were determined: one conformational specific and one pointing to a 24 kDa subunit.

Keywords: Electron microscopy; Single particle analysis; Antibody; Epitope

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G.7. A novel, enzymatically active conformation of the *Escherichia coli* NADH:ubiquinone oxidoreductase (Complex I)

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Electron microscopy has demonstrated the unusual L-shaped structure of the respiratory complex I consisting of two arms which are arranged perpendicular to each other. We found that the *Escherichia coli* complex I has an additional stable conformation with the two arms arranged side by side resulting in a horseshoe-shaped structure. The structure of both conformations were determined by means of electron microscopy of gold-thioglucose stained single particles. They were distinguished from each other by titration of the complex with poly(ethylene glycol) and by means of analytical ultracentrifugation. The transition between the two conformations is induced by the ionic strength of the buffer and is reversible. Only the horseshoe-shaped complex I exhibits enzyme activity in detergent solution, which is abolished by the addition of salt. Therefore, it is proposed that this structure is the native conformation of the complex in the membrane.

Keywords: Complex I; NADH dehydrogenase; *Escherichia coli*; Conformation; Analytical ultracentrifugation; Electron microscopy

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G.8. Rotenone inhibits the mitochondrial permeability transition-induced cell death in U937 and KB cells

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The Permeability Transition Pore (PTP) is a mitochondrial inner membrane Ca^{2+}-sensitive channel that plays a key role in different models of cell death. Since functional links between the PTP and the respiratory chain complex I have been reported, we have investigated the effects of rotenone on PTP regulation in U937 and KB cells. We show that rotenone was more potent than cyclosporin A (CsA) at inhibiting Ca^{2+}-induced PTP opening in digitonin-permeabilized cells energized with succinate. Consistent with PTP regulation by electron flux through complex I, the effect of rotenone persisted after oxidation of pyridine nucleotides by duroquinone. Tert-butylhydroperoxide (TBH) induced PTP opening in intact cells (as shown by mitochondrial permeabilization to calcein and cobalt), as well as cytochrome *c* release and cell death. All these events were prevented by rotenone or CsA. These data demonstrate that respiratory chain complex I plays a key role in PTP regulation in vivo, and confirm the importance of PTP opening in the commitment to cell death.

Keywords: PTP; Complex I; Rotenone; Cyclosporine A; Cell death

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G.9. Alternative NADH:ubiquinone oxidoreductase of *Yarrowia lipolytica*

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Alternative NADH:ubiquinone oxidoreductases (NDH2) are found in the respiratory chain of plants, fungi and many bacteria. In contrast to complex I, NDH2 does not contribute to the generation of a proton gradient across the membrane. In the obligate yeast *Yarrowia lipolytica* as an eukaryotic model system, NDH2 is a small, single subunit enzyme of about 50–60 kDa. It contains one molecule noncovalently bound FAD and its active site is located at the outer face of the inner mitochondrial membrane. Redirection of NDH2 to the matrix side of the membrane by means of an N-terminally attached mitochondrial targeting sequence rescues the lethality of complex I deletion mutants and enables the cells to grow in the presence of the complex I inhibitor 2-decyl-4-quinazolinyl amine.

Kinetic measurements on mitochondrial membranes show that NDH2 employs a ping-pong reaction mechanism.

1-hydroxy-2-dodecyl-4(1H)chinolon was found to be an effective inhibitor for NDH2, which causes a 50% inhibition at a concentration of 200 nM.

In the NDH2 sequences, highly conserved regions can be found, e.g. two dinucleotide binding motifs as well as two apolar/aromatic regions. Site-directed mutagenesis showed that these domains may be involved in the binding of NADH and FAD and ubiquinone, respectively.

Keywords: Alternative NADH dehydrogenase; *Yarrowia lipolytica*; Site-directed mutagenesis

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G.10. The NADH:menaquinone oxidoreductase from the thermohalophilic bacterium *Rhodothermus marinus*

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Rhodothermus marinus is a strict aerobic thermohalophilic bacterium which possesses an unusual respiratory chain: besides a succinate:menaquinone oxidoreductase [1], *R. marinus* has a unique complex III [2] and a caa_3 oxidase with properties different from the oxidases of purple bacteria [3,4]. *R. marinus* also possesses a rotenone-sensitive NADH:menaquinone oxidoreductase (Complex I or NDH-I), which purification, biochemical and spectroscopic characterization is reported here.

The *R. marinus* Complex I was purified without loss of its integrity. Three of its subunits react with antibodies against 78, 51 and 21.3c kDa subunits of *Neurospora crassa* Complex I. NADH dehydrogenase activity is maximum at 50 °C and pH 8.1 and the k_M for NADH is 9 μM. The enzyme also displays NADH:quinone oxidoreductase activity with two menaquinone analogues, 1,4-naphtoquinone and 2,3-dimethyl-1,4-naphtoquinone. The last one is rotenone-sensitive and, when incorporated in liposomes, it is stimulated by dissipation of the membrane potential. The purified complex contains 13.5 ± 3.5 iron atoms and ~ 3.7 menaquinone per FMN. At least two $[2Fe-2S]^{2+/1+}$ and three $[4Fe-4S]^{2+/1+}$ centres are observed by EPR spectroscopy. By fluorescence spectroscopy, a still unidentified chromophore was detected in the purified protein.

Keywords: *Rhodothermus marinus*; Complex I; Menaquinone; Rotenone

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G.11. ATR-FTIR difference spectroscopy of mitochondrial Complex I from beef heart and *Yarrowia lipolytica*

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We have used attenuated total reflection (ATR) FTIR perfusion-induced difference spectroscopy to investigate wild-type mitochondrial Complex I (NADH:ubiquinone oxidoreductase) from beef heart and the yeast *Yarrowia lipolytica*. A signal at 1745 cm^{-1} and 1751 cm^{-1} in the bovine and yeast enzymes, respectively, has been tentatively assigned to a redox-linked carboxylic acid, which may be associated with iron–sulfur centre N2. ATR-FTIR analysis of the *Y. lipolytica* Complex I D99N and D115N (PSST subunit) site-directed mutants [1] is in progress and results will be compared to the wild-type enzyme. We have also obtained ATR-FTIR difference spectra of well-defined subfragments [2,3] of wild-type bovine Complex I. Redox difference spectra of the 1-lambda fragment (corresponding to the hydrophilic extramembranous domain), 1-alpha (consisting of the 1-lambda fragment and assorted membranous subunits) and 1-beta fragments (hydrophobic membranous components) will be presented and discussed. Additionally, we have investigated the 300–400 cm^{-1} spectral region of wild-type bovine Complex I and compared it to that of parsley ferredoxin, allowing tentative identification of Fe–S modes.

Keywords: Complex I; FTIR spectroscopy; *Yarrowia lipolytica*; Iron–sulfur protein

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G.12. Identification of a tyrosine as part of the proton pathway coupled with the redox reaction of cluster N2

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The proton-pumping NADH:ubiquinone oxidoreductase couples the transfer of electrons from NADH to ubiquinone with the translocation of protons across the membrane. The *Escherichia coli* complex I is made up of 13 subunits named NuoA to N. The FeS cluster N2 is located on NuoB and its redox reaction is most likely coupled with proton translocation across the membrane [1]. Electrochemically induced FT-IR difference spectra of complex I from *E. coli* have shown that the redox reaction of cluster N2 is coupled with the protonation/deprotonation of a tyrosine [2]. We changed three conserved tyrosines of NuoB to cysteines by means of site-directed mutagenesis. Though the mutations did not alter the electron transport activity of complex I in the membrane, the EPR signal of cluster N2 was shifted to a lower magnetic field in the complex I isolated from all three mutants. Electrochemically induced FT-IR difference spectra revealed that the tyrosine signal was diminished in one mutant while it did not significantly change in the other mutants. These data indicated that this tyrosine is involved in the proton-transfer reaction coupled with the redox reaction of cluster N2.

Keywords: Complex I; NADH:ubiquinone oxidoreductase; *Escherichia coli*; FT-IR spectroscopy; FeS cluster N2; Proton pathway

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G.13. Proton-translocating activity of NADH:ubiquinone oxidoreductase from *Rhodobacter capsulatus*

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NADH:ubiquinone reductase (Complex I) catalyzes the reduction of ubiquinone by NADH coupled with proton translocation across the membrane. To understand the coupling mechanism, it is essential to know an exact $H^+/2e^-$ stoichiometric ratio. Sub-bacterial particles from aerobically grown *Rhodobacter capsulatus* were capable of fast uncoupler-sensitive inside-directed proton translocation when a certain amount of NADH was oxidized by excess of exogenous ubiquinone analogues. Alkalinization of the external medium and acidification of the internal space of particles during NADH oxidation by ubiquinone was demonstrated as in Ref. [1]. By applying different methods [1,2] the $H^+/2e^-$ stoichiometry for the NADH to quinone region of *R. capsulatus* was determined as $2H^+/2e^-$. Type II NADH dehydrogenase was not present in our preparations, thus we conclude that transfer of two electrons in *R. capsulatus* Complex I is coupled with translocation of two protons, which is different from the stoichiometry previously observed in mammalian enzyme ($4H^+/2e^-$ [1,3]). In the presence of rotenone, the particles were able to oxidize added NADH with relatively high rates but proton translocation was completely prevented. Hence, in *R. capsulatus* Complex I, the mechanism of interaction with exogenous ubiquinone homologues and/or localization of the internal proton-translocating machinery is distinct from that of the mammalian enzyme [1,4].

Keywords: NADH:ubiquinone reductase; Complex I; Energy transduction; Proton pumping; *Rhodobacter capsulatus*

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G.14. Two aspartic acid residues in subunit PSST are essential for catalytic activity in complex I of *Yarrowia lipolytica*

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Complex I catalyzes electron-transfer from NADH to ubiquinone and translocates protons across the inner mitochondrial membrane. The PSST subunit most likely carries iron–sulfur cluster N2 which has been proposed to play a crucial role in ubiquinone reduction and proton pumping [1,2]. To explore the function of this subunit, we have generated site-directed mutants of all highly conserved acidic residues in the PSST subunit of *Yarrowia lipolytica* [3].

Mutation D99N has only 5% of the wild-type catalytic activity and D115N only 8%. In both cases complex I is stably assembled and no significant change in the EPR spectra of cluster N2 or other iron–sulfur centers was observed. Almost identical results were obtained if the aspartates were changed to glutamate.

In our structural model of the PSST and 49 kDa subunits, based on homology to known X-ray structures of [Ni–Fe] hydrogenases [4], the two residues are located not far from cluster N2 at the interface between the two subunits. Remarkably, both are close to D458 of the 49 kDa subunit. Mutations at this position exhibit pronounced resistance to hydrophobic inhibitors of complex I [5]. We suggest that D99 and D115 play a critical role in the catalytic mechanism of complex I.

Keywords: Complex I; PSST subunit; *Yarrowia lipolytica*

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G.15. Sodium cycle mediates energy coupling between complex I and ATP synthase

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The oxidation of NADH by complex I generates a proton motive force that drives the synthesis of ATP by the F_1F_0 ATP synthase. Here we demonstrate that these two complexes, co-reconstituted into proteoliposomes, are fully coupled via an electrochemical gradient. Using bacterial homologues of complex I and the F_1F_0 ATPase that transport sodium ions rather than protons, a sodium cycle is established that drives the synthesis of ATP in the course of NADH oxidation. We also demonstrate for the first time the reversal of complex I activity, namely the ATP-driven reduction of NAD by ubiquinol, with purified and reconstituted complex I. From kinetic and transport studies with these co-reconstituted vesicles, we conclude: (i) No Q cycle-type of mechanism is operative in complex I, since the Na^+-transporting, enterobacterial complex I does not translocate protons in addition to Na^+. (ii) The reduction of NAD by complex I (reverse electron transfer) is obligatorily dependent on the transmembrane voltage, but not on the concentration difference of Na^+ ions. We therefore propose that the generation of an electrical potential by complex I is accomplished by the transport of H^+ or Na^+ from the negatively to the positively charged side of the membrane.

Keywords: Na^+-translocating complex I/F_1F_0 ATP synthase/reverse electron transfer

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G.16. FT-IR spectroscopic characterization of the quinone binding site of the NADH:ubiquinone oxidoreductase (complex I) from *Escherichia coli*

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The NADH:ubiquinone oxidoreductase, also known as respiratory complex I, couples the transfer of electrons from NADH to ubiquinone with the translocation of protons across the membrane.

While the binding site for NADH and most of the cofactors have been located, the quinone binding site and the amino acids involved in binding of the quinone are still not known.

FT-IR difference spectroscopy was used to search for possible binding partners involved in the binding of both possible quinones, ubiquinone and menaquinone.

Electrochemically induced FT-IR difference spectra of the isolated complex devoid of any quinone and in the presence of externally added ubiquinone and menaquinone were obtained.

The double difference spectra include the vibrational modes of the quinones bound to the protein in the oxidized and in the reduced and fully protonated form.

For assignment of the vibrational modes of the protein-bound quinone, the data were compared to data from quinone in aqueous solution.

The most interesting part of the spectra include modes reflecting alterations on the protein site upon the quinone binding.

Tentative assignments to individual amino acids like aspartic or glutamic acids as well as arginines are presented and discussed as binding partners for quinone binding and concomitant proton transfer.

Keywords: Complex I; *E. coli*; FTIR; protein electrochemistry; quinones

G.17. Mitochondrial NADH:ubiquinone oxidoreductase (complex I)

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Complex I (NADH:ubiquinone oxidoreductase) is the largest, and least understood member of the respiratory electron transport chain. Mitochondrial complex I is composed of at least 43 different polypeptides, and probably contains eight iron–sulfur clusters, in addition to an FMN active site where NADH is reduced, and one or more bound quinones. The Complex I Group in Cambridge has adopted a number of approaches to further our understanding of both the structure and function of mitochondrial complex I. Recent new results include the following:

–a complete definition of the subunit composition of this multisubunit system is now in sight. In particular, a new subunit, B16.6, has been identified and found to be a direct homolog of 'GRIM-19', the product of a cell-death regulatory gene induced by interferon-β and retinoic acid [1].

–adopting the 'component' approach, we have begun to characterise the redox characteristics of the individual iron–sulfur clusters of complex I, using protein-film voltammetry [2]. Interestingly, the 24 kDa subunit, close to the FMN-active site, contains a [2Fe–2S] cluster with unusual pH- and ionic-strength dependence.

This poster will discuss selected new results on mitochondrial complex I from this group.

Keywords: Complex I; NADH:ubiquinone oxidoreductase; Protein–film voltammetry

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G.18. Cysteine scanning of helices 3, 9, 13 and 14 of domain II in *E. coli* transhydrogenase

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Although a topology model of proton-translocating *E. coli* transhydrogenase with 13 transmembrane helices has been established [1], the helix packing of membrane domain, dII, remains unknown. Four of the 13 helices, i.e. helices 3, 9, 13 and 14, have conserved amino acids to a high extent. These helices are thought to be involved in the formation of a channel in dII, through which a proton is transported in the redox reaction between NAD(H) in domain I, dI, and NADP(H) in domain III, dIII [2]. In order to determine the helix packing of dII and the function of the individual helices 3, 9, 13 and 14, cysteines were introduced in the cysteine-free transhydrogenase. All mutants made in helix 3, i.e. βS255C, βT257C, βN258C, βA259C, βI260C, βS261C and βG262C, were combined with βH91C in helix 9. The double mutants were studied with cross-linking and activity measurements. Helix 13 and 14 were cysteine scanned from βP215C–βS260C. The activity measurements and cross-linking in the absence and presence of NADPH indicated a conformational communication between dII and dIII. The accessibility of cysteine residues introduced in dII to cysteine specific probes and cross-linking with βH91C provided indications for the organization of the proton channel.

Keywords: Transhydrogenase; Cysteine scanning; Membrane protein

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G.19. *Yarrowia lipolytica*, a yeast genetic system to study mitochondrial complex I

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The obligate aerobic yeast *Yarrowia lipolytica* is a powerful new model for the structural and functional analysis of mitochondrial complex I. The respiratory chain of *Y. lipolytica* contains complexes I–IV, one single "alternative" NADH-dehydrogenase (NDH2) and a non-heme alternative oxidase (AOX). Because the NADH binding site of NDH2 faces the mitochondrial intermembrane space rather than the matrix, complex I is essential in *Y. lipolytica*. Nevertheless, complex I deletion strains could be generated after redirecting NDH2 to the matrix side by N-terminally attaching the targeting sequence of a matrix protein. To allow fast and efficient purification of complex I by affinity-chromatography, a hexa-histidine tag was C-terminally attached to the NUGM (30 kDa) subunit and the his-tagged allele integrated into the genome by homologous replacement with the resident gene copy. The purified complex I has lost most of its NADH:ubiquinone oxidoreductase activity, but is almost fully reactivated by adding 400–500 molecules of phosphatidylcholine per complex I. The established set of genetic tools has proven useful for the site-directed mutagenesis of individual subunits of *Y. lipolytica* complex I. Characterization of a number of mutations already allowed for the identification of several functionally important amino acids demonstrating the usefulness of this approach.

Keywords: *Yarrowia lipolytica*; Complex I; Alternative NADH:ubiquinone oxidoreductase; Alternative NADH dehydrogenase; Alternative oxidase

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G.20. Studying the importance and role of two strictly conserved Glu residues within trans-membrane helices of the nuoK subunit (ND4L equivalent) of *E. coli* NDH-1 by site-directed mutagenesis

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Complex I is the largest and least understood of the mitochondrial respiratory complexes. The bacterial homologue, NDH-1, is more amenable to studies on specific residues within this multi-subunit complex using genetic manipulations, and we have used the enzyme from *E. coli* for such studies. We have noticed that its nuoK subunit (mitochondrial ND4L subunit counterpart) contains two strictly conserved acidic residues predicted to reside in middle of two putative trans-membrane helices [1]. Such conserved residues within the membrane domain of NDH-1 may play an important role in the enzyme's function, and we set up to study this by site-directed mutagenesis. Two subunits, nuoJ and nuoK were thus deleted from the bacterial genome and replaced with streptomycin-resistance gene. The deletion was then complemented by wild-type genes that also contain a His-tag, and later by genes with several different point mutations. The effects of mutations on growth rate in different media were analyzed. In addition, activities of the membrane-bound enzyme were assayed directly using different electron acceptors. The results indicate that the presence of two acidic residues are indeed essential in this domain, but some changes in their exact nature (Glu vs. Asp) and location may be accommodated by the enzyme.

Keywords: NADH–ubiquinone oxidoreductase; Proton pumping; Membrane domain

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G.21. Bioenergetic consequences of homoplasmic LHON mutations

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In the present report, we investigated the bioenergetic consequences of three different homoplasmic Leber's hereditary optic neuropathy (LHON) mutations affecting the mitochondrial-encoded subunits of NADH:CoQ oxidoreductase ND2 (C4640A [1]), ND4 (G11778A [2]) and ND6 (T14484C [3]) for human skeletal muscle and brain (for the ND2 mutation only). All mutations caused in patients and non-affected carriers of the mutation a similar decrease of citrate synthase-normalized activities of complex I being most severe for the ND6, less severe ND4 and close to the detection limit for the ND2 mutation. This enzyme activity change was observed to be responsible for decreased respiration rates with NAD-dependent substrates detected in saponin-permeabilized muscle fibers, isolated skeletal muscle mitochondria and digitonin-treated parahippocampal homogenates (for the ND2 mutation).

Titrations of the activity of NADH:CoQ oxidoreductase with the complex I inhibitors amytal, rotenone and piericidin A revealed with all mutations no difference to controls indicating no alteration of kinetic properties of the CoQ reduction site by any of the investigated mutations. Since all mutations led to a considerable increase of amytal sensitivity of mitochondrial respiration our data are compatible with the concept that the investigated mutations lead to decreased quantities of the active NADH:CoQ oxidoreductase enzyme complex.

Keywords: NADH:CoQ oxidoreductase; Leber's hereditary optic neuropathy; ND2 mtDNA mutation; ND4 mtDNA mutation; ND6 mtDNA mutation; Amytal; Rotenone; Piericidin A

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G.22. Investigation of the quinone binding site in Complex I of *Escherichia coli*

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Very little is known about the location and number of quinone binding sites in complex I and subunits ND1 [1], ND4 and ND5 [2], PSST and TYKY [3] and 49 kDa [4] have been suggested as possible locations. It is possible that the quinone-binding site is shared between some of these subunits. Kinetic assays, measuring complex I activity, have been used to study new and known complex I inhibitors and inhibition by *decyl*-ubiquinol in strain ANN001 (ndh2$^-$). Inhibitors of complex I have been shown to act as mixed inhibitors while *decyl*-ubiquinol is a competitive inhibitor. Attempts are being made to create inhibitor-resistant mutants in *E. coli* and the model organism *Chlamydomonas reinhardtii* by growth on media containing complex I inhibitors. We are currently making mutations in the ND4 and 5 subunits of complex I in ANN001. Inhibitor assays will then be used to characterise the effects of these mutations. The results of these experiments will be presented.

Keywords: Complex I; *Escherichia coli*; Quinone binding site

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G.23. Complex I studied by pulsed EPR spectroscopy

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The proton-pumping NADH:ubiquinone oxidoreductase is the initial complex (Complex I) of the respiratory chain of many bacteria and also of mitochondria from most eukaryotes. It couples the transfer of electrons (from NADH to ubiquinone) with the concomitant translocation of protons across the membrane.

One flavin monucleotide (FMN), up to nine iron–sulfur (FeS) clusters and up to three quinones participate in this redox chain and it is generally accepted that the immediate electron donor to ubiquinone is a Fe_4S_4 cluster known as N2 [1]. Very little is known about the function, location and direct ligation of cluster N2. Recent comparisons with hydrogenases have suggested that a histidine may form its fourth ligand to the protein backbone [2].

A pulsed-electron paramagnetic resonance (EPR) study to characterise several of these paramagnetic centres is presented dealing with

(i) the selective study of cluster N2 with respect to the whole EPR signal using T_1-selected EPR;
(ii) the ligation-sphere of cluster N2 using electron spin envelop echo modulation (ESEEM) spectroscopy;
(iii) the characterisation of semiquinone radicals arising in Complex I.

Keywords: Iron–sulfur; Quinone; Pulsed-EPR spectroscopy; ESEEM; Relaxation

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G.24. Active/de-active transition of respiratory complex I in bacteria, fungi and animals

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Mammalian complex I exists as a mixture of interconvertible active (A) and de-active (D) forms. The A-form is capable of NADH:quinone-reductase catalysis, while the D-form is unable to catalyze quinone reduction [1]. Complex I from *Paracoccus denitrificans* exists only in the A-form [2]. This bacterial complex I consists of 17 subunits and contains FMN and 6–8 [Fe–S] centers, while bovine heart complex I has 26 additional subunits. The question remains how the structural complexity of complex I of organisms from different evolutionary levels correlates with its ability to undergo the A/D transition. To distinguish A and D forms of the enzyme, NADH:quinone-reductase activity was assayed in bacterial and mitochondrial membranes. We have shown that complex I from *Escherichia coli* and nonvertebrate organisms (ringworm, lobster, cricket) do not show the A/D transitions. The vertebrate organisms (carp, frog, chicken) exhibit the A/D transition similar to well characterized beef complex I. *Neurospora crassa* and *Yarrowia lypolytica* enzymes showed a very distinguished A/D transition with much lower activation barriers for the transitions compared to beef enzyme. We discuss possible roles of the A/D transition of complex I in the molecular mechanism and in regulation of enzymatic activity.

Keywords: NADH:Q oxidoreductase; Complex I; Respiration; Activation; Regulation

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G.25. The antiporter-like subunits of respiratory Complex I

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Respiratory NADH:quinone oxidoreductase (Complex I) contains three large membrane spanning subunits, denoted ND5/NuoL, ND4/NuoM and ND2/NuoN in mitochondrial and bacterial enzyme, respectively. These subunits show great primary sequence similarity to one class of Na^+/H^+-antiporters. This makes these subunits prime candidates for providing the H^+ channels for the Complex I H^+ pumping machinery. We have determined the transmembrane topology of the NuoL subunit and built a 2-D structural model for the protein family [1]. Phylogenetic analyses of primary sequences in the family indicate that the three homologous Complex I subunits could have different roles in the Complex I functional mechanism. The bona fide antiporters of this class diverge into two distinct groups, MrpA-type and MrpD-type antiporters, where ND5 polypeptides group with MrpA antiporters and ND4/ND2 polypeptides group with MrpD antiporters. This suggests that ND5 may be functionally related to MrpA, and ND4/ND2 to MrpD. To experimentally test this prediction, we have created two *Bacillus subtilis* deletion strains lacking MrpA and MrpD, respectively, with distinct pH and salt-sensitive growth phenotypes, similar to the *B. subtilis* strains described in Ref. [2]. These strains are exploited to investigate the function of the homologous Complex I subunits.

Keywords: NADH:quinone oxidoreductase; Complex I; ND5; NuoL; ND4; NuoM; ND2; NuoN; Antiporter; Energy coupling; H^+ pumping

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G.26. A single subunit NADH–quinone oxidoreductase as the rescue molecule for neurodegenerative diseases with complex I deficiency

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Abnormalities of mitochondrial function, and NADH–ubiquinone oxidoreductase (complex I) in particular, have been linked to neurodegenerative diseases with the most notable case being Parkinson's disease [1]. Mitochondria of *Saccharomyces cerevisiae* contain a single-subunit NADH–quinone oxidoreductase (Ndi1). We have previously shown that using a recombinant adeno-associated virus vector carrying the *NDI1*-gene (rAAV-NDI1), the *NDI1* gene is capable of compensating deficiencies caused by complex I defects in the host cells [2 3 4]. It seems likely that the *NDI1* gene provides a potentially useful tool for treatment, in the form of gene therapy, for neurodegenerative diseases associated with complex I defects. As a first step, we attempted functional expression of the *NDI1* gene in nerve cell lines, rat PC12 and mouse MN9D using rAAV-NDI1. It was shown that the *NDI1* gene was successfully introduced into the neuronal cells in vitro and the expressed Ndi1 protein protected cells from inhibitory effects of complex I inhibitors such as rotenone and pyridaben. In addition, rAAV-NDI1-infected cells were able to differentiate morphologically as characterized by neurite formation. Also, the differentiated cells could be infected with rAAV-NDI1 and the expression of the Ndi1 protein was confirmed both in the cell body and in neurites.

Keywords: Complex I defect; Neurodegenerative disease; Gene therapy

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G.27. Hybrid ubiquinone; novel mitochondrial complex I inhibitor

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We synthesized novel ubiquinone analogs by hybridizing natural ubiquinone ring (2,3-dimethoxy-5-methyl-1,4-benzoquinone) and hydrophobic phenoxybenzamide unit, named hybrid ubiquinone. The hybrid ubiquinones worked as electron transfer substrates with bovine heart mitochondrial complexes II and III, but not with complex I. With complex I, they rather worked as inhibitors in a noncompetitive manner against short chain ubiquinones irrespective of the presence of natural ubiquinone ring. Structure/activity study showed that high structural specificity of the phenoxybenzamide moiety is required to act as an inhibitor of complex I. These findings indicate that binding of the hybrid ubiquinones to complex I is mainly decided by the interaction of phenoxybenzamide moiety to the enzyme. It is noteworthy that analogous bulky and hydrophobic substructure can be commonly realized in recently announced synthetic pesticides action site of which is complex I. The present study supports the recently proposed idea that the inhibitor binding domain in bovine complex I is comprised of large hydrophobic cavities.

Keywords: Complex-I; Ubiquinone; Respiratory inhibitor

G.28. EPR and thermodynamic characterization of multi-component semiquinone species in bovine heart complex I in coupled SMP and in the isolated enzyme

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Complex I contains one FMN and eight iron–sulfur clusters as intrinsic redox components in its promontory part [1]. Ubiquinone is most likely the key player in the proton pumping reactions in the membrane-spanning domain. Three distinct semiquinone species have been spectrally resolved in the NADH–Q oxidoreductase (complex I) segment of the respiratory chain in the tightly coupled bovine heart submitochondrial particles [2,3]. Their physico-chemical properties differ considerably, specifically in their spin relaxation behavior, allowing them to be designated as SQ_{Nf} (fast), SQ_{Ns} (slow), SQ_{Nx} (very slow) species. We found that SQ_{Nf} is highly sensitive to $\Delta\mu_{H^+}$ while the other two species are not. Thermodynamic parameters of these SQ species in the mitochondrial membrane system could not be accurately determined. However, using isolated bovine heart complex I [4,5], we have conducted potentiometric redox titrations of the $\Delta\mu_{H^+}$ insensitive slow relaxing SQ species. Partially overlapping two SQ titration curves were obtained. The major component gave an E_m value in the range of +40 to +90 mV with a "Q_i" and "Q_B" type intermediate K_{stab} value. We will extend this work for other SQ species, and their functional roles will be discussed.

Keywords: Complex I; Bovine heart; Semiquinone; Thermodynamic property

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G.29. Redox-sensitive loops D and E regulate NADP(H) binding in domain III and domain I–domain III interactions in proton-translocating *Escherichia coli* transhydrogenase

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In order to examine the roles of loop D and E in domain III from membrane-bound *E. coli* transhydrogenase, conserved residues in loop D and E were selected for cysteine mutagenesis. Mutants βA398C, βS404C, βI406C, βG408C, βM409C and βV411C in loop D and βY431C in loop E were constructed. Only the βI406C and βY431C mutants exhibited significantly altered forward and reverse reaction rates, which was confirmed by increased k_{off} NADP(H) values for those mutants. All mutants, except βS404C, exhibited a lowered affinity for *R. rubrum* domain I, and the βI406C-βV411C and βY431C mutants all displayed markedly decreased hydride transfer rates. Incubation of βI406C with the thiol-reactive probes NEM and MIANS gave wild-type reverse activity, indicating a change of loop D conformation. That loop D is redox-sensitive was demonstrated by the different results obtained when $NADP^+$ or NADPH was added to the MIANS-modified mutants. $NADP^+$ proved to be a slow but effective quencher of MIANS fluorescence, whereas NADPH produced only minor quenching. NAD(H) did not produce any effect. Along with previously constructed mutants in these loops, the results indicate an important regulatory role for loops D and E in both NADP(H) binding as well as in domain III–domain I interactions.

Keywords: Transhydrogenase; NADP; Proton pump; Membrane protein

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G.30. Isolation and characterization of NADH:ubiquinone oxidoreductase (complex I) from the hyperthermophilic eubacterium *Aquifex aeolicus*

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Knowledge of the complete genome sequence of *Aquifex aeolicus*, a hyperthermophilic eubacterium that diverged early in evolution, provides an opportunity to understand adaptation to extreme environmental conditions. A proton-translocating NADH:ubiquinone oxidoreductase (complex I) has been purified from *A. aeolicus* by extraction of the enzyme from the membrane with dodecyl-β-D-maltoside, followed by anion exchange chromatography and gel filtration. The activity of the purified enzyme is readily detectable above 50 °C. A high specific activity of 29 U/mg for electron transfer from NADH to decylubiquinone is observed at 80 °C. The activity shows a linear Arrhenius plot at 50–85 °C (activation energy at 22.05 kJ/mol). The *A. aeolicus* complex I appears completely sensitive to rotenone and 2-*n*-Decyl-Quinazolin-4-yl-Amine (DQA). SDS-PAGE shows that it may contain up to 14 subunits. The isolated enzyme complex is stable and highly active in a temperature range from 50 to 90 °C, with a half-life at 80 °C of about 10 h. Gel filtration indicates that the enzyme complex is stable between pH 4.5 and 9.0. Single particle electron microscopy shows that the *A. aeolicus* complex I has the typical L-shape which has been observed for other species. A possible relationship between the high activity of the enzyme and the properties of *A. aeolicus* membranes is discussed.

Keywords: NADH:ubiquinone oxidoreductase; Complex I; Electron microscopy; *Aquifex aeolicus*; Rotenone sensitivity

G.31. Complex I assembly in mutants of *Chlamydomonas reinhardtii* defective in mitochondria-encoded ND1, ND4, ND5 and ND6 subunits

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In *Chlamydomonas*, only five subunits (ND1, ND2, ND4, ND5 and ND6) of complex I are encoded by the mitochondrial genome. The impact of mutations that affect four of these genes and that lead to inactivation or drastic reduction of complex I activity has been investigated. Solubilized mitochondrial complexes were separated by blue-native PAGE or sucrose gradient centrifugation. Complex I was identified by NADH:NBT and NADH:$K_3Fe(CN)_6$ dehydrogenase activity and by immunodetection. We found that (1) the absence of ND1 or ND6 prevents the assembly of the 850 kDa complex I; (2) the decrease of *nd5* transcript reduces the amount of assembled complex I; (3) the loss of ND4 or ND4/ND5 leads to the formation in reduced amount of a 650 kDa subcomplex; (4) the loss of amino acid residues 199–200 from ND1 does not prevent a correct assembly. Moreover, a soluble 160–210 kDa fragment comprising the hydrophilic 49 and 76 kDa subunits of the complex I peripheral arm and retaining NADH dehydrogenase activity is assembled in all the strains analyzed. Implications of our findings for the possible role of the four ND subunits on the activity of complex I and on the structural organization of the membrane arm are discussed.

Keywords: Complex I; ND subunit; Mitochondrial mutant; *Chlamydomonas*

G.32. Photo-labelling of *Rhodobacter capsulatus* NADH:ubiquinone oxidoreductase with azidoquinones

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NADH:ubiquinone reductase (Complex I) catalyses the reduction of ubiquinone by NADH coupled with proton translocation across the membrane. To understand the coupling mechanism, it is essential to know the number and exact location of quinone binding sites participating in the reaction. To enhance the possibility of labeling all quinone binding sites in the enzyme, we have synthesized azidoquinones (2-methyl-3-azido-5,6-dimetoxy-1,4-benzoquinone and 2-methyl-3-(5-azidopentyl)-5,6-dimetoxy-1,4-benzoquinone) closely resembling the soluble ubiquinone analogues Q_0 and Q_1. Both azidoquinones function as substrates for Complex I; the former with an apparent k_m of 51 μM (compared to 105 μM for Q_0) and the latter with very similar apparent k_m as Q_1 (15–20 μM). In the presence of azidoquinone, the membrane-bound Complex I is inactivated upon UV-illumination in a light- and time-dependent reaction following first order kinetics. The rotenone and piericidin A sensitivity of the two azidoquinone reductase reactions is also similar to those of the corresponding Q_0 and Q_1 reductase reactions, allowing us to study the protective effect of inhibitors during labeling. Thus, these azidoquinone compounds are suitable for identifying quinone binding—and inhibitor binding—sites in Complex I.

G.33. Structural characterisation of complex I from *E. coli*

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We use bacterial complex I as a 'minimal' model of mammalian complex I of respiratory chains (NADH:ubiquinone oxidoreductase). A modified [1,2] procedure for purification of complex I from *E. coli* (BL21) has been developed. Highly pure and monodisperse enzymes can be obtained in large amounts, suitable for structural studies. Electron micrographs show the familiar L-shape of the complex under a variety of conditions. Two different forms of two-dimensional crystals, with p2 and p3 symmetry, were obtained. Imaging in negative stain indicates that crystals are likely to contain intact complex I and thus provide a good starting point for structure determination by electron cryo-crystallography.

Keywords: Complex I; Respiratory chain; Two-dimensional crystallisation; Electron microscopy

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G.34. The crystal structure and reaction kinetics of the dIII component of human proton-translocating transhydrogenase in complex with the analogue thio-NADP$^+$

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Transhydrogenase is found in the inner mitochondrial membrane of mammals and the cytoplasmic membrane of bacteria. It has a tridomain structure: dI contains the binding site for NAD(H), dIII binds NADP(H) and dII spans the membrane. Transhydrogenase couples proton translocation across the membrane with hydride-ion transfer between the NC4 position of NAD(H) and the NC4 position of NADP(H):

$$\mathrm{NADH} + \mathrm{NADP}^+ + \mathrm{H}^+(\mathrm{out}) \Leftrightarrow \mathrm{NAD}^+ + \mathrm{NADPH} + \mathrm{H}^+(\mathrm{in})$$

Thio-NADP$^+$ is used in the study of many nucleotide-binding enzymes as an analogue of NADP$^+$, though its redox potential is 40 mV higher and the H-bonding properties of the pyridine ring substituent are altered. Here we present the crystal structure of dIII-thioNADP$^+$ solved to 2.4 Å resolution with an R-factor of 22%. The most prominent difference between this structure and that of human dIII-NADP$^+$ [1] is observed in the nucleotide conformation: the 3′-carbothiamide group in thioNADP$^+$ is twisted by 25° relative to the pyridine plane. Despite this, dIII-thioNADP$^+$ has similar kinetic characteristics to dIII-NADP$^+$. The implications of these findings for the nature of the redox reaction catalysed by transhydrogenase will be discussed.

Keywords: Transhydrogenase; X-ray structure; Mitochondria; Thionicotinamide adenine dinucleotide (thio-NADP$^+$)

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G.35. Properties of a transhydrogenase with a C-terminal plasma membrane Ca-ATPase tag and an N-terminal his-tag purified by affinity chromatography

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In order to develop a single chromatography step purification procedure for *E. coli* transhydrogenase, a calmodulin-binding peptide from human plasma membrane Ca^{2+}-ATPase was fused C-terminally to the beta subunit of transhydrogenase. A construct with a fusion peptide of 30 amino acids (pHEK1) was not expressed. However, extending the peptide to 150 amino acids (pHEG1) gave a good expression. The pHEG1 construct was purified by affinity chromatography on calmodulin–Sepharose and eluted with EGTA. The specific activity of the purified protein was similar to that of wild-type transhydrogenase. In wild-type the amount of alpha and beta subunits are equal. However, HEG1 contained a higher amount of alpha relative to beta. In order to investigate if the distribution between the subunits was affected by the purification procedure, a construct, pAKT1, with an N-terminal his-tag on the alpha subunit was made based on pHEG1. pAKT1 was successfully expressed and purified by immobilized metal (Ni^{2+}) affinity chromatography. Characterization of AKT1 gave the same results as for pHEG. The altered subunit distribution compared to the wild-type is hence an effect of expression rather than an effect of the purification process.

Keywords: Transhydrogenase; NADP; Affinity purification

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G.36. Substitution of Gln132 with Asn in the NAD(H)-binding component of proton-translocating transhydrogenase leads to inhibition of hydride transfer and to symmetry changes in the X-ray structure

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Transhydrogenase, from bacterial and mitochondrial membranes, couples the redox reaction between NAD(H) and NADP(H) to proton translocation (reviewed in Ref. [1]). It comprises three components: dI, which binds NAD(H), and dIII, which binds NADP(H), protrude from the membrane, whereas dII spans the membrane. The crystal structure of a complex formed from a mixture of recombinant dI and dIII from *Rhodospirillum rubrum* transhydrogenase is profoundly asymmetric [2], indicating that, in the complete enzyme, the catalytic sites alternate during turnover. The conformation of invariant Gln132 differs in the two subunits of dI in the complex. It was suggested that the sidechain of Gln132 participates in the mutual positioning of the nicotinamide rings of NAD(H) and NADP(H) at the hydride-transfer step. We have mutated Gln132 to Asn in isolated dI. The mutant protein binds NADH normally and it readily forms a tight complex with isolated dIII. However, the apparent first-order rate constant for hydride transfer between nucleotides in the complex is greatly decreased relative to wild-type and the asymmetries in its crystal structure are altered. The role of Gln132 in regulating the nucleotide conformation before and after the hydride transfer step to permit efficient coupling of transhydrogenase to proton translocation are explained.

Keywords: Transhydrogenase; Proton-translocation; X-ray structure; Hydride transfer; Nucleotide binding

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G.37. Preparation of partially purified Complex I from *E. coli* with stable quinone reductase activity

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Complex I from *E. coli* (NDH-1) purified by means of the commonly used nonionic detergent, dodecyl maltoside, has low ubiquinone reductase activity in comparison with the artificial hexammineruthenium reductase activity [1]. With the use of two different zwitterionic detergents, SB12 and CHAPS, a preparation of partially purified NDH-1 was obtained. This preparation showed high and stable NADH::ubiquinone 1 (decylubiquinone, ubiquinone 2) oxidoreductase activity. The purification of NDH-1 on sucrose gradient demonstrated that the preparation with high ubiquinone reductase activity was co-purified with bo_3 oxidase. Further purification of NDH-1 with the use of ion exchange chromatography resulted in loss of ubiquinone reductase and bo_3 activities but not of hexammineruthenium reductase. We conclude that a high quinone reductase activity of solubilized Complex I may be obtained only by operation of NDH-1 in supercomplexes with other membrane proteins.

Keywords: NDH-1; *E. coli*; Quinone reductase

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G.38. Exploring roles of membrane domain in the proton-translocating NADH–quinone oxidoreductase

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Fenpyroximate is a potent inhibitor of the proton-translocating NADH–quinone oxidoreductase of bacteria and mammals (complex I) [1]. We synthesized a photoaffinity analogue of fenpyroximate, [^{3}H](trifluoromethyl)diazirinylfenpyroximate. In the presence of NAD(P)H, the analogue exclusively labeled a Mr = 50,000 band of bovine submitochondrial particles (SMP) upon UV-illumination. It was shown, by blue native gel analyses, that the modified band belonged to complex I. The p*I* of the labeled band was approximately 10 similar to that of the ND5 subunit (10.01). These results suggested that the target may be the ND5 subunit. This was confirmed by immunoblotting experiments with the antibody against a C-terminal oligopeptide of the bovine ND5 subunit. In the range up to 10 nM, the labeling was parallel with inactivation of the NADH oxidase. The labeling was completely displaced by known complex I inhibitors (such as rotenone, piericidin, pyridaben, capsaicin) and Na^+/H^+ antiporter inhibitors. It should be noted that the Na^+/H^+ antiporter inhibitors inactivate the NADH oxidase activity of bovine SMP. It has been reported that the PSST, ND1, and 49 kDa subunits constitute the inhibitor-binding pocket(s) [2 3 4]. It seems likely that the ND5 subunit is also involved in construction of inhibitor-binding pocket(s) in complex I.

Keywords: Inhibitor; Complex I; ND5; Photoaffinity analogue

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G.39. Monoclonal antibodies for the location of individual subunits in complex I

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Complex I is the largest and least understood enzyme of the respiratory chain. Electron microscopy has revealed an L-shaped structure [1]. However, little details like individual subunits or binding sites for substrates or prosthetic groups have been assigned.

We have raised monoclonal antibodies for decoration of native complex I to map subunits and epitopes in the electron microscopy structure of complex I. The 49 kDa subunit has been shown to play a major role in the catalytic mechanism [2]. In the enzyme from *E. coli*, the N-terminus of this subunit is fused with the C-terminus of the 30 kDa subunit. We have used two monoclonal antibodies recognizing different epitopes to localize the 49 kDa subunit in the peripheral arm of complex I. In addition, we have used a commercial antibody to locate the His-tag that has been attached to the C-terminus of the 30 kDa subunit of complex I from *Yarrowia lipolytica* for affinity chromatography [3]. The implications of the position of the 49 and 30 kDa subunits for the catalytic mechanism are discussed.

Keywords: Electron microscopy; *Yarrowia lipolytica*; Ubiquinone; Epitope mapping

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G.40. Complex I of *Yarrowia lipolytica*: EPR spectroscopic characterization of redox centers and enzymatic activity

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NADH ubiquinone oxidoreductase (complex I) from *Yarrowia lipolytica* is a particularly suitable model system to study this important enzyme complex of the mitochondrial respiratory chain. Using established tools derived from yeast genetics, the organism allows application of a His-tag-based enzyme purification and site-directed mutagenesis. EPR-spectroscopic characterization of the redox centers of *Y. lipolytica* complex I revealed similar properties as the enzyme from bovine heart mitochondria or *Neurospora crassa*. In mitochondrial membranes as well as in the purified enzyme, the redox properties of iron sulfur centers of complex I were monitored by EPR spectroscopy. The special role of iron sulfur cluster N2, which is supposed to be the electron donor for the quinone, was also evident from its pH-dependent midpoint potential. The enzymatic activity of the purified complex could be stimulated about 10-fold by addition of specific lipids to the enzyme. Reconstitution of complex I into liposomes resulted in a similar increase of enzymatic activity which was sensitive to uncouplers. These complex I proteoliposomes showed an EPR-detectable radical signal having characteristic properties of semiquinone radicals. The origin of this radical and its significance for the catalytic mechanism need further investigations.

Keywords: Complex I; EPR spectroscopy; Iron sulfur cluster; Reconstitution

G.41. Proton translocation catalyzed by *Paracoccus denitrificans* NADH:quinone oxidoreductase (NDH-1)

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The stoichiometry quotient n (H^+/2e) for vectorial translocation of protons coupled with NADH:ubiquinone oxidoreduction is a key parameter for any model of energy transduction catalyzed by Complex I. The value of 4 has been determined for both rotenone-sensitive and rotenone-insensitive NADH:Q_1 oxidoreduction in tightly coupled bovine heart submitochondrial particles [1]. The experimental procedure similar to that previously used for the mitochondrial system was developed to determine n for much simpler prokaryotic *Paracoccus denitrificans* dehydrogenase operating with decyl ubiquinone as electron acceptor within the membranes of tightly coupled sub-bacterial particles [2]. The same protonophoric uncoupler-sensitive proton translocation as for the mammalian enzyme ($n = 4$) was found. Prepulsing of the enzyme with NADH under aerobic conditions (several cycles of NADH oxidase reaction) and presence of Mg^{2+} were needed to obtain maximal stoichiometry ($n = 4$). Valinomycin markedly decreased proton uptake by coupled particles and stimulated their protonmotive force-controlled NADH oxidase.

Keywords: NADH–ubiquinone oxidoreductase; Complex I; NDH-1; Energy transduction; Respiratory chain; *Paracoccus denitrificans*

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G.42. Kinetics of the spectral changes during reduction of the Na^+-motive NADH:quinone oxidoreductase from *Vibrio harveyi*

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The Na^+-translocating NADH:ubiquinone oxidoreductase (Na^+-NQR) generates an electrochemical Na^+ potential driven by aerobic respiration. Two radical signals are seen in the Na^+-NQR by means of EPR spectroscopy. The first radical was observed in the oxidised enzyme, and was assigned as a neutral flavosemiquinone. The second radical was observed in the reduced enzyme and was assigned to be the anionic form of flavosemiquinone. The time course of Na^+-NQR reduction by NADH, as monitored by stopped-flow optical spectroscopy, shows three distinct phases, the spectra of which suggest that they correspond to reduction of three different flavin species. The first phase was fast both in the presence and absence of sodium, and is assigned to reduction of FAD to $FADH_2$. The rates of the other two phases were strongly dependent on sodium concentration, and these phases were attributed to reduction of two covalently bound FMN's. It was shown that a neutral FMN flavosemiquinone species preexists in the oxidized enzyme, and that NADH reduces it to the fully reduced form. The other FMN moiety is initially oxidised, and is reduced to the anionic flavosemiquinone form. One-electron transitions of two discrete flavin species are thus assigned as sodium-dependent steps of the catalytic cycle of Na^+-NQR.

Keywords: Na^+-NADH:quinone oxidoreductase; Flavin cofactors; Na^+-pump; Respiratory enzyme; Bacteria; *Vibrio harveyi*

Reference

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G.43. A truncated NuoL (ND5) subunit of complex I (NDH I) from *E. coli* induces passive Na^+ influx

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The pathway of the coupling cation through the membranous fragment of complex I has not been identified so far. The problem is addressed using the Na^+-transporting complex I (NDH I) from *E. coli* [1]. The N-terminal segment of the NuoL (ND5) subunit from *E. coli* NDH I exhibits high sequence similarity to Na^+/H^+ antiporters. Expression of this fragment (NuoL′) in *E. coli* resulted in a NaCl-dependent growth inhibition. The presence of NuoL′ increased the rates of passive Na^+ uptake into membrane vesicles in an *E. coli* strain devoid of two Na^+/H^+ antiporters. NuoL′ was partially purified by affinity chromatography and reconstituted into proteoliposomes. Na^+ uptake along a Na^+ concentration gradient into proteoliposomes was inhibited by amiloride, but was not affected by a transmembrane potential (inside positive), indicating electroneutral Na^+ transport. A ΔpH established in native membrane vesicles containing NuoL′ was diminished by Na^+, demonstrating that the truncated NuoL′ subunit catalyzes Na^+/H^+ antiport. It is proposed that the NuoL (ND5) subunit participates in Na^+ (or H^+) transport through complex I.

Keywords: Complex I; NADH:quinone oxidoreductase; Na^+ transport; Cation pathway

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G.44. Location and function of FeS cluster N1c in *E. coli* complex I.

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The NADH:ubiquinone oxidoreductase (complex I) of *Escherichia coli* consists of 13 subunits named NuoA to NuoN. One FMN and 7 FeS clusters have so far been detected in the preparation. The cluster N1c was uniquely detected in the preparation of *E. coli* complex I and has been assigned to a binuclear cluster [1]. This cluster is most likely located on NuoG containing an additional cysteine motif for the binding of an FeS cluster. This motif is also present in NuoG from other sources like *Salmonella typhimurium, Thermus thermophilus*, and *Aquifex aeolicus* [2, 3]. It was shown that an overexpressed fragment of NuoG from *T. thermophilus* containing this additional cysteine motif contained an FeS cluster [4]. However, it was proposed to be a tetranuclear cluster. We localized cluster N1c on NuoG by means of EPR-spectroscopy of site directed mutants. To discriminate between a binuclear or tetranuclear type of cluster we measured the EPR-spectra of N1c in whole cells under aerobic and anaerobic conditions.

Keywords: Complex I, *Escherichia coli*, FeS clusters, assembly

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Session H: Cytochrome be Complexes

H.1. Crystal structure of the yeast cytochrome bc_1 complex with its bound substrate cytochrome *c*

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Small, diffusible redox proteins facilitate electron transfer in respiration and photosynthesis by alternately binding to integral membrane proteins. The mobile electron carrier cytochrome *c* shuttles electrons from the cytochrome bc_1 complex to cytochrome *c* oxidase. We present the crystal structure of the complex critical for electron transfer between cytochrome *c* and the cytochrome bc_1 complex from *Saccharomyces cerevisiae.* The complex was crystallized with the help of antibody Fv fragments and its structure was determined at 2.97 Å resolution. Cytochrome *c* is bound to subunit cytochrome c_1 of the enzyme. The tight and specific interactions are mediated mainly by nonpolar forces. The close spatial arrangement of the c-type hemes unexpectedly suggests a direct and rapid heme-to-heme electron transfer at a calculated rate of up to 8.3×10^6 s^{-1}. Remarkably, cytochrome *c* binds to only one recognition site of the homodimeric complex. Interestingly, the occupancy of quinone in the Qi site is higher in the monomer with bound cytochrome *c* suggesting coordinated binding and reduction of both electron-accepting substrates. Cytochrome *c* reduction by the cytochrome bc_1 complex might be regulated in response to the respiratory conditions.

Keywords: Cytochrome bc_1 complex; Cytochrome *c*; Electron transfer; X-ray structure; *Saccharomyces cerevisiae*

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H.2. Conformations of the ubiquinones in the doubly occupied Q_o/Q_p-pocket of the cytochrome bc_1 complex provided by solid state NMR measurements

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The reaction most critical for energy conservation in the cytochrome bc_1 complex is an obligatory bifurcation of the electron path linked to the two electron oxidation of ubiquinol to ubiquinone. This unique reaction occurs in the Q_o (or Q_p) pocket of the cytochrome bc_1 complex [1].

We have recently shown by High Resolution Magic Angle Spinning NMR spectroscopy that there are two ubiquinones bound in the Q_o site [2]. Additional solid-state NMR measurements presented here provide direct experimental evidence for the existence of two ubiquinones in the Q_o pocket. The resulting distance restraints define the conformations of the two ubiquinones relative to each other. The obtained structures were used for modeling of the PDB entry 2BCC [3] of the chicken enzyme as starting structure. Therefore, at the Q_o pocket, the inhibitor stigmatellin was replaced by the obtained structure of the pair of ubiquinones and the system was subject to energy minimization and molecular dynamics simulations. Preliminary results of these calculations will be shown.

Keywords: Cytochrome bc_1 complex; Ubiquinone; Q_o/Q_p-pocket; Solid-state NMR; Modelling

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H.3. The bc_1 complex of the iron-grown acidophilic chemolithotrophic bacterium *Acidithiobacillus ferrooxidans* functions in the reverse but not in the forward direction. Is there a second bc_1 complex?

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Acidithiobacillus ferrooxidans is an acidophilic chemolithotrophic bacterium that can grow in the presence of either a weak reductant, Fe^{2+}, or reducing sulfur compounds which provide more energy for growth than Fe^{2+}. We first review the latest findings about the uphill electron transfer pathway established in iron-grown *A. ferrooxidans*, that has been found to involve a bc_1 complex [1,2]. We then provide evidence that this bc_1 complex cannot function in the forward direction (exergonic reaction), even with an appropriate substrate. A search for the sequence of the three redox subunits of the bc_1 complex in the complete genome sequence of the *A. ferrooxidans* ATCC 23270 strain showed the existence of two different bc_1 complexes in *A. ferrooxidans*. Cytochrome *b* and Rieske protein sequence comparisons allowed us to point out some sequence particularities of these proteins in *A. ferrooxidans*. It has been suggested that in the case of sulfur-grown cells, sulfur oxidation may involve a bc_1 complex functioning in the forward direction [3]. Might each of these two bc_1 complexes be specifically involved in either reverse functioning (iron-grown cells) or forward functioning (sulfur compounds-grown cells)?

Keywords: *Acidithiobacillus ferrooxidans*; bc_1 complex; Reverse electron transfer; Cytochrome *b*; Rieske protein; Acidophilic chemolithotrophic bacteria

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H.4. Q_O site deficiency can be compensated by extragenic mutations in the hinge region of the FeS Rieske protein in the *Saccharomyces cerevisiae* bc_1 complex

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The mitochondrial bc_1 complex catalyses electron transfer between quinol and cytochrome *c*, which is coupled to proton translocation across the inner membrane. The cyt *b* mutant A144F is respiratory deficient and exhibits less than 10% bc_1 complex activity. It is affected in the high potential electron transfer pathway and the measured k_{min} for ubiquinol is lowered. This substitution is located at 6 Å of the ubisemiquinone analogue stigmatellin in the bc_1 complex structure [1]. One intragenic revertant (A144F + F279L) recovers 30% activity and the secondary mutation is located at 4.5 Å from the original mutation, suggesting that a short hydrophobic residue is required is this region. Extragenic suppressor mutations were also obtained and are all located in the hinge region of the FeS Rieske protein. This flexible linker is required for the extrinsic domain movement of the FeS protein during catalysis [2]. These extragenic mutations (FeS: T85A, A90G, A92T, A92D) are located about 20 Å away from the original mutation in the cyt. *b* Q_O region. These revertants recover 20–30% of the bc_1 activity. Their analysis shows that the catalytic activity is most likely restored by repositioning the [2Fe2S] cluster domain in the Q_O site, which allows better quinol oxidation and electron transfer to cyt. c_1.

Keywords: bc_1 complex; FeS hinge region; Suppressor mutation; Long distance interaction; Quinol binding

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H.5. Construction and characterisation of yeast mutants with modified Q_o and Q_i sites in cytochrome *b*

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The mitochondrial bc_1 complex contains two distinct quinone binding sites: Q_o and Q_i. It has been previously well documented that mutations in the mitochondrial cytochrome *b* gene, coding for amino acid substitutions in these sites, confer resistance to several inhibitor compounds, including myxothiazol and antimycin, which bind to the Q_o and Q_i site, respectively. Cytochrome *b* mutations have also been associated with a number of human diseases. Using a biolistic transformation approach, we have been modelling several mutations in *Saccharomyces cerevisiae* to establish their effects on the respiratory growth competence, on the bc_1 complex assembly, activity and sensitivity to inhibitors. One of the first mutations introduced was to change the yeast coding $CCV_{133-135}$ to $VLP_{133-135}$, which is typical of the majority of eukaryotes, and the myxothiazol resistance mutation G143A observed in pathogenic fungi.

Keywords: *Saccharomyces cerevisiae*; Cytochrome *b*; Cytochrome bc_1; Resistance mutation; Biolistic transformation; G143A

H.6. Redox transitions in bovine cytochrome bc_1 complex studied by perfusion induced ATR-FTIR difference spectroscopy

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Perfusion-induced attenuated total reflection Fourier transform infrared (ATR-FTIR) spectroscopy [1] was used to investigate redox transitions of hydrophobic films of purified bovine cytochrome bc_1 complex. Selective reductants and oxidants were used to resolve individual redox spectra. Visible difference spectra of the protein layer were simultaneously recorded with a scanned visible beam reflected off the top surface of the prism and provided a definitive means of establishing which prosthetic groups were undergoing redox changes. The technique produces data of high signal/noise and extends previous FTIR studies on bacterial bc_1 complex [2]. Assignments of vibrational features were made by comparison with spectra of subfragments of the complex, other haem proteins and model compounds. Assignments could be made for features due to the haem groups of cytochromes c_1 and b and ubiquinone. A range of other vibrational changes could be ascribed to changes in amino acids, particularly of carboxylic acid groups linked to the haems b_H and b_L. These data will be discussed in the light of structural and functional features of the complex.

Keywords: bc_1 complex; Electron transfer; FTIR spectroscopy; ATR-FTIR spectroscopy

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H.7. Study of acquired resistance to atovaquone in *Pneumocystis carinii*, using yeast as a model system

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Pneumocystis carinii is an opportunistic pathogenic fungus which causes pneumonia and is life-threatening in immune-compromised individuals. Spontaneously acquired resistance to Atovaquone, a hydroxynaphthoquinone that is used to treat *P. carinii* infections, has been linked to mutations in the mitochondrially encoded cytochrome *b* gene [1,2]. Because *P. carinii* cannot be easily cultivated, we are developing *Saccharomyces cerevisiae* as an alternative system in which to study Atovaquone resistance. Yeast and *P. carinii* cytochrome *b* are highly similar, a high-resolution structure of yeast bc_1 complex is available, and mutations can be introduced in the yeast cytochrome *b* gene by biolistic transformation [3]. We have constructed in yeast seven mutations linked to Atovaquone resistance in the pathogen. These mutations are located in the Q_0 binding site. Characterization of the effects of the mutations on bc_1 activity and drug binding is in progress.

Keywords: bc_1 complex; *Pneumocystis carinii*; *Saccharomyces cerevisiae*; Atovaquone resistance

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H.8. Flash-induced turnover of the cytochrome bc_1-complex in chromatophores of *Rhodobacter capsulatus:* the bc_1-dimer is not able to accumulate more than one electron on its two high-potential b_h hemes

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The coupling in the cytochrome-bc_1 complex (bc_1) of *Rhodobacter capsulatus* chromatophores was investigated under their excitation by flashing light. We studied how the variation of temperature, pH and the degree of ubiquinone pool reduction affected the redox-reactions of the high-potential heme b_h (as monitored at 561–570 nm), of cytochrome c_1 (as monitored at 552–570 nm) and the generation of transmembrane voltage (monitored by electrochromism at 522 nm). To unmask the otherwise ''invisible'', because of its faster oxidation than reduction, redox changes of heme b_h, we applied Zn^{2+}, which retarded the heme b_h oxidation without blocking the enzyme turnover [1].

Under all conditions tested, the reduction of heme b_h remained much faster than the voltage generation and the reduction of cytochrome c_1. These observations indicate (1) that the transmembrane electron transfer towards heme b_h is effectively ''neutralized'' by an internal charge re-distribution and (2) that the ''postponed'' electrogenic proton release from bc_1 is coupled with the allosterically(?) linked reactions of heme b_h oxidation and cytochrome c_1 reduction. Under no conditions were we able to successfully flash-reduce more than the half of the heme b_h content. The latter observation might indicate that bc_1 is a functional dimer, as outlined elsewhere [2].

Keywords: Electron transfer; Proton transfer; Coupling; Protonmotive force; *Rhodobacter sphaeroides*

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H.9. The new type of respiratory chain in the amino acid-producing Gram-positive bacterium *Corynebacterium glutamicum*

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Respiratory enzyme complexes and their whole structural genes of *Corynebacterium glutamicum* have been identified and characterized [1 2 3]. Among them, cytochrome *c*-subunit of cytochrome *bc*-type quinol–cytochrome *c* oxidoreductase contains two c-type cytochrome domains, one of which may directly transfer electrons to a terminal oxidase. Here, cytochrome aa_3-type oxidase was found to contain an extra cluster, which is rich in both negatively and positively charged amino acid residues. Since the cluster is located in or close to the substrate-binding site, the cluster might play an important role in interacting with the new type of cytochrome *c* fused to bc complex. To reveal the whole respiratory enzyme complexes in the *C. glutamicum*, the proteins were fractionated by DEAE-Toyopearl amino-charge chromatography after solubilization with *n*-dodecyl beta-D-maltoside (DM). The a, b, c-type cytochrome was eluted in the same fraction, and this showed quinol oxidase activity. These findings suggest that *bc* complex and cytochrome aa_3 may form a supercomplex. This is confirmed by native polyacrylamide gel electrophoresis. Mass spectrometry indicated that the haem of the oxidase is haem As. Edman degradation and mass spectrometry suggested that the N-terminal signal sequence of subunit II is cleaved and that the new N-terminal cysteine residue might be diacylglycerated.

Keywords: Dihaem cytochrome; High-G + C Gram-positive bacteria; Respiratory chain; MALDI-TOF/MS

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H.10. The role of midpoint potentials in electron transfer reactions in the cytochrome bc_1 complex

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The Q cycle mechanism involves two quinone reaction centers, which contain four different redox centers, the Rieske iron–sulfur protein, cytochrome c_1 and the di-heme cytochrome b with a high potential and a low potential heme b. We examined the influence of the midpoint potential of the Rieske center on cytochrome b reduction rates by oxidant-induced reduction. Purified bc_1 complexes with decreased midpoint potential of the Rieske center [1] were treated with antimycin to block center N and decylubiquinol as electron donor was added. A mixture of cytochrome c and cytochrome oxidase was added by stopped-flow mixing, and oxidant-induced reduction of cytochrome b was monitored. Second order rate constants were calculated and showed that the oxidant-induced reduction rates decreased as the midpoint potential of the Rieske center decreased. To evaluate the effect of the midpoint potentials of the cytochrome b hemes on reduction of cytochrome b, we also examined pre-steady state reduction kinetics of bc_1 complexes of mutants with cytochrome b hemes of altered potentials. One of the mutations raises the midpoint potential of heme b_L by $+60$ mV, while the other drops the midpoint potential of heme b_H by -90 mV.

Keywords: bc_1 complex; Midpoint potential; Electron transfer reaction

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H.11. Cofactor knockout strategies uncover the minimal functional unit of the primary energy conversion steps of cytochrome bc_1

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Redox cofactor chains and electron transfer steps through the cytochrome bc_1 complex have been functionally and structurally identified. However, the mechanistic features of the discrete electron transfer steps and the quinone/quinol catalytic stages in the Q_o (oxidation) and Q_i (reduction) sites remain to be elucidated. The reaction sequence most difficult to access is in the Q_o site where the primary energy conversion takes place. This site catalyzes two-electron quinol oxidation and delivers one electron along the high potential iron–sulfur cluster(FeS)/heme c_1 redox chain and one electron along a low potential heme b_L/b_H Q_i redox chain. We use selective cofactor knockouts to progressively shorten both the high and low potential chains that meet at the Q_o site until the primary charge separating reaction is isolated. This allows us to reveal the action of the minimal quinol oxidation unit formed by quinol, FeS and heme b_L. We observe that the reaction has been engineered to be reversible, with near-zero driving force and unaffected by transmembrane electric field. The reaction is driven to completion by a second oxidizing center in either of the chains indicating that they operate as independent redox contacts with the redox pools that meet at the cytochrome bc_1.

Keywords: Electron transfer; Cytochrome bc_1; Quinol oxidation; Heme; Iron–sulfur cluster; *Rhodobacter capsulatus*

H.12. Crystallographic analysis of the quinol-oxidizing (Q_o) site in the yeast cytochrome bc_1 complex

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The cytochrome bc_1 complex from *Saccharomyces cerevisiae* (ubiquinol:cytochrome *c* oxidoreductase, E.C.1.10.2.2, QCR) couples electron transfer to translocation of protons across the inner mitochondrial membrane and contributes to the proton gradient, which drives the production of ATP [1]. This multisubunit membrane protein complex operates as a functional homodimer and contains three essential catalytic subunits: cytochrome *b*, cytochrome c_1, and the Rieske protein. Ubiquinol, delivers electrons at the Q_o site, whose transfer to cytochrome *c* involves domain movement of the extrinsic part of the Rieske protein [2]. For crystallographic analysis, it is beneficial to fix the movement of the Rieske protein, and so far, the inhibited enzyme is the only available structure of the yeast QCR [3]. While main features of catalysis are understood and described with the modified Q cycle, important aspects of the molecular mechanism, for instance of quinol oxidation, are still in question. Inhibitors provide an important tool to analyse the active site. Recently, the structure of the co-complex of yeast QCR and an antibody Fv fragment with the Q_o site inhibitor heptyl-HDBT, a candidate for anti-malarial drug development, was determined to 2.5 Å resolution. The observed differences at the Q_o site, with respect to the reported stigmatellin-inhibited structure will be described.

Keywords: Cytochrome bc_1 complex; Quinol-oxidizing site; Inhibitor; X-ray crystallography; *Saccharomyces cerevisiae*

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H.13. Characterization of the quinone binding sites of the cytochrome bc_1 complex from *Paracoccus denitrificans* and *Saccharomyces cerevisiae* by electrochemically induced FTIR difference spectroscopy and specific inhibitors

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Ubiquinol–cytochrome *c* oxidoreductase (cytochrome bc_1 complex) is one of the fundamental components of the respiratory and photosynthetic electron transfer chains located in the inner mitochondrial or in the bacterial cytoplasmatic membrane. It catalyzes the electron transfer from ubiquinol to cytochrome *c* coupled to translocation of protons across the membrane. Both bacterial and mitochondrial complexes work through the modified Q-cycle mechanism [1] which postulates two separate binding sites for quinones, Q_o and Q_i. Although the Q-cycle mechanism is well supported, not all aspects of the quinol/quinone binding mechanism are yet fully understood.

Here, we investigate the bc_1 complexes from *Paracoccus denitrificans* and *Saccharomyces cerevisiae* by a combination of electrochemistry and FTIR difference spectroscopy [2]. Difference spectra were obtained for a potential step from the fully reduced to the fully oxidized state and for the reverse process in the presence or absence of the Q_o-, and Q_i-site inhibitors stigmatellin and antimycin A, respectively. Fully reversible spectra could be observed indicating a full reaction at the electrode. Tentative assignments of the vibrational modes of bound ubiquinone and inhibitor specific changes in the spectra are presented. On this basis, the local environment of the quinone- and inhibitor-binding sites is discussed.

Keywords: bc_1 complex; Quinone binding sites; Inhibitor; FTIR spectroscopy; Protein electrochemistry; *Paracoccus denitrificans*; *Saccharomyces cerevisiae*

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H.14. Structure–function relationships in the cytochrome bc_1 complex of *Saccharomyces cerevisiae*

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The mitochondrial cytochrome bc_1 complex transfers electrons from ubiquinol to cytochrome *c*. This redox reaction is coupled to a translocation of protons across the inner mitochondrial membrane. Molecular details of these processes are not understood. Proton pathways towards the quinone reduction site have been proposed [1,2]. With the help of the structural data from the cytochrome bc_1 complex of *S. cerevisiae* [1], amino acid residues, which are presumably important for catalytic activity and proton transport, are defined. Site-directed mutagenesis of the chosen residues combined with biophysical measurements will give further insight into the mechanism of the complex.

Keywords: Electron pathway; Proton translocation; Site-directed mutagenesis

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H.15. The involvement of arginine-84 of subunit IV in ionic interaction with core subunits of the cytochrome bc_1 complex from *Rhodobacter sphaeroides*

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Subunit IV, the only supernumerary subunit in *Rhodobacter sphaeroides* bc_1 complex, has been successfully overexpressed in *E. coli* [1]. Purified recombinant subunit IV is functionally active as it can restore bc_1 activity of the three-subunit complex (reconstitutive activity) to the same level as that of wild-type, four-subunit complex. Incorporation studies indicate that reconstitutive activity of subunit IV requires interaction with the core complex after it is incorporated into the bc_1 complex through the transmembrane helix region (residues 86–109) [2]. Deletion mutational studies identified two putative interacting regions of subunit IV: residues 41–53 (region I) and 77–85 (region II). Substitution mutational studies indicate that the amino acid specificity in region II, but not region I, of subunit IV is essential for interaction with the core complex to restore bc_1 activity. Alanine scans of region II identified arginine-84 as the essential residue. The positively charged group in arginine-84 is involved in ionic interaction with the negatively charged group in core subunits to restore the bc_1 activity. E425, E426, and D427 of cytochrome *b* are likely candidates for interaction with R-84 of subunit IV.

This work was supported in part by a NSF grant #0077650.

Keywords: Supernumerary subunit; Ionic interaction; Bacterial bc_1 complex

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H.16. The temperature-dependency of the charge transfer kinetics in cytochrome bc_1-complex of *Rhodobacter sphaeroides* chromatophores

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The temperature dependencies of the charge transfer kinetics in *Rhodobacter sphaeroides* cytochrome bc_1-complex were studied using the photovoltage technique [1]. The single-turnover catalytic cycle of the bc_1-complex was initiated by the rapid formation of ubiquinol in photosynthetic reaction centers upon laser flash illumination of chromatophores. In the presence of antimycin A under the conditions of high redox potential, the kinetics of the electrogenic phase accompanying the oxidation of a ubiquinol molecule (time constant, 4–6 ms, +25 °C, pH 7.5) was highly activated (activation energy, 65–70 kJ/mol). The amplitude of the phase was temperature-independent (20–22% from the primary charge separation phase). The data were interpreted as that the transfer of the electron kinetically coupled to the conformational changes of Rieske FeS-protein is the rate-limiting step of the whole reaction.

Keywords: bc_1-complex; Electrogenesis; Charge transfer; Ubiquinone

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Session I: Topics in Photosynthesis

I.1. Relation between mitochondrial oxidative phosphorylation and photosynthetic activity in respiratory-deficient mutants of *Chlamydomonas reinhardtii*

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Chlamydomonas mitochondrial mutants of respiratory-chain complexes (I,III,IV,I + III,I + IV) were analyzed for photosynthetic activities. Photosynthetic oxygen evolution curves showed a positive relationship between the apparent yield of linear electron transport in the chloroplast and the number of oxidative phosphorylation active sites (0–3) in mitochondria. Moreover, the distribution of excitation energy between chlorophylls of the two photosystems showed that mutants displayed a preferential excitation of PSI (state 2). The extent of the change was correlated with the expected loss of the ATP synthesis capability of mitochondria and with phosphorylations of light-harvesting complexes II (LHCII) polypeptides, indicating the preferential association of LHCII with PSI. The transition to state 1, which normally occurs in wild-type exposed to far red-light or to white-light + DCMU, was only observed in white-light + DCMU conditions for mutants. This suggests a weaker rate of net oxidation of the plastoquinone pool in the mutants due to either enhanced cyclic electron flow around PSI, coupled to ATP synthesis, or enhanced glycolysis.

The present results suggest that the main role of the state transitions is to modify the excitation energy distribution and to modulate cyclic versus linear transport rate in response to intracellular ATP concentration.

Keywords: State transition; Mitochondrial mutant; Photosynthesis; Chlamydomonas

I.2. Control of electron transport in Photosystem I by the iron–sulfur cluster F_X in response to intra- and inter-subunit interactions

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Photosystem I (PSI) is a trans-membrane multisubunit complex that mediates light induced electron transfer from plactocyanine to ferredoxin. The electron transfer proceeds from an excited chlorophyll *a* dimer (P700) through a chlorophyll *a* (A_0), a phylloquinone (A_1), a [4Fe–4S] iron–sulfur cluster F_X all located on the core subunits PsaA and PsaB to iron–sulfur clusters F_A and F_B, located on subunit PsaC. Earlier, it was attempted to determine the function of F_X in the absence of $F_{A/B}$ by chemical dissociation of subunit PsaC. However, not all of PsaC subunits could be removed from the PSI preparations by this procedure without partially damaging F_X. We therefore removed subunit PsaC by deletion of *psaC* gene of PS I in the cyanobacterium *Synechocystis* sp. PCC 6803. Cells could not grow under photosynthetic conditions when subunit PsaC was deleted. Yet the PsaC$^-$ mutant cells grew under heterotrophic conditions and assembled the core subunits of PS I in which light-induced electron transfer from P700 to A_1 occurred. The photo-reduction of F_X was substantially reduced as seen from direct measurement of the extent of electron transfer from A_1 to F_X. Replacement of the cysteine ligand of F_X by serine in site-directed mutation C565S/D566E in psaB also caused a slowdown in electron transfer from the quinone to F_X. Based on these and other results, we propose that F_X might have a major role in controlling electron transfer through PS I.

Keywords: Photosystem I; Iron–sulfur cluster; Quinone; Electron transfer; Suppressor mutant

I.3. Study of interaction and electron transfer between the ferredoxin and two ferredoxin dependent enzymes: ferredoxin NADP+ reductase and nitrite reductase

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The reaction center of Photosystem I (PSI) reduces soluble ferredoxin which is involved in many bioenergetic pathways in cyanobacteria and chloroplasts. We studied interactions and electron transfers between ferredoxin and two soluble partners: Ferredoxin NADP[+] reductase (FNR), a FAD protein involved in carbon assimilation, and nitrite reductase (NiR), a [4Fe–4S] and siroheme protein implicated in nitrogen assimilation. Structures of FNR are available [2,3]. The structure of *E. coli* sulfite reductase hemoprotein, which is similar to NiR, is also available. Two main methods were used: optical spectral perturbations [1,4,5] induced by formation of complexes and flash absorption spectroscopy. By these methods, dissociation constants have been calculated. Flash absorption spectroscopy experiments also permitted to study the electron transfers. The different reactions leading eventually to reduction of FNR (or NiR) were observed after photoexcitation of PSI on a reconstituted system containing PSI, Fd and the soluble partner. In particular, we could identify dissociation rates between PSI and Fd and the association between Fd and soluble partners. The catalytic mechanism of enzymes can be also studied. In particular, the storage of electrons on the different cofactors was observed.

Keywords: Protein interaction; Electron transfer; Ferredoxin; Ferredoxin NADP+ reductase; FNR; Nitrite reductase; NiR; Flash absorption spectroscopy

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I.4. Acclimation of chloroplasts from north- and south-exposed canopy sectors of chestnut (*Castanea sativa* Mill.)

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Chestnut is characterized by a high vigour of their canopies. The aim of this work is to characterize the photosynthetic apparatus from different canopy regions.

In canopies of adult plants, it is possible to identify, a shady (north) and a sunny (south) sides. The south side has 70–80% of total photonic radiation as opposed to the north side where just 10–20% of total light is available [1,2]. In consequence, significant differences of photosynthesis/transpiration compromises are detected [1].

Energization capacity of chloroplasts from north and south sides were measured with 9-aminoacridine [3,4] at different temperatures [1]. Results suggest that nonexcited chloroplasts from the north side have more free negative charges at the thylakoid surface than those from the south side. However, when they are excited, chloroplasts from the south side generate a higher charge density at temperatures above 18 °C. The lipid phase transition of the north side chloroplasts is at 16 °C, while the south chloroplast it is at 20 °C. These facts are in accordance with the more heliophilic character of south (Chla/Chlb = 3.42) than north (Chla/Chlb = 3.31) chloroplasts, showing that chloroplasts have enough sensibility in the presence of different environmental conditions as can be found around the canopy. In consequence, fruits from the north are 20% smaller than those from the south [1].

Keywords: Chestnut; *Castanea sativa* Mill.; Chloroplast; Photosynthesis; Membrane potential; 9-aminoacridine; Sunny and shady leaves

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I.5. Pathways for control of the redox state of the thylakoid plastoquinone pool in the dark

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Observations of non-photochemical plastoquinone reduction/oxidation in plants have led to proposals of various 'chlororespiratory' oxidases and reductases that interact directly with the plastoquinone pool. One possible pathway for dark reoxidation is via the Immutans protein, a thylakoid homologue of the mitochondrial alternative oxidase. We assessed plastoquinone pool oxidation rates in thylakoids from *Arabidopsis thaliana* by monitoring fluorescence induction curves and measured effects of proposed inhibitors of the Immutans protein, octyl gallate and propyl gallate, and also several mitochondrial inhibitors. This work suggests that the Immutans protein is not the major oxidation pathway. However, the pH and oxygen dependency of the dominant pathway was not consistent with simple non-enzymatic plastoquinol autoxidation by molecular oxygen and another enzymatic reaction with oxygen appears likely. The ndha–ndhk genes of NDH dehydrogenase in higher plant chloroplasts are similar to those of subunits of mitochondrial Complex I and have led to a proposal for a thylakoid NAD(P)H-plastoquinone oxidoreductase. The role of the NDH dehydrogenase has been assessed by monitoring plastoquinone pool reduction rates in wild-type and mutant tobacco whole leaf and thylakoids with the re-reduction of $P700^+$ after far-red illumination and also with chlorophyll fluorescence induction experiments. Data on these experiments will be presented.

Keywords: Chlororespiration; Immutans; NDH dehydrogenase; Plastoquinone

I.6. Cyclic electron transfer in plant leaf

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The turnover of linear and cyclic electron flows has been determined in fragments of dark-adapted spinach leaf by measuring the kinetics of fluorescence yield and of the transmembrane electrical potential changes under saturating illumination. When Photosystem (PS) II is inhibited, a cyclic electron flow around PSI operates transiently at a rate close to the maximum turnover of photosynthesis. When PSII is active, the cyclic flow operates with a similar rate during the first seconds of illumination. The high efficiency of the cyclic pathway implies that the cyclic and the linear transfer chains are structurally isolated ones from the others. We propose that the cyclic pathway operate within a supercomplex including one PSI, one cytochrome *bf* complex, one plastocyanin and one ferredoxin. The cyclic process induces the synthesis of ATP needed for the activation of the Benson–Calvin cycle. A fraction of PSI (~ 50%), not included in the supercomplex, participates in the linear pathway. The illumination would induce a dissociation of the supercomplexes that progressively increases the fraction of PSI involved in the linear pathway.

Keywords: Cyclic electron flow; Supercomplex; Plant leaf

I.7. The intermediates in nitrite reduction to ammonia by ferredoxin-dependent plant nitrite reductase

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Plant nitrite reductase (NiR) catalyses six-electron reduction of nitrite to ammonia with reduced ferredoxin as an electron donor [1]. It contains one siroheme and one [4Fe–4S] cluster, acting as separate one-electron carriers. Nitrite binds to the siroheme, its nitrogen acting as the sixth coordinate for the heme iron [1]. In the present work, we demonstrate by EPR spectroscopy the enzyme turnover by two-electron steps and substrate depletion using ferredoxin reduced by Photosystem I as an electron donor. Ferrous siroheme complex with NO is proved to be an intermediate in enzyme catalytic cycle formed on two-electron reduction of enzyme complex with nitrite, as previously proposed in Ref. [2]. The enzyme cycle contains a branching point between NO and ammonia where in the absence of reductant, the enzyme returns to NO less than in 1 min. Hydroxylamine, another putative intermediate in nitrite reduction by NiR [3], reacts with oxidised nitrite reductase, giving ferrous siroheme–NO state with characteristic time of about 15 min. The bottleneck of this reaction is the hydroxylamine binding, the hydroxylamine to NO conversion being fast and probably representing the return to NO from the abovementioned branching point.

Keywords: Electron transfer protein; EPR; Ferredoxin; Hydroxylamine; NO

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I.8. Light harvesting complexes 2 investigated by high-resoltion atomic force microscopy

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Light harvesting complexes 2 (LH2) are the accessory antenna proteins in the bacterial photosynthetic apparatus and are built of $\alpha\beta$-heterodimers containing three bacteriochlorophylls and one carotenoid each [1]. We have used atomic force microscopy (AFM, [2]) to investigate reconstituted LH2 2D-crystals from *Rubrivivax gelatinosus* and *Rhodobacter sphaeroides*. The complexes of both organisms form nonameric rings integrated in the lipid bilayer protruding strongly on one side, and weakly on the other side of the membrane. Topographs of thermolysin-digested *R. gelatinosus* LH2 reconstituted revealed a height reduction of the strongly protruding surface and a change of its surface appearance. These results allowed the periplasmic surface to be assigned [3]. Topographs of 2D crystals of *R. sphaeroides* LH2 allowed the subunit packing of the proteo-pigment complexes to be investigated in detail. Most interestingly, the barrels are tilted integrated into the membrane [4]. Models of interaction between LH2, LH1 and the RC have been established. However, the organization of LH2 and LH1 complexes around RCs is still under debate. The AFM with its high signal-to-noise ratio might be the appropriate tool to study the supramolecular organization of the photosynthetic apparatus at submolecular resolution.

Keywords: Photosynthetic protein; Membrane protein; Atomic force microscope; 2D crystal; Light harvesting complex

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I.9. Fusion of *Rhodobacter capsulatus* chromatophores with proteoliposomes

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Fusion of chromatophores of phototrophic bacteria with liposomes is used to change the chemical composition of the chromatophore membrane and to study the impact of such changes on bioenergetic reactions [1,2]. So far, the fusion of chromatophores with liposomes was achieved either by the addition of high amounts of Ca^{2+} [3] or by decreasing the pH value [4] or by freeze–thaw sonication [5]. Here, we describe a new technique which allows us to fuse chromatophores of *Rhodobacter capsulatus* with (proteo)liposomes from soybean asolectin at neutral pH, physiological concentration of divalent cations, and room temperature.

Keywords: ATP synthase; Membrane transporter; Proteoliposome; *Rhodobacter capsulatus*

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I.10. Reduction of tellurite to elemental tellurium and its effect on both membrane redox components and electron transport of *Rhodobacter capsulatus* photosynthetically grown cells

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Potassium tellurite (K_2TeO_3) is highly toxic to eukaryotic and bacterial cells [1]. Intrinsic low-level resistance to TeO_3^{2-} is present in a few Gram-positive bacteria while a constitutive high-level of resistance has been described for anaerobic- and aerobic-photosynthetic Gram – negative bacteria [2]. Resistance to tellurite (Te^R) is linked to at least five determinants, all apparently unrelated to one another at either DNA or protein level [1]. Most Te^R bacteria convert TeO_3^{2-} to Te^0 ($4e^-$ reaction) which is stored intracellularly as black inclusions [1]. The role assumed by the plasma membrane redox enzymes in Te^R remains elusive [3]. The extent of tellurite reduction in *Rhodobacter sphaeroides* was shown to depend on $FADH_2$ oxidation activity [3] while the membrane-bound NarG and NarZ reductases were found to reduce TeO_3^{2-} and contribute to Te^R of *E. coli* [4]. Respiration in *P. aeruginosa* has been correlated with TeO_3^{2-} reduction although at a rate considerably lower than the rate of oxygen reduction [5]. Here, we report on the effect of tellurite on both plasma membrane organization and arrangement of photosynthetic and respiratory components in phototropically grown cells of *Rhodobacter capsulatus*. Further, by X-ray microanalysis linked to TEM, we unequivocally show the presence of Te-crystallites located near the ICM system.

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Keywords: Tellurite oxyanion; Plasma membrane; Electron transport; Reaction center; *Rhodobacter capsulatus*

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I.11. The inhibition of the proton transport in Zn(2+)-treated reaction centers of *Rhodobacter sphaeroides* induces the kinetic isotope effect of the QB reduction in chromatophores

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The illumination of the *Rhodobacter sphaeroides* reaction center (RC) by the second laser flash causes the second reduction step of QB coupled to the binding of two protons. The proton delivery to QB is accompanied by an electrogenic effect (phase B2) whose kinetics (kB2) was studied using the photovoltage technique [1] in chromatophores. Zn^{2+} ions were shown to inhibit the proton transfer to QB [2]. In the absence of the metal ions, the B2 kinetics measured in H_2O and that in D_2O were identical. This evidently agrees with the postulated mechanism of the second QB-reduction step where the rate-limiting electron transfer to QBH($^{\bullet}$) follows fast, equilibrium proton binding to QB($^{\bullet -}$). In the presence of Zn^{2+} the kinetic H/D isotope-effect (KIE) was observed above pH 6 (its value was 1.5 at pH 7 and it rose at higher pH). In the presence of Zn^{2+}, the high activation energy ($\sim$ 60 kJ/mol) of kB2 was observed both for D_2O and H_2O. The KIE oberved, indicates that in the presence of Zn^{2+}, the proton transfer rate cannot be rate-limited by any changes in conformation of proton-transfer groups within the proton transfer chain(s).

Keywords: Bacterial reaction center; Electrogenesis; Charge transfer; Ubiquinone; Divalent cation

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Session J: Mitochondrial Transporters

J.1. Regulation of uncoupling proteins

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Uncoupling proteins (UCPs), widely distributed in animals and plants as a subgroup of mitochondrial carrier family, facilitate physiological uncoupling of oxidative phosphorylation. UCPs are activated by fatty acids (FA) and inhibited by nucleotides. The physiological regulation of UCP will be elucidated on the basis of reconstituted systems. The activation by FA is widely accepted but control by nucleotides is ignored assuming constant binding. However, debinding is facilitated by a nearly 100-fold decrease of affinity of UCP1 for ATP^{4-} per unit pH increase. In cells, small Na^{+} movements can cause strong pH shift via Na/H-exchanger to remove inhibition of UCP1. The pH control involves several *carboxyl*- and one His-group identified by mutagenesis. These residues are well conserved within the UCP variants. A superimposed control by ATP/ADP ratio varies according to different functions of UCP1,2 and 3. UCP1,2 are controlled primarily by ATP^{4-} but UCP3 by ADP^{3-}. Another control occurs by coenzyme Q as obligatory cofactor in addition to FA. Oxidised Q but not QH_2 activate UCP1. In this context, function of UCP3 as a facilitator of FA oxidation will be discussed. A distinct small activation of UCP1 by oxygen radicals in reconstituted system under particular conditions will be demonstrated.

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J.2. Expression of UCP1 in mouse muscle selectively affects muscles at rest

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UCP1 is responsible for heat production in brown adipose tissue (BAT). Expression of UCP1 in muscle and/or heart, at similar level to that normally found in BAT, was obtained in transgenic mice. These mice showed a lower body weight. The activity of UCP1 was verified using muscle or heart mitochondria. A specific reduction of muscle mass, with a shift in fiber type promoting types IIa and IIx at the expense of type IIb, was observed for the gastrocnemius and plantaris muscles. On the opposite, the heart and the soleus were found unaffected. This can be related to the different contractile activities of muscles and to the dependence of UCP1 protonophoric activity to mitochondrial membrane potential: UCP1 has no influence on muscles undergoing repetitive contraction (heart, soleus), whose mitochondria are under state 3 with a lowered membrane potential and a high respiratory activity. In muscles ordinarily at rest (gastrocnemius, plantaris), the ATP turnover is low, therefore mitochondria tend to reach state 4, and in these muscles UCP1 probably prevents the establishment of a normal high state 4 membrane potential, this would be at the origin of the decrease in the expression of specific genes for the glycolytic muscle fiber subtype.

Keywords: UCP1; Mitochondria; Transgenic mouse; Muscle

J.3. The *Saccharomyces cerevisiae* ATP/ADP carrier fused to cytochrome *c* is functional

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The *Saccharomyces cerevisiae* adenine nucleotide carrier (Anc2p) is a 35 kDa nuclear encoded protein which mediates the one-to-one exchange of ATP against ADP across the inner mitochondrial membrane. ATP/ADP transport can be blocked by specific inhibitors belonging to two families: the atractylosides and the bongkrekic acids. These inhibitors bind to distinct conformers involved in the transport process, leading to the formation of stable complexes. Therefore, structure investigation of both complexes affords a particular opportunity to elucidate the transport mechanism.

We report the biochemical characterisation of chimeras in which the *iso*-1-cytochrome *c* (Cyc1) was fused either to the N- or to the C-terminus of Anc2p in order to increase its polar area for crystallographic approaches.

The two Anc2p–cyc1 chimeras are able to restore the growth on glycerol medium of a yeast strain devoid of any adenine nucleotide carrier, with growth rates similar to that of the wild-type strain. This demonstrates that the function of Anc2p is not impaired by fusion to Cyc1. In addition, the amount of both fusion proteins in mitochondria was similar to native Anc2p, as assessed by atractyloside binding experiments.

Anc2p–Cyc1 fusion proteins display spectral characteristics of cytochrome *c*, consistent with the presence of heme.

Keywords: ATP/ADP carrier; Mitochondria; Fusion protein

J.4. The human mitochondrial adenine nucleotide carriers: kinetic properties and importance of the N-terminus region for their expression in yeast

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The mitochondrial adenine nucleotide carrier (Ancp) exchanges cytosolic ADP for matrix ATP. In humans, three distinct genes have been characterized, the expression of which depend on the tissue and the cellular differentiation state. Abnormal *HANC* transcription or HAnc1p inactivation have been associated with cardiomyopathies or ophtalmoplegia. In order to investigate HAncp kinetic properties, we have set up a system for efficient human *ANC* expression in a yeast strain inactivated for its endogenous *ANC*. The three *HANC* were able to restore yeast growth on a nonfermentable carbon source. *HANC3* was the most efficient, though its growth level was lower than in the presence of *ScANC2*. *HANC1* and *HANC2* restored growth to equivalent level, but smaller than that of *HANC3*. For the first time, kinetic exchange properties of the three HAncp have been characterized. HAnc3p distinguishes by higher Vm and Km, whereas HAnc1p and HAnc2p have similar kinetic constants. Growth characteristics of *HANC*-expressing yeast together with in vitro import experiments suggested that HAncp import into yeast mitochondria could be limiting. Although import signal for mitochondrial carriers is intricate, we could identify a negatively charged residue in the N-terminus part of HAnc1p that is crucial for its expression in yeast.

Keywords: ADP/ATP carrier; Human ANC; Yeast; Mitochondria; Heterologous expression

J.5. Heterologous expression of the uncoupling protein: his tagging or fusion with MBP to improve the purification of the recombinant protein

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Uncoupling protein 1 (UCP1) is a 33 kDa mitochondrial membrane protein present into brown adipose tissue (BAT). Its physiological role is redox free energy-dissipation into heat. It belongs to the mitochondrial carrier family and has a well-known topology constituted by six transmembrane α-helices connected by hydrophilic segments. We report the expression and purification of two recombinant forms of UCP1 with the purpose of crystallographic studies. First, we cloned the UCP1 gene from rat BAT into a pMal-c2 vector (NEB). This allowed expression in *Escherichia coli* of a chimeric protein made by UCP1 fused to the C-terminal end of the prokaryotic soluble maltose-binding protein (MBP). Several conditions of induction, such as *E. coli* strains and temperatures were tried. We pointed out a soluble form of the chimera at 16 °C, representing about 15% of expressed MBP-UCP1. The soluble form was purified by affinity chromatography using amylose resin. Second, we cloned the UCP1 gene into a new type of vector, a Topo-TA cloning vector pCRT7/CT-TOPO (Invitrogen). We introduced a His-tag sequence. This His-UCP1, accumulated in inclusion bodies, was purified after solubilization by sarkosyl.

This work was supported by grants of the Belgian F.R.F.C and P.D. is recipient of a F.R.I.A. fellowship.

Keywords: Uncoupling protein; Fusion protein; Heterologous expression

J.6. Limited proteolysis of the membrane-embedded ADP/ATP carrier of *Saccharomyces cerevisiae* mitochondria

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The ADP/ATP carrier (Ancp) is a 30 kDa nuclear-encoded protein located in the inner mitochondrial membrane. It catalyses the one-to-one transmembrane exchange between ATP generated inside mitochondria by oxidative phosphorylation and cytosolic ADP.

ADP/ATP transport can be specifically inhibited by carboxyatractyloside (CATR) and bongkrekic acid (BA) which bind to two different conformers of Ancp involved in the nucleotide transport process [1]. Topographical changes undergone by the carrier during transport have been studied by limited proteolysis carried out on the *Saccharomyces cerevisiae* Anc2p isoform, in the presence of CATR or BA.

Site-specific endoproteases have been used on mitochondria and on inside-out submitochondrial particles to cleave sites exposed either to the cytosolic or to the matrix faces of the membrane, respectively. Identification of the cleavage sites was assessed by immunodetection of the generated fragments after SPAGE, using polyclonal antipeptide antibodies directed against specific regions of the carrier and by amino acid microsequencing.

The results obtained are consistent with a six transmembrane α-helices arrangement of Anc2p in the mitochondrial membrane. In addition, we have identified a re-entrant loop predicted to face the matrix, but cleavable by externally added proteases, depending on the carrier conformation, and therefore probably involved in the ADP/ATP transport process.

Keywords: ADP/ATP carrier; Mitochondria; Limited proteolysis

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J.7. Functional studies of UCP2 using mitochondria of mice which have been made null for the *Ucp2* gene

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Several observations suggest that UCP2 has an uncoupling activity, nevertheless its physiological function is still unknown. Macrophages from mice whose *Ucp2* gene has been invalidated show increased production of reactive oxygen species [1]. Moreover, higher ATP levels were observed in pancreatic islets from *Ucp2*(−/−) mice resulting in the stimulation of insulin secretion [2]. Both phenotypes are consistent with an increased mitochondrial membrane potential in *Ucp2*(−/−) mice and thus with an uncoupling activity of UCP2.

Mitochondrial proton leak from wild-type (*Ucp2*(+/+)) and *Ucp2*(−/−) mice was measured in organs expressing UCP2 (spleen and lung) or not (liver). However, no difference was observed for either of the organs analysed between *Ucp2*(+/+) or *Ucp2*(−/−) mitochondria under basal conditions. Similar results were obtained after LPS injection to mice, which increased UCP2 expression. Moreover, the use of several activators of UCP2 such as retinoic acid and superoxide did not make it possible to distinguish *Ucp2* (−/−) from wild-type mitochondria. These results suggest a compensatory mechanism in these organs or inappropriate experimental conditions, including the lack of an activator required for UCP2 proton transport activity lost during mitochondrial isolation. Alternatively, the possibility remains that the physiological function of UCP2 is the transport of other ions than protons.

Keywords: UCP2; Mitochondria; Proton leak; Knockout mice

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J.8. Anatomy of mitochondrial anion carrier sequences

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We have analyzed sequences coding for mitochondrial anion carrier proteins (MACPs) in human genome and other available eukaryotic genomes. Sequences were sorted out according to a homology-based phylogeny tree into >40 subfamilies and representative subfamily sequences were analyzed and compared. A transmembrane spanning model of Klingenberg [1] was used for all of them, similarly as previously for uncoupling proteins [2,3]. This revealed unique features among subfamilies, namely the shifts in the positive and negative charges or polar residues along the defined putative transmembrane segments or in relations to the MACP family signatures. Hence, the main features by which subfamilies differ from each other are the number and nature of charges and polar residues in the transmembrane segments and their relative locations. On the contrary, the hydrophobic residues are mostly conserved in exact locations in the predominantly hydrophilic matrix or cytosolic segments.

Keywords: Mitochondrial family of anion carrier; Sequence analysis

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J.9. Interaction of mitochondrial uncoupling protein UCP2 with fatty acids and other ligands

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Ubiquitously expressed mitochondrial uncoupling protein UCP2 might contribute to body weight regulation, decreased reactive-oxygen-species production, to adaptive thermogenesis including fever, and to balance of *pro*- and *anti*-apoptotic processes. Pathologically distorted UCP2 regulations might cause the development of obesity, type 2 diabetes, and heart failure. We expressed human recombinant UCP2 in yeast and screened efficiency of fatty acid (FA) activation of its protonophoric function, the inhibitory ability of purine nucleotides as well as ^{3}H-GTP binding. We demonstrated that all natural long-chain FAs activate UCP2-mediated H^+ uniport, whereas coenzyme Q_{10} had no further significant activating effect. FA activation kinetics (FA cycling) revealed the highest apparent affinity to UCP2 (lowest K_m) for ω-6 polyunsaturated FAs: all-*cis*-8,11,14-eicosatrienoic and all-*cis*-6,9,12-octadecatrienoic acid. Since these ω-6 polyunsaturated FAs are also the most potent agonists [1] of nuclear receptor PPARβ which activates UCP2 transcription, this finding suggests their possible dual role in activating both, transcription of UCP2 as well as its uncoupling activity. Nucleotides were found to inhibit UCP2-mediated H^+ uniport and compete with ^{3}H-GTP binding to isolated UCP2. Hence, they are suggested to bind and inhibit UCP2 also in vivo.

Keywords: Mitochondrial uncoupling protein UCP2; Fatty acid-induced uncoupling; Polyunsaturated fatty acid; Purine nucleotide

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J.10. Peripheral-type benzodiazepine receptor polymerisation leads to drug ligand activated cholesterol transport

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The peripheral benzodiazepine receptor (PBR) is an 18,000 Mr membrane protein expressed in the central and peripheral nervous systems with high densities found in steroid-producing tissues. In all tissues examined, PBR proteins were mostly located in the outer membranes of the mitochondria. A key role of PBR in the regulation of steroidogenesis is cholesterol transport and multiple protein complex have been suggested [1].

Recombinant mouse PBR was overexpressed in *E. coli* and successfully reconstituted into proteoliposomes. We demonstrated that this protein alone could bind with nanomolar affinity cholesterol and PBR's drug ligand, PK11195 (isoquinoline carboxamide) [2]. Reconstituted PBR could be induced to form polymers after reactive oxygen species generation by UV photoirradiation. Spectroscopic analysis revealed the formation of dityrosine as the covalent cross-linker between PBR monomers. Electron microscopy of freeze-fractured proteoliposomes revealed intramembraneous particles consistent with polymer formation. Photoirradiation increased PK11195 binding and reduced cholesterol binding. Simultaneous addition of PK11195 and cholesterol to polymers exhibit a time dependent increased of cholesterol binding to proteoliposomes suggesting transport. We concluded that PBR polymer is the functional unit responsive for ligand-activated cholesterol transport.

Keywords: Peripheral benzodiazepine receptor; Polymer; Cholesterol transport

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J.11. The importance of the Pho84 transmembrane segment XI for phosphate transport into *Saccaharomyces cerevisiae*

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Inorganic phosphate uptake is essential to all cells meeting metabolic requirements for growth and replication. In the yeast *Saccharomyces cerevisiae* uptake of phosphate is regulated by the phosphate-repressible *PHO* system, which during phosphate starvation mobilizes a high-affinity phosphate uptake system (reviewed in Ref. [1]). One of the gene products in the high-affinity system is the Pho84 protein which catalyses phosphate uptake in symport with H^+ at an optimum of pH 4.5. This 65 kDa protein is composed of 587 amino acids and contains 12 putative transmembrane segments, with extended hydrophilic N- and C-termini.

The Pho84p as well as other Pi transporters in fungi and plants contain in the N-terminal part of the protein a partly conserved sequence motif (GKxGxxI). The importance of these conserved amino acids for the transport ability was analyzed by site-directed mutagenesis. In contrast to the Gly491Ala mutant, the Lys492Arg, Gly494Ala and Ile497Leu mutations resulted in essentially unperturbed transport function. In the light of these results, an extended study including 36 (amino acids 478–512) mutants covering the predicted span of helix XI was carried out in order to investigate their importance for the protein function. Their participation in a putative phosphate transport pathway will be discussed.

Keywords: Phosphate transporter; Pho84; *PHO* gene; Plasma membrane; Yeast; Site-directed mutagenesis

Reference

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J.12. Immunological study of the uncoupling protein 2

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UCP2 belongs to the mitochondrial carrier family of the inner mitochondrial membrane. Study of the Ucp2(− / −) mice revealed a role of UCP2 in immunity by regulating the production of reactive oxygen species (ROS). In contrast to UCP1, whose expression is restricted to the brown adipose tissue, UCP2 mRNA is widely expressed. We generated a highly sensitive polyclonal anti-human UCP2 antibody and we detected UCP2 protein in spleen, lung, stomach and white adipose tissue mitochondria, but not in heart, skeletal muscle, liver and brown adipose tissue mitochondria. Quantitative comparisons revealed that the level of UCP2 in spleen mitochondria is less than 1% of the level of UCP1 in brown adipose tissue mitochondria. Starvation and LPS treatments increased UCP2 protein level up to 12 times in lung and stomach, which support the hypothesis that UCP2 responds to oxidative stress situations. Surprisingly, up-regulation of UCP2 protein was achieved without any changes of UCP2 mRNA levels. Transient transfection of COS cells with mouse UCP2 cDNA demonstrated that the UCP2 gene is regulated at the translational level. We showed that an upstream open reading frame, located in exon two of the UCP2 gene, strongly inhibits the expression of the protein.

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J.13. The *Yarrowia lipolytica* yeast offers a fertile field for studying proton- and sodium-coupled phosphate transport systems

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In this study, we have used a newly isolated *Yarrowia lipolytica* yeast strain with a unique capacity to grow over a wide pH range (3.5–10.5), which makes it an excellent model system for studying proton- and sodium-coupled phosphate transport systems. In cells grown at pH 4.5, inorganic phosphate (Pi was accumulated by two kinetically discrete proton/Pi cotransport systems. The low-affinity system is most likely constitutively expressed and operates at high Pi concentrations. The high-affinity system comes into play during Pi starvation, being under the control of both extracellular Pi availability and intracellular polyphosphate stores. In cells grown at pH 9.5–10, Pi uptake is mediated by several kinetically discrete sodium-dependent systems that are specifically activated by sodium ions and insensitive to the protonophore CCCP. Of these, a low-affinity transporter operates at high Pi concentrations, and is to our knowledge, kinetically characterized for the first time. The other two, derepressible, high-affinity, high-capacity systems, function during Pi starvation, and appear to be controlled by the availability of extracellular Pi. They represent the first examples of high-capacity, sodium-driven Pi transport systems in an organism belonging to neither the animal nor bacterial kingdoms. The interplay between proton- and sodium-coupled phosphate transport systems is discussed.

Keywords: Yeast; Phosphate; Transport

J.14. The mitochondrial adenine nucleotide carrier from *S. cerevisiae*: structural and functional properties of each monomer within the functional dimer

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The adenine nucleotide carrier (Ancp) is a mitochondrial protein located in the inner membrane. Ancp allows the exchange of ADP versus ATP between matrix and cytoplasm. This transport is electrogenic and depends on the membrane potential. Ancp is a 32 kDa protein with potentially six transmembrane segments organized in α-helix linked by hydrophilic loops. The N- and C-termini are both exposed to the intermembrane space. Numerous studies have shown a dimeric state of Ancp in vivo. To study the structural and functional properties of each monomer within the dimer, we have constructed genes encoding covalent tandem dimers of wild-type and *op1* mutant of *S. cerevisiae ANC2*. Growth on nonfermentable carbon sources, restored by the homodimeric (Wt–Wt) and heterodimeric (Wt–*op1*, *op1*–Wt) genes were similar to the one restored by the wild-type *ANC2*, while the *op1*–*op1* homodimer did not restore growth. Expressions of these four dimeric genes led to syntheses of stable 64 kDa proteins in vivo. Their proper membrane insertion was investigated by proteolytic degradation experiments. Their ATR binding properties, a specific inhibitor of Ancp, as well as their adenine nucleotide exchange properties were compared with those of the wild-type Anc2p protein.

Keywords: ADP/ATP carrier; Topography; Subunit stoichiometry; Covalent tandem dimer; *Saccharomyces cerevisiae*

J.15. High-throughput method for the measurement of the rate of respiration in isolated yeast mitochondria. Application to the investigation of the uncoupling protein UCP1

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We have developed a method to determine the respiratory rate of isolated *S. cerevisiae* mitochondria in standard 96-well microtiter plates. The main advantages are the low protein concentration required, the small sample volume and the possibility of simultaneously analysing a large number of samples. Since yeasts are widely used for recombinant expression of eukaryotic proteins, the procedure can be adapted for high-throughput screenings when the rate of mitochondrial respiration is the parameter under evaluation. As an example, the experimental protocol has been applied to the analysis of the regulation of the uncoupling protein UCP1. The procedure is validated by comparing the determinations with oxygen electrode measurements.

J.16. Fatty acid-induced uncoupling in brown-fat mitochondria from wild-type and UCP1-ablated mice

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Initial studies of brown-fat mitochondria from uncoupling protein 1 (UCP1)-ablated mice indicated that the difference in fatty acid sensitivity between brown-fat mitochondria with or without UCP1 was minor [1,2]. However, with more optimal substrates (pyruvate and palmitoyl carnitine), marked effects of the presence of UCP1 are noticeable, with UCP1-containing mitochondria being 10-fold more oleate-sensitive and reaching maximal oleate-induced rates of thermogenesis more than double those seen in brown-fat mitochondria without UCP1. Similar results are obtained using the non-metabolizable fatty acid analogue, *β,β′*-methyl-hexadecane α,ω-dicarboxylic acid (MEDICA16). Remarkably, UCP1-dependent thermogenesis induced by oleate in brown-fat mitochondria is competitive with GDP. As no direct competition between fatty acids and the GDP-binding site exists, the competition must be functional. Albumin is less capable of reducing oleate-induced respiration in UCP1(+/+) mitochondria than in UCP1(−/−) mitochondria. Inhibition by carboxyatractyloside shows that the ATP/ADP-antiporter mediates oleate-induced uncoupling to an equal extent in brown-fat mitochondria from wild-type and UCP1-ablated mice. The inhibitor of permeability transition cyclosporin A did not affect the sensitivity to oleate. We conclude that UCP1 is responsible for the high fatty acid sensitivity of brown-fat mitochondria and that the ATP/ADP-antiporter cannot substitute for UCP1 function in mitochondria lacking that protein.

Keywords: Uncoupling protein 1; Fatty acid; Brown-fat mitochondria; ATP/ADP-antiporter

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J.17. Structural analysis of bovine ADP/ATP translocase by infrared spectroscopy

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The secondary structure of bovine heart ADP/ATP translocase (AAT) has been investigated by Fourier transform infrared (FT-IR) spectroscopy after purification and reconstitution into liposomes. The spectra indicate that protein is predominantly α-helical, but also contains an appreciable amount of *β*-sheet. A study of the rate of H/D exchange revealed that the peptide backbone is highly accessible to the bulk solvent. Inhibition of the AAT with atractyloside drastically reduces the solvent accessibility of the peptide backbone, demonstrating a significant conformational change. Polarised attenuated total reflection FT-IR spectroscopy has been used to study the transmembrane α-helical tilt angle.

Keywords: Infrared spectroscopy; Mitochondria; Transporter

J.18. The Ca^{2+}-transport system of yeast mitochondria. *Endomyces magnusii* mitochondria show no permeability transition (pore)

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Recently, we have shown that tightly coupled *Endomyces magnusii* mitochondria are endowed by two independent systems for Ca^{2+} uptake and release [1,2]. Ca^{2+} uptake by the uniport mechanism was specifically activated by low concentrations of ADP, NADH, spermine, and Ca^{2+} itself. Added together, physiological modulators provided very high (record) rates of ion uptake and an ability of yeast mitochondria to maintain a set-point at extramitochondrial Ca^{2+} concentrations comparable to the lower limit of sensitivity of the Ca^{2+} assay. The Na^{+}-independent system responsible for Ca^{2+} efflux from Ca^{2+}-preloaded yeast mitochondria was driven the proton gradient, insensitive to cyclosporin A and Na^{+}, inhibited by La^{3+}, TPP^{+}, Pi, and nigericin, while being activated by spermine and hypotonicity, thus displaying similarity with the Na^{+}-independent pathway for Ca^{2+} release from mitochondria in nonexcitable mammalian tissues. Thus, it was shown that *E. magnusii* mitochondria have a highly efficient system for Ca^{2+} uptake that is under control of low concentrations of physiological modulators. This suggests its role in regulation of intramitochondrial Ca^{2+} and, ultimately, of mitochondrial oxidation. Here we present, that *E. magnusii* mitochondria have no Ca^{2+}-dependent permeability transition (MPT, pore). All known inductors of MPT, respiratory inhibitors, depletion of mitochondria by adenine nucleotides, did not stimulate swelling of mitochondria in the presence of Ca^{2+}.

Keywords: Yeast; Mitochondria; Ca^{2+}

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J.19. Energy status of *Yarrowia lipolytica* cells grown at alkaline conditions

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In this study, we have used a *Yarrowia lipolytica* strain with a unique capacity to grow over a wide pH range (3.5–10.5). Previously [1 2 3], it has been shown that in these cells, phosphate (P_i) is transported by proton- or/and sodium-coupled systems. In cells grown at pH 4.5–6.0, phosphate is transported primarily via H^+-coupled systems. However, in cells grown at 9.5–10, P_i uptake is driven essentially by Na^+-symport, via several kinetically discrete Na^+-dependent systems that are insensitive to the protonophore CCCP. The plasma membrane potential was not significantly affected by CCCP, suggesting that a hyperpolarization of the plasma membrane visualized with DiO_6 [3] was not due to the proton electrochemical gradient. The $\Delta\psi$-fluorescence related to the mitochondrial potential was sensitive to the uncoupler CCCP (50 μM). In the respiratory chain all three points of energy conservation were functional and the bulk of electron flux was mediated through the cytochrome pathway with minor contribution of the alternative oxidase. *Y. lipolytica* cells grown at pH 9.5–10.0, displayed no glucose repression. The oxidative phosphorylation system played the predominant, if not exclusive, role in supporting cell growth and development. Therefore, cells grown at alkaline pH represent a unique model, combining Na^+-bioenergetics (transport of nutrients with Na^+-symport) at the level of the plasma membrane with classical, proton bioenergetics at the mitochondrial level.

Keywords: Phosphate; Transport; Yeast

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J.20. Systematic investigation of the uncoupling protein UCP1 by replacement with divergent UCP2 motifs

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The structure–function relationships in the uncoupling protein from brown adipose tissue UCP1 have been extensively studied. However, the molecular mechanism of transport and its regulation is still poorly understood. UCP1 is regulated by nucleotides (inhibitors) and fatty acids (activators) but fundamental aspects of their mode of action are still under debate. The mitochondrial transporter family include a growing number of proteins closely related to UCP1 (reviewed in Ref. [1]). Few details are available on their bioenergetic properties and some published data show conflicting results. We have shown that, in isolated yeast mitochondria, UCP2 appears as an uncoupling protein when it is activated by retinoic acid [2]. These data have been corroborated in intact thymocytes [3]. In contrast to UCP1, UCP2 activity does not appear to be influenced by purine nucleotides or fatty acids. To investigate the domains involved in the determination of the specific features of the different UCPs, we have created chimeric proteins by systematic substitution of divergent sequences in UCP1 by their UCP2 counterparts. We will present chimeras where UCP1 properties have been shifted to those characteristic of UCP2.

Keywords: Uncoupling protein; Mutagenesis; Regulation; Mitochondria; Transport

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J.21. Transmembrane arrangement of the uncoupling protein UCP1 and topological considerations on the nucleotide binding site

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The increasing number of amino acid sequences of membrane proteins and the difficulty in obtaining high-resolution structures have resulted in an increasing effort to develop methods of structure prediction. The available bioinformatic tools have made the prediction of membrane-spanning regions nearly an elementary task but modelling the packing of these transmembrane segments is still a difficult assignment. Nevertheless, the membrane environment imposes important restrictions to the number of possible folds and therefore tertiary structure predictions are easier than those for globular proteins. To date, modelling is still the only possible way to visualize potential structures for most membrane proteins but those models can subsequently be tested experimentally. We will discuss a model for the transmembrane arrangement of the UCP1 and we will consider the possible location of residues that may be important for nucleotide binding. Our main effort is directed towards the analysis of the structural elements that are important for nucleotide binding and how these elements can be understood in a 3-D model.

Keywords: Uncoupling protein; Structure; Sequence analysis; Nucleotide binding; Topology

J.22. Interactions of potassium channel effectors with mitochondria

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Intracellular targets for potassium channel openers and antidiabetic sulfonylureas such as glibenclamide have recently attracted attention due to the identification of potassium channels in the inner mitochondrial membrane. These include mitochondrial ATP-regulated and big conductance (BK) potassium channels. Especially, the cardiac mitochondrial ATP-regulated potassium channels play an important role in protecting cardiomyocytes during ischemia/reperfusion. In the present work, we have analyzed effects of potassium channel effectors on mitochondrial function. We will show the effects of BK channel openers such as CGS7184 and CGS7181 on human glioma cells mitochondria leading to calpain-dependent cell death. Further, we will report activation of cyclosporin A sensitive permeability transition pore by glibenclamide in skeletal muscle mitochondria. Finally, the effects of mitochondrial potassium channel opener-diazoxide on expression of pro- and anti-apoptotic proteins in rat neonatal cardiomyoctes will be presented.

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Keywords: Mitochondria; Ion channel; Potassium channel opener

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J.23. ATP/ADP antiporter participates in fatty acids mediated uncoupling in potato mitochondria

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The effects of in vivo cold treatment and in vitro treatment with laurate, carboxyatractylate, atractylate, nucleotides and BSA in potato tuber mitochondria have been studied. It was found that 48–96 h cold exposure of the tubers results in some uncoupling which could be completely reversed by BSA and partially by ADP, ATP, GDP, UDP, carboxyatractylate and atractylate. UDP, GDP and ATP is less effective than ADP, and atractylate is less effective than carboxyatractylate. The recoupling effects of nucleotides were absent when nucleotides were added after carboxyatractylate. Some catalytic properties of nucleotide and fatty acid transportation were studied and inhibition constants for attactiloside and carboxyattractiloside were calculated. It is concluded that the cold-induced fatty acid-mediated uncoupling in potato mitochondria is partially (to essential part) due to operation of the ATP/ADP antiporter.

This study was partially supported by INTAS and RFBR.

Keywords: ATP/ADP antiporter; Fatty acid; Uncoupling; Plant mitochondria

Session K: Mitochondrial Physiology

K.1. UCP1 and UCP2 in mice

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In brown adipose tissue, the uncoupling protein UCP1 is the main $\Delta\mu H^+$ consumer, and is responsible for heat production in this thermogenic organ of mammals. Studies with transgenic mice have demonstrated that expression of UCP1 in muscle could lead to resistance to the obesity induced by a hyperlipidic diet [1]. Using a model of transgenic mice produced in the laboratory [2], we observed a specific reduction of muscle mass. It seems that the muscles are differently affected according to their workload, and for example, the heart could support very high level of UCP1 expression without showing obvious phenotype. This indicates that expression of a UCP is not per se deleterious to ATP formation, but could influence muscle differentiation, when muscles are poorly recruited for contraction.

Ucp2 and *Ucp3* are two genes coding for proteins closely related to UCP1. Many experiments using recombinant expression or reconstitution support the hypothesis that UCP2 and UCP3 like UCP1 are able to transport protons and therefore to uncouple partially mitochondria. However, our studies with isolated mitochondria from *Ucp2* knockout mice hardly support this hypothesis [2]. This contradiction will be discussed.

Keywords: Uncoupling protein; Mitochondrial carrier; Energy expenditure; Muscle

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K.2. Control of mitochondrial function in permeabilized cardiac fibers by the calcium-induced sarcomere contraction

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To investigate whether the structural changes in sarcomere during Ca^{2+}-induced contraction may influence the regulation of mitochondrial function in the heart, mitochondria in permeabilized cardiomyocytes and myocardial fibers were investigated in situ by oxygraphy and confocal microscopy. The results showed that apparent K_m for exogenous ADP decreased with increase of free Ca^{2+} concentration from 320 μM at 0.4 μM $[Ca^{2+}]_{free}$ to 50 μM at 3 μM $[Ca^{2+}]_{free}$. V_{max} decreased only two times. The fibers transferred from 3 to 0.1 μM Ca^{2+} solution again displayed the high apparent K_m(ADP) and recovered V_{max}, demonstrating the reversibility of these effects. Similar decline of membrane potential observed in the presence and absence of CsA excluded permeability transition. Apparent K_m(ADP) in ghost fibers after myosin extraction (300 μM) did not change by the increasing $[Ca^{2+}]_{free}$, indicating the involvement of contractile apparatus. Thus, the changes in apparent K_m(ADP) upon increase of $[Ca^{2+}]_{free}$ are related to contraction and structural reorganization of mitochondria within their functional complexes, visible by confocal microscopy in normal but not ghost cardiomyocytes.

Conclusion: Mitochondrial regulation can be reversibly modulated by Ca^{2+}, demonstrating a high sensitivity to the structural state of intracellular energetic units (ICEU) as an important determinant of this regulation in vivo.

Keywords: In situ mitochondria; Regulation of respiration; Sarcomere contraction; Calcium

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K.3. Confocal immunofluorescence microscopic study of mitochondrial–cytoskeletal interactions in isolated cardiomyocytes

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Mitochondria in heart cells in vivo are linked by some cytoskeletal proteins to sarcomeres and sarcoplasmic reticulum to form intracellular energetic units (ICEU). In this study, we investigated which component of cytoskeleton may be responsible for this binding. Plectin, tubulin, MAP-2 and desmin localizations were studied before and after treatment of permeabilized cells by trypsin by immunofluorescence confocal microscopy.

Methods: Permeabilized cardiomyocytes were incubated with Trypsin 100 nM or 1 μM (15 min, 4 °C). After cell washes, MitoTracker® Green FM was added (45 min, 4 °C). Then, fixed cardiomyocytes were incubated with primary antibody followed by secondary antibody-TRITC. Imaging of cardiomyocytes was performed by biphotonic confocal microscope (LSM510-NLO, Zeiss, 40 × oil immersion lens, NA 1.2).

Results: MitoTracker® Green FM appears to preferentially accumulate in mitochondria and gives an organisation of mitochondria in cells with a punctuated staining and linear position. If cardiomyocytes were treated with trypsin (5 μM), this organisation was destroyed and mitochondria were released from the cell structure. Tubulin was still present in cardiomyocytes after saponin permeabilisation but not after trypsin (1 μM) treatment. The same effect was observed with plectin but not with desmin.

Conclusion: Two cytoskeletal proteins—plectin and tubulin—may participate (with others) in structural organization of ICEUs.

Keywords: Mitochondria; Cytoskeleton; Cardiomyocyte; Plectin; Tubulin; Desmin; MAP-2

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K.4. Influence of H_2O_2 on protein phosphorylation in mitochondria isolated from rat brain and muscle. Consequences on oxydative phosphorylation

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In addition to their role in ATP synthesis, mitochondria are also a source of reactive oxygen species (ROS) [1]. ROS can modulate the activity of the enzymes involved in the mitochondrial signalisation: kinases and phosphatases [4,5] which are responsible for the reversible phosphorylation of some respiratory chain complexes [2,3]. Our aim was to study: (i) the ROS production of mitochondria from different tissues, (ii) the phosphorylation of detergent-extracted mitochondrial proteins resulting from addition of ATP and also during respiration at state 3 (ADP added) and ROS produced by complex III inhibition, (iii) the influence of H_2O_2 and dibutyryl-AMPc on respiration.

Results showed that ROS production was lower in the brain than in the muscle, and the activity of MnSOD and glutathion peroxidase was higher.

Phosphorylations of mitochondrial proteins were detected on both serine and tyrosine residues, with muscle mitochondria showing no phosphorylated proteins above 100 kDa, in contrast to brain mitochondria. The phosphorylation of a protein at about 75 kDa was sensitive to H_2O_2, phosphatase inhibitors and to ATP produced during state 3 respiration in both tissues. H_2O_2 and dibutyryl-AMPc decreased respiration at state 3. These results confirm that OXPHOX enzymes can be regulated by phosphorylation and are sensitive to oxydative stress.

Keywords: ROS; Signalisation; Tissue specificity; Kinase; Phosphatase; Ageing; cAMP

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K.5. Nitric oxide and calcium together inactivate mitochondrial complex I and induce cytochrome *c* release

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Cellular nitric oxide and calcium levels have been reported to increase during various pathologies, including particularly ischaemia. In this study, we investigated whether elevated nitric oxide and calcium levels can synergistically damage isolated rat heart mitochondria. We found that NO and calcium together inhibited the oxygen consumption of mitochondria respiring on pyruvate but not on succinate. In the same conditions, complex I activity was synergistically inhibited by NO and calcium and this inhibition was completely prevented by superoxide dismutase or urate suggesting that the inhibition was mediated by peroxynitrite. The inhibition of complex I activity by NO and calcium was reversed by reduced thiols or light (as was complex I inhibition by S-nitrosothiols or peroxynitrite) suggesting that the inhibition may involve S-nitrosation of complex I. However, NO and calcium caused loss of mitochondrial cytochrome *c*, and the induced inhibition of respiration was partially reversed by addition of exogenous cytochrome *c*. Thus, NO and calcium appear to synergistically inhibit mitochondrial respiration, partly by inactivation of complex I and partly by inducing cytochrome *c* release.

Keywords: Mitochondria; Complex I; Nitric oxide; Respiration; Cytochrome *c*

K.6. Mitochondrial biogenesis, aging and oxidative stress in *Saccharomyces cerevisiae*: investigation on the function of Uth1p

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From different approaches, a new gene family termed the SUN family (*SIM1*, *UTH1*, *NCA3* and *SUN4*) has been identified, the products of which are highly homologous. The remarkable feature of the four gene products is that they share a common C-terminal domain of 258 amino acids bearing 75–85% identity. These C-terminal regions comprise a putative Fe-binding domain consisting in Cys residues organized in a Cys-X5-Cys-X3-Cys-X24-Cys motif. Despite this remarkable homology, the SUN family gene products are involved in various cellular functions [1 2 3]. Sun4p, Uth1p and Sim1p were found to be cell wall proteins extractable by DTT-treatment of intact cells. In addition, Uth1p and Sun4p were found to have a mitochondrial localization. Sub-localization experiments show that Uth1p is inserted in the outer mitochondrial membrane and that Sun4p is preferentially a matrix protein [4].

UTH1 is a yeast aging gene that has been isolated on the basis of a better stress resistance and longer life span of mutants. It was further shown to participate in both oxidative-stress response and mitochondrial biogenesis. In cells inactivated for *UTH1* gene (Δ*uth1*), cytochromes aa_3, *c* and *b* were lowered by 25%, 20% and 15%, respectively, and measurements on whole lysed cells showed that another mitochondrial enzyme, citrate synthase, was also lowered. Electron micrographs showed no difference in global mitochondria content, and cardiolipid and mtDNA quantifications were equivalent in parental and Δ*uth1* strains. These results suggest that the protein equipment could be lowered in mitochondria from these strains (Δ*uth1*). Despite this decreased content in mitochondrial complexes, Δ*uth1* strains were not affected in their generation time and exhibited an extended life span. It has also been observed that the expression of a proapoptotic gene (*BAX*) in the Δ*uth1* strain did not lead to apoptosis. On the other hand, the overexpression of *UTH1* gene under the control of a high promoter (promoter gal) in a wild-type strain was lethal.

Keywords: *S. cerevisiae*; Aging; Mitochondrial biogenesis

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K.7. Mitochondrial reactive oxygen species as negative regulators of white adipose tissue development

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Reactive oxygen species (ROS) generated in cells are involved in different events as subcellular messengers in gene regulatory and signal transduction pathways. Accumulating evidence suggests that the site of ROS production is crucial in determining their effect. We studied the consequences of a moderate mitochondrial ROS production in white preadipocyte growth and differentiation.

Respiratory chain inhibitors (rotenone or oligomycin) increased ROS, inhibited growth and ERK1/2 phosphorylation and activated p38 MAPK activity in 3T3-L1 preadipocytes and human stroma vascular cells. These effects are partly prevented by radical scavengers (butyl-hydroxianisole, BHA or trolox).

Differentiation of 3T3-L1 preadipocytes was decreased by rotenone, antimycin that generate mitochondrial ROS. On the contrary, radical scavengers as ascorbic acid, BHA, butyl-hydroxytoluene, BHT and trolox or uncoupler of oxidative phosphorylation, carbonyl cyanide *m*-chlorophenyl-hydrazone (CCCP) increased differentiation. This modulation of differentiation paralleled with modification of transcription factors as peroxisome proliferator-activated receptor (PPAR gamma 2).

This work demonstrates that endogenous mitochondrial ROS act as negative regulators of preadipocyte growth and differentiation. These effects are mediated via the key regulators of these events (MAPK, PPAR gamma 2). However, the physiological importance of such regulation remains to be determined.

Keywords: Reactive oxygen species; Mitochondria; Adipose tissue

K.8. Differential effect of triiodothyronine on mitochondrial energy coupling in piglet glycolytic and oxidative muscles

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Thyroid hormones play a pivotal role in mitochondrial biogenesis, energy metabolism and differentiation. Recently, it has been suggested that UCP3 may act as a molecular determinant in the regulation of resting metabolic rate (RMR) by T3 [1] in the mixte gastrocnemius muscle of rat.

The aim of this study was to get some insights into the effect of T3 on mitochondrial metabolism in a fast glycolytic (longissimus thoracis, LT) and a slow oxidative (rhomboideus, RH) muscles of piglets.

We have determined RMR, mitochondrial respiratory capacity, membrane potential and UCP2 and UCP3 mRNA levels in skeletal muscles of hypothyroid, euthyroid or hyperthyroid piglets. As expected, T3 increased RMR but effect on mitochondrial metabolism differed according to skeletal muscle metabolic type. Nonphosphorylating respiration rate (state IV), proton leak and UCP3 were significantly increased in LT of T3 treated piglets, whereas the treatment had no effect in RH muscle. UCP2 mRNA level was not modified by the treatment. Our results show that the promoting uncoupling effect of T3 on skeletal muscle mitochondrial metabolism is both gene- and muscle-specific.

Keywords: T3; Skeletal muscle; Mitochondrial metabolism; UCP; Piglet

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K.9. Hydrogen peroxide production during the mitochondrial respiration in the *Taenia crassiceps* metacestode

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In *Taenia crassiceps* metacestode, the oxygen consumption during mitochondrial respiration is quite resistant to most classical respiratory inhibitors, suggesting the presence of an alternative pathway. We have also found a spectral signal for a carbon monoxide complex, with the spectral features of a cytochrome-*o*-CO-like complex. The presence of a respiratory branch, with this kind of cytochrome has been frequently proposed in this group of parasites.

Nevertheless, by photodissociation experiments at subzero temperatures, or by HPLC analysis of the haem groups of metacestode mitochondria, we demonstrate that this putative cytochrome oxidase is absent and therefore is not responsible for the inhibitor-resistant pathway.

Under physiological conditions, mitochondrial respiration produces 1.0–2.0% of superoxide radical in addition to water. By dismutation, this superoxide forms hydrogen peroxide. In the metacestode, we demonstrate that from the total of the oxygen consumed in the electron transport, at least 10 times more H_2O_2 is produced as compared with rat liver mitochondria, measured under the same conditions, this suggests that it could be responsible for the alternative pathway observed.

This work was supported by DGAPA-UNAM IN-212600.

Keywords: Hydrogen peroxide; Electron transport; Parasite

K.10. Biochemical threshold effect and tissue specificity: molecular basis and case of the adenine nucleotide translocator

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The molecular basis of the biochemical threshold effect able to explain why deficiencies in the OXPHOS complexes do not necessarily affect the mitochondrial respiration or the ATP synthesis and thus not lead to pathology remains unclear [1]. Furthermore, this new mechanism of threshold effect is also able to explain the tissue specificity observed in mitochondrial pathologies [1]. We have determined in isolated mitochondria from five different rat tissues, therefore in whole fibroblast cells, the functional amount of adenine nucleotide translocator (ANT) [2] involved in different steady state of respiration by using the carboxyatractyloside (CATR). By increasing the respiratory rate, this amount of carriers appears increased in muscle, brain and fibroblasts but not in heart and liver. This may be due to either a mobilization of free subunits to constitute new functional carriers or a mobilization of carriers taking part in macrocomplexes. These findings were confirmed (i) by two-dimensional gel electrophoresis where a coexistence between monomers and oligomers of ANT was detected in muscle but not in heart, (ii) and by using Cyclosporine A able to increase this amount of carriers involved in the respiratory rate. In this work, we evidence that OXPHOS complexes like ANT for instance may mobilize some enzymes not initially taking part in the respiratory rate. Such a molecular recruitment could constitute a first explanation of the biochemical threshold effect phenomenon in addition to the metabolic network attenuation.

Keywords: OXPHOS; Mitochondrial disease; Threshold effect; ANT; Mobilization

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K.11. Mitochondrial permeability transition and diabetes

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The mitochondrial permeability transition (MPT) has been related to some forms of mitochondrial dysfunction. In this study, we evaluated the effect of diabetes on MTP susceptibility. Liver mitochondria were isolated from STZ (type 1 diabetes), 3 and 9 weeks after treatment with streptozotocin, and from Goto–Kakizaki (GK) rats (type 2 diabetes), with 3 and 6 months of age. Control animals were nondiabetic Wistar rats of similar age.

We observed that MPT induction was significantly decreased both in GK and STZ rats and that this decrease was related to the progression of the disease. We also observed that liver mitochondria from diabetic rats presented a higher content of CoQ_9 and CoQ_{10} when compared with control rats. Additionally, diabetic rats exhibited a higher respiratory transmembrane electric potential. Our results suggest that the decreased susceptibility of MPT was inversely related to CoQ content and membrane potential, and may correspond to an adaptative response to the increased oxidative stress in diabetes.

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K.12. Preconditioning alters mitochondrial permeability transition pore opening in the rabbit heart

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There is increasing evidence that mitochondria are involved in the molecular events leading to the tissue damage in different pathophysiological situations, including ischemia/reperfusion. Recent studies suggest that preconditioning, which has antinecrotic and antiapoptotic effects, may protect the myocardium through modulation of mitochondrial physiology, specially ion fluxes. The aim of the present work was to determine whether preconditioning might influence mitochondrial permeability transition pore opening (MPT).

Anesthetized NZW rabbits underwent either no intervention (sham; $n = 10$) or a test episode of 10 min ischemia followed by 5 min of reperfusion. Before this test ischemia/reperfusion, rabbits underwent either no intervention (control, $n = 8$) or preconditioning (5 min ischemia/5 min reperfusion; PC $n = 9$). Some animals were treated with ciclosporin perfusion (CsA, Novartis); 10 mg/kg; 10 min before preconditioning (PC-CsA; $n = 8$) or before the test ischemia/reperfusion (control-CsA; $n = 8$).

For all hearts, myocardium from the area at risk was rapidly harvested at the end of protocol. Mitochondria were isolated after homogenisation in a buffer containing 70 mM sucrose, 210 mM mannitol, 1 mM EDTA in 50 mM Tris/HCl pH 7.4 with a Kontes tissue grinder. After differential centrifugation, mitochondria were washed in the same buffer containing 0.1 mM EDTA and aliquots of 6 mg of mitochondria were prepared for calcium overload analysis. Quality of the preparation was assessed by marker enzyme analysis and electron microscopy. Mitochondrial Ca^{2+} uptake and release was measured as a change in the free calcium concentration within a suspension of 6 mg mitochondria in a buffer containing 150 mM sucrose, 50 mM KCl, 2 mM KH_2PO_4, 5 mM succinic acid in 20 mM Tris/HCl pH 7.4, using a calibrated Ca^{2+} selective mini electrode. MPT pore opening was defined as the massive release of Ca^{2+} by mitochondria in the suspension, following repeated Ca^{2+} pulses. Specifically, we measured total Ca^{2+} overload necessary to open the MPT pore.

In the control group, total Ca^{2+} overload responsible for MPT pore opening was significantly reduced versus sham. Interestingly, preconditioned mitochondria needed a higher Ca^{2+} overload to open the MPT. Mitochondria prepared from animals treated with ciclosporin were particularly resistant to calcium overload.

These data suggest that preconditioning may modulate mitochondrial permeability transition pore opening in the rabbit heart and that ciclosporin protected mitochondrial physiology in regard to calcium fluxes.

Keywords: Heart; Mitochondria; Ischemia; Preconditioning; Calcium

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K.13. Genotype–phenotype relations in skeletal muscle of patients with deletions and point mutations of mtDNA

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To characterize genotype–phenotype relations we performed quantitative analysis of biochemical, genetic and morphological findings in skeletal muscles of patients with mutations of mtDNA [1 2 3]. Three types of mutations were compared. At large-scale deletions, tRNA's as well as several proteins were missing, causing defects at the level of transcription and translation (I). At the A3243G mutation, $tRNA^{Leu}$ is affected (II, translation defect), whereas G11778A mutation affects ND4 (III, transcription defect).

In patients with deletions, CS related activities of respiratory chain complexes (I, I + III, II + III, III, COX) and state 3 respiration were decreased in the same extent, linearly correlating with heteroplasmy. Changes did not correlate with the deletion size. Apparently all mitochondrial coded proteins were affected in the same manner. Citrate synthase activity and number of COX-negative fibers linearly increased with heteroplasmy of deletion.

A3243G mutations caused similar changes on the enzymatic and functional levels but to a lesser extent in comparison to large-scale deletions. The smallest changes we observed were in a patient with G11778A mutation despite of a 100% heteroplasmy. These differences have implications for the thresholds of heteroplasmy causing functional deficits. Thresholds are low or absent in group I-, higher in group II- and highest in group III-mutations of mtDNA.

Keywords: Skeletal muscle; Mitochondria; MtDNA; Deletion; Mitochondrial function

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K.14. Influence of insulin, insulin-dependent cytoplasmic regulator on energy metabolism of rat thymocytes

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The activity of insulin-dependent cytoplasmic regulator (IDCR) is increased by insulin injection [1]. IDCR increases Ca^{2+} capacity, decreases substrate oxidation in isolated suspension of rat liver mitochondria and similar to insulin, reduces $[Ca^{2+}]_{in}$ in thymocytes by means of stimulation of Ca^{2+} transport into mitochondria and endoplasmic reticulum [2].

In this work, we investigated the influence of insulin and IDCR on energy metabolism of the rat thymocytes, depending on oxidation substrate and level of $[Ca^{2+}]_{in}$.

It was established that insulin in concentrations 100 μU/ml and IDCR in concentration 10^{-5} μg/ml inhibit respiration of rat thymocytes by 30–40%. The receptor-dependent increase in cytosolic Ca^{2+} concentration by concanavalin A (Con A) stimulates the respiration due to uncoupling of oxidative phosphorylation by 40% in thymocytes. The action of insulin and IDCR before and after Con A action causes a decrease in the rate of pyruvate and glucose oxidation and restoration of respiration and ADP phosphorylation and degree of conjugation in mitochondria of rat thymocytes. The high concentrations of insulin (1–10 mU/ml) and IDCR (10^{-4} μg/ml) cause an increase in thymocyte respiration rate and uncoupling of oxidative phosphorylation. Based on the fact that the effects of insulin in vitro are shown only on cellular level and IDCR on cellular and subcellular level in mitochondria of rat's thymocytes, it is suggested that the IDCR is a mediator of insulin action on thymocyte energetics.

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Keywords: Energetic metabolism; Insulin; Insulin-dependent regulator; Concanavalin A; Thymocyte

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K.15. Functional properties of mitochondria isolated from type I and type II skeletal muscle in pigs

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Kinetics of regulation of respiration by ADP or ATP have been shown to differ markedly in situ according to muscle fiber types [1,2], but the persistence of some of these functional differences on isolated mitochondria is controversial. Therefore, the functional properties of intermyofibrillar mitochondria isolated [3] from pig rhomboideus (68% type I) and longissimus dorsi (89% type II) muscles were compared. Maximal respiration capacity of rhomboideus mitochondria in the presence of malate plus pyruvate represented 120% of that of longissimus dorsi mitochondria. State III respiration with either malate plus pyruvate or succinate plus rotenone was also 1.3-fold higher in rhomboideus than in longissimus dorsi mitochondria, whereas no differences were observed during state IV. Therefore, rhomboideus mitochondria exhibited higher RCR values, in agreement with a 1.3-fold higher ATP synthesis rate. The K_m for ADP did not differ significantly between the two mitochondrial population, but respiration of longissimus dorsi mitochondria was more sensitive to ATP inhibition than that of rhomboideus mitochondria. These findings demonstrate that, although the specificity of regulation by ADP according to fiber type is lost, mitochondria isolated from type I and type II muscles still differ substantially in their functional properties.

Keywords: Isolated mitochondria; Respiration; Adenine nucleotide; Skeletal muscle

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K.16. Metformin prevents the mitochondrial permeability transition-induced cell death in KB cells

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Antihyperglycemic agent Metformin is widely used in the treatment of type 2 diabetes. Its glucose-lowering effect results from an inhibition of gluconeogenesis [1] and an increase in glucose utilisation, but its cellular mechanism of action is largely unknown. A beneficial effect of metformin on fatty liver has been reported [2], while activation of AMP-activated protein kinase [3] was also suggested [3]. The permeability transition pore (PTP) is involved in different models of cell deaths and recently we have reported a functional link between PTP, complex I of the respiratory chain and cellular death [4]. Since metformin inhibits complex I [5], we have investigated its effect on PTP regulation and cellular death. The present work confirms in KB cell lines the inhibitory effect of metformin on complex I, this effect prevents the PTP opening after *tert* butyl-hydroperoxyde addition, both in permeabilized cells (calcium retention) and in intact cells (calcein fluorescence). Finally, metformin significantly prevents cell death as assessed by trypan blue exclusion, annexin V and cytochrome *c* release. These data demonstrate that metformin, by inhibiting respiratory chain complex I, plays a key role in PTP regulation and confirms the importance of PTP opening in the commitment of cell death.

Keywords: Metformin; PTP; Complex I; Cytochrome *c*; Cell death

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K.17. Mitochondrial regulation in myoglobin-deficient mouse heart

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The role of redox relationships in the regulation of cellular respiration in intact organs can be studied nondestructively my means of optical probes. In heart muscle, their usefulness is hampered by the high concentration of myoglobin swamping the cytochrome spectra and quenching the fluorescence of optical probes during large excursions of intracellular oxygen concentration. A myoglobin knockout (myo $-/-$) mouse strain has been established [1].

We set out to investigate the steady-state redox state of fluorescent flavoproteins and cytochrome aa_3 upon changes in work output and oxidizable substrate in isolated hemoglobin-free perfused myoglobin-deficient heart.

It was shown that step increases in perfusate calcium within the range of 0.5–2.5 mM caused oxidation of mitochondrial matrix NADH and reduction of cytochrome aa_3 concomitantly with an increase in oxygen consumption. Also with electrical pacing, an increase in work output resulted in matrix NADH oxidation and reduction of cytochrome aa_3. Only under oxygen-limiting conditions brought about by pacing at high frequency did a reduction of NAD occur concomitantly with cytochrome aa_3 reduction.

The results indicate that metabolic substrate-level regulation has a subordinate role in the regulation of cell respiration and most of the control is exerted in the respiratory chain proper.

Keywords: Myoglobin; Cellular respiration; Cytochrome *c* oxidase; Flavoprotein; Nicotinamide-adenine dinucleotide

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K.18. Occurrence, properties and protein expression of uncoupling protein and alternative oxidase of *Dictyostelium discoideum* (Mycetozoa) during vegetative life and starvation-induced early development

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Alternative oxidase and uncoupling proteins are free energy-dissipating systems which both divert energy from ATP synthesis. Uncoupling protein dissipates the H^+ gradient built by the respiratory chain and alternative oxidase consumes the reducing power without energy conservation into a H^+ electrochemical gradient. In this study, we have shown that *Dictyostelium discoideum* alternative oxidase is stimulated by purine mononucleotides, is monomeric (non-plant type) and can efficiently divert energy from oxidative phosphorylation in state 3 respiration. Uncoupling protein of *D. discoideum* is stimulated by free fatty acids, is insensitive to GTP and can induce a decrease in transmembrane potential in state 4 and in ADP/O ratio in state 3 respiration, respectively. It has also been shown that both proteins can collaborate in energy-dissipation as they are present together. Alternative oxidase expression in free-living amoeboid cells decreases strongly from exponential phase to stationary phase but is maintained quite high during starvation-induced aggregation. On the contrary, uncoupling protein expression is constant in all conditions indicating permanent need. Our results favor a peculiar role of AOX in *D. discoideum* development and differentiation.

This work was supported by the Polish K.B.N., the Belgian F.N.R.S. and the Polish–Belgian Joint Research Project.

Keywords: *Dictyostelium discoideum* development; Alternative oxidase; Uncoupling protein

K.19. How sensitive are in situ cardiac mitochondria to stimulation by ADP?

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Mitochondria in saponin-skinned cardiac fiber bundles were reported to have an order of magnitude lower apparent affinity to ADP than isolated mitochondria [1]. Although ADP was measured outside the bundles, it was thought that the low affinity was not caused by diffusion gradients, because diffusion distances between the core of the bundle and incubation medium are relatively short. Recently, we demonstrated that increasing the intrafiber ATP splitting activity in rat heart skinned fiber bundles using yeast hexokinase and glucose may result in a 4-fold increase in the apparent affinity to ADP of the bundles, because the production of ADP inside the bundles diminishes existing diffusion gradients between the incubation medium and mitochondria. We also observed similar effects for mouse heart and rat soleus skinned fiber bundles. Our results show that the mitochondrial outer membrane is not a major diffusion barrier in these bundles as proposed previously. We conclude that the affinity to ADP of in situ and probably also in vivo mitochondria is of the same order of magnitude as that of isolated mitochondria.

Keywords: Heart; Soleus; Diffusion; ATPase

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K.20. Mitochondrial defect and effects of creatine on the survival of hippocampal neurons with defective mitochondria in an animal model of epilepsy

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Mitochondrial function is a key determinant of both excitability and viability of neurons [1,2]. We demonstrate pathological changes in mitochondrial oxidative phosphorylation and the influence of creatine supplementation on the neuronal survival and mitochondrial function in the epileptic rat hippocampus. In pilocarpine-treated epileptic rats, we observed a decline of the activities of complex I and complex IV of respiratory chain in the CA3 and CA1 hippocampal subfields. In addition, high-resolution respirometry revealed an increased flux control of complex I on respiration in the CA1 and CA3 subfields and decreased maximal respiration rates in the more severely affected CA3 subfield. After 20 days of feeding with a diet containing 2% creatine, epileptic rats showed a more pronounced loss of pyramidal neurons and a nonspecific decrease of total mitochondrial enzyme activities in all investigated hippocampal regions. Nevertheless, the citrate synthase-normalized activity ratios of complex I and complex IV in CA1 and CA3 approached upon creatine treatment almost control values. Thus, our results demonstrate that epileptic seizure activity results in the defect of mitochondrial respiratory chain complexes and creatine feeding resulted in a preferential loss of neurons with defective mitochondria which should be considered for treatment of mitochondrial diseases presenting with epileptic phenotypes.

Keywords: Hippocampus; Mitochondrial function; Oxidative phosphorylation; Creatine treatment

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K.21. Study of the creatine kinase role in the cellular resistance to tumor necrosis factor-alpha

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The aim of this work was to look for the existence of the creatine kinase (CK) isoenzymes in two cell lines which display opposite sensitivities to tumor necrosis factor-alpha (TNF-alpha). This absence or presence could partly explain the difference of cellular sensitivity to TNF-alpha cytotoxicity. CK isoenzymes were only present in the TNF-α resistant C6 but not in the sensitive WEHI-164 cells. The B-CK isoform was the major enzyme having a dimeric structure composed of 40–52 kDa subunits and localized in cytosol and at a lesser extent in mitochondria. The mitochondrial CK (Mt-CK) had a dimeric structure composed of 70 kDa subunits. TNF-α treatment did not change the CK oligomeric state, but significantly enhanced the activity of the B-CK isoenzyme present in mitochondria; any induction of CK did not occur in WEHI-164 cells. The study of the cardiolipin's acyl chains, the mitochondrial receptor of Mt-CK, did not show any major differences in both cell lines. In C6 cells, TNF-α induced an increase of double bond content of the cardiolipin's acyl chains which could indirectly act on the mitochondrial B-CK activity through the protein kinase C signal pathway.

Keywords: Mitochondria; Creatine kinase; TNF-alpha; Cardiolipin; Cell

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K.22. The role of lipid oxidation in Bax-induced cell death in yeast

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Heterologous expression of pro-apoptotic protein Bax in the yeast *Saccharomyces cerevisiae* leads to cell death. Cytochrome *c* is released from mitochondria during the process [2] via the formation of a high conductance channel in the outer mitochondrial membrane [3]. However, cytochrome *c* release is not absolutely required for Bax-induced cell death [5].

As in mammalian apoptotic cells, Bax-expression in yeast is accompanied by ROS-production and oxygen is required for Bax-induced cell death [1]. In the present study, we show that Bax also induces an oxidation of mitochondrial lipids and that this oxidation, but not ROS production, is required for Bax-killing effects.

In addition, although inducing cell death, Bax-expression is able to protect plasma membrane permeability properties against a physiological ethanolic stress, known to involve lipid oxidation [4]. This indicates that Bax is able to shift from a ''necrotic-type'' form of death towards an ''apoptotic-type'' form of death. Lipid oxidation is also involved in this effect of Bax.

Taken together, these results suggest that Bax acts at different levels on lipid oxidation and we hypothesize that it may regulate the activity of yet unidentified enzymes regulating lipid oxidation.

This can be related to activation of a plastid lipoxygenase during hypersensitive cell death in plants [6].

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Keywords: Apoptosis; Bax; Active oxygen species; Lipid oxidation; Yeast

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K.23. Virtual mitochondria and their control

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Inside the eukaryotic cell, mitochondria is the place of oxidative phosphorylation (OXPHOS), i.e. of an ATP synthesis (cell energy supply) coupled to the respiratory chain function. The control of mitochondrial ATP production is a complex problem with different patterns according to different tissues and organs.

We have already developed computer dynamic models for muscle and liver oxidative phosphorylation, tested and used them to understand and simulate metabolism perturbations as observed in the case of mitochondrial diseases, with a typical threshold effect.

Now, our aim is to continue the modelling of oxidative phosphorylation in different tissues in order to simulate their functioning and to understand the basis in their control differences.

In addition, mitochondria hold a significant part of cellular metabolism: Krebs cycle, β-oxidation of fatty-acids, etc. The second aim of our work will be to model this metabolism. In a third step, we will include this virtual mitochondria in a virtual cell by modelling the exchanges of metabolites, energy and signals (calcium signals) between the cell and mitochondria.

In constructing the complete metabolic map of mitochondria, we will take advantage of the sequenced genomes of eukaryotic organism, from which a significant part concerns mitochondria.

Keywords: Mitochondria modelling; Mitochondrial metabolism; Metabolic control analysis

K.24. Effects of bradykinin on mitochondrial respiratory chain function in an animal model of global myocardial ischemia

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The impact of Bradykinin (BDK) on cardiac mitochondrial function during acute ischemia is not yet fully known. The aim of our study was to assess this impact in an animal model of global myocardial ischemia, using a Lagendorff perfusion system. We used three experimental groups—A (control), B (ischemic) and C (ischemic with BDK). Mitochondrial fraction was isolated, and the activity of complexes I, II–III and IV of the mitochondrial respiratory chain (MRC) were measured, as well as the RCR, state 3 O_2 consumption (S3), electrical potential variation after phosphorylation and phosphorylative lag phase (PLP).

For every evaluated parameter, group A (control) showed results that were significantly higher than those of group B (ischemia). BDK (group C) always showed a significantly higher potential than group B (with the initial potentials identical in both groups), demonstrating a better recovery of electrical potential after a phosphorylative cycle. The BDK group always had a lower S3 respiration when compared with group B, without any significant differences between the RCR values in both groups. No significant differences were also found between groups B and C regarding PLP. These results can help us understand the mechanisms underlying the positive effects of BDK in acute myocardial ischemia.

Keywords: Ischemia; Bradikinin; Mitochondria; Heart

K.25. Bradykinin's protective effects on cardiac mitochondria are nitric oxide-dependent

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Bradykinin (BD) is an important vasodilator peptide, whose effects on endothelium are modulated by nitric oxide release. BD was also proposed to induce preconditioning, improving myocardial resistance to several forms of stress; however, it remains to be determined the role of cardiac mitochondria and nitric oxide in those processes.

We used an animal model of ex vivo global myocardial ischemia, after perfusion in a Lagendorff system. We used four experimental groups—A (control), B (ischemia), C (ischemia + BD) and D (ischemia + BD + L-NAME). Mitochondria were isolated and the activity (EA) of complexes I, II–III and IV was evaluated, as well as the RCR, state 3 respiration (S3), membrane electrical potential, phosphorylative lag phase (PLP) and energetic charges (EC).

When compared with group C, group D had a behavior nonsignificantly different, with the exception of the induction of an even higher increase in the EA of Complexes II–III and a decrease in the EA of Complex IV, without any important changes regarding O_2 consumption; this could mean that nitric oxide plays a key role in promoting a balanced activity of MRC during cardiac ischemia, and that can be further achieved in the presence of BD.

Keywords: Bradykinin; Ischemia; Mitochondria; Heart

K.26. Energy conservation and dissipation in mitochondria isolated from tomato non-ripening mutants (nor, rin, Nr) during development in plants: comparison with wild-type and effects of ethephon

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Tomato mitochondria contain both alternative oxidase (AOX) and uncoupling protein (UCP) as energy dissipating systems that can decrease the efficiency of oxidative phosphorylation. Tomato ripening is a suitable model to follow mitochondrial energetic evolution. As ethylene is a very important hormone in ripening process, we have worked with C.V. Pearson wild-type climacteric tomato (WT) and three isogenic mutant cultivars related to ethylene perception (never ripe—Nr) and sensitivity (non-ripening—nor and ripening inhibitor—rin) that produce non-climacteric fruits. Bioenergetics of tomato development on plant was followed from early growing stage to senescence. The measured parameters, cytochrome pathway-dependent respiration sustained by ATP synthesis, UCP activity-sustained respiration, AOX-mediated respiration, as well as the protein expression of UCP and AOX and free fatty acid content, exhibited different evolution patterns in WT and mutants cultivars that can be attributed to their climacteric/non-climacteric properties, respectively. Supplementary experiments were undertaken to point out the role of ethylene in the bioenergetic difference between WT and mutants. For this purpose, we used ethephon which can induce ripening in rin mutant.

This work was supported by the Belgian F.N.R.S. and the Polish–Belgian Joint Research Project. R.N. is recipient of a F.R.I.A. fellowship.

Keywords: Alternative oxidase; Uncoupling protein; Tomato ripening

K.27. Ciprofibrate treatment reduces susceptibility of liver mitochondria to calcium-induced permeability transition in hypertrigliceridemic transgenic mice

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Fibrates are ligands for peroxisome proliferator and activator receptors that increase expression of enzymes involved in fatty acid beta-oxidation. We investigated ciprofibrate effects on energy-linked functions of liver mitochondria in hypertrigliceridemic apo-CIII transgenic mice. The drug or placebo were given daily (10 mg/kg BW) for 21 days. Ciprofibrate reduced plasma triglycerides from 429 ± 134 to 288 ± 140 mg/dl ($P < 0.05$) but did not change FFA and total cholesterol plasma levels. Hypertriglyceridemic mice presented lower mitochondrial respiratory control as compared to normotriglyceridemic control mice (2.89 ± 0.39 vs. 3.4 ± 0.62, respectively). In contrast with normotriglyceridemic controls, mitochondria isolated from transgenic mice did not retain the membrane potential and underwent extensive swelling after accumulation of 100 nmol calcium per mg protein. Both effects were abolished by cyclosporin A, characterizing mitochondrial permeability transition (MPT). The ciprofibrate treatment reversed both the low mitochondrial respiratory control to 3.59 ± 0.49 and the susceptibility to calcium-induced MPT to control values.

Keywords: Hypertriglyceridemic transgenic mouse; Liver mitochondria; Permeability transition; Fibrate

K.28. Critical role of mitochondrial protein thiol groups in the inhibitory action of carvedilol on the mitochondrial permeability transition

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The mitochondrial permeability transition (MPT) is a widely studied phenomenon in mitochondrial bioenergetics. The role of the MPT in myocardial cell death after ischemia and reperfusion has already been described. Carvedilol, a beta-blocker with anti-oxidant properties, was shown providing a degree of cardioprotection not afforded by other beta-blockers. The aim of this work was to determine the precise mechanism of carvedilol's inhibition of the MPT, using three different inducers, each one with different mechanisms.

Hallmarks of MPT induction as membrane depolarization, swelling and decrease of calcium loading capacity were tested to study the effect of carvedilol. The effect on mitochondrial protein thiol groups was also studied as well. We showed that carvedilol was only effective when the oxidation of mitochondrial protein thiol groups was a primary event in MPT induction, and not a mere pos-MPT consequence. Carvedilol inhibited the oxidation of thiol groups with calcium plus phosphate and with calcium plus *t*-butylhydroperoxide, but not with calcium plus carboxyatractyloside. The carvedilol concentrations had no effect on the total amount of calcium loaded into mitochondria.

The antioxidant properties of carvedilol may account for the observed effect on the mitochondrial protein thiol groups.

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Keywords: Mitochondria; Permeability transition; Carvedilol; Thiol group

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K.29. Superoxide detection in membrane preparations by lucigenin is hampered by several pitfalls—coelenterazine performs better, although not ideally

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Lucigenin is widely used as a probe in superoxide production assays due to its high sensitivity, even though it has been claimed to produce superoxide via an auto-oxidative process [1,2]. It has been postulated that a one-electron reduction of luc^{2+} precedes the chemiluminescent reaction with $O_2^{\cdot -}$ [3]. We have observed that in *P. denitrificans* cytoplasmic membranes, the rotenone-insensitive activity of NDH-1 (Complex I) plays a major role in lucigenin one-electron reduction that precedes the detection of hypoxanthine/xanthine oxidase produced superoxide. It thus appears that changes in the pre-reduction of lucigenin may result in significant misinterpretation of the extent of superoxide production. Under a similar set of conditions, the probe coelenterazine displayed no dependence of its sensitivity on pre-reduction. In addition, the lucigenin signal in bacterial membranes was found to be markedly SOD-insensitive, while that of coelenterazine is largely SOD-sensitive, supporting the notion that it is a more reliable probe. On the other hand, the coelenterazine sensitivity in bacterial membranes is several folds lower than that of lucigenin, and it is not suitable for studying dodecyl maltoside-solubilised membrane due to an apparent interaction between the detergent and the probe.

Keywords: NADH–ubiquinone oxidoreductase; Superoxide; Lucigenin; Coelenterazine

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K.30. Influence of a decrease of the mitochondrial DNA on the oxidative phosphorylation

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Mitochondria possess its own circular double strand DNA (mtDNA) that is present in multiple copies in mammalian cells. However, this number can vary according to the nature and to the steady state of studied tissues [1,2].

In our study, we investigated the influence of a decrease of the mtDNA content on the respiratory rate, on ATP production, and on the enzymatic activities of various mitochondrial respiratory chain complexes. We also searched for the possible existence of a threshold in the number of mtDNA copies on the mitochondrial metabolism. For these studies, we used a set of lymphoblastoid cell lines with various degrees of mtDNA depletion, obtained by ddC treatment [3].

We have shown that mtDNA depletion causes an almost proportional decrease of the activities of the various complexes of the respiratory chain as well as of the respiration and ATP synthesis.

These results show that there is no threshold in the number of mtDNA copies on the mitochondrial energetic metabolism and that mtDNA amount in the cell seems to be one of the parameters which should be taken into account in OXPHOS regulation. Contrary to OXPHOS complexes [4], it does not seem to exist in excess of mtDNA in cells, which could compensate for a loss of a part of this DNA.

Keywords: Mitochondria; MtDNA depletion; OXPHOS

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K.31. Substrate regulation of mitochondrial form and function in human living cells

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In human living cells, mitochondria form a large network that can take several conformations [1] and in tissues, biochemical and morphologic characteristics of mitochondria differ strongly, possibly due to differences in energy metabolism [2]. Changes of mitochondrial ultrastructure can also be observed in patients harboring an impairment of mitochondrial energy production. To understand the relations between oxidative phosphorylation and mitochondrial network morphology, we used human cell lines grown in conditions where the major source of energy was derived most likely from either glycolytic ATP or mitochondrial ATP. This was obtained when the sugar present in the culture medium was, respectively, glucose or galactose. In both conditions, we analyzed the conformation of the mitochondrial network in relation to its function by targeting to the matrix space two new ratiometric GFP-biosensors that act like a pH or a redox state indicators. In these cells, we also determined the proliferation rate, mitochondrial content, respiratory rate and cytochrome *c* oxydase activity, mitochondrial internal organization, and the expression level of the respiratory chain complexes. Our results show substantial differences in cells grown in galactose compared to glucose, suggesting that mitochondrial function is highly related to and controls the morphology of the organelle via protein biogenesis.

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K.32. Lipopolysaccharide and TNFα-stimulation of uncoupling protein UCP2 mRNA in rat liver and hepatocytes

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A major role in inducing a febrile response is played by pyrogenic cytokines IL1α and IL1β, TNFα, IL6 and IFN. Upregulation of UCP2 transcription by pyrogenic cytokines and lipopolysaccharide (LPS, simulating bacterial infection) have been previously found [1,2]. We quantified UCP2 mRNA using the RT PCR on a Real Time PCR instrument (Lightcycler, Roche) with own designed set of primers and hybridization probes, while calibration with dilution series of the amplicon ligated into the AdvanTAge vector (Clontech) was performed. LPS led to a high increase in UCP2 mRNA protected by indomethacine in rat liver. Cytokine-stimulation was documented by observing the direct stimulating effects of TNFα on UCP2 transcription in isolated hepatocytes, sensitive to indomethacine. Also hepatocytes isolated from LPS-treated rats exhibited high levels of UCP2 mRNA in comparison to controls. All these results suggest a role of mitochondrial UCP2 as a terminal heat-producing effector during fever.

Keywords: UCP2 transcription; Pyrogenic cytokine; TNFalpha; Fever

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K.33. Mitochondrial respiratory function within intracellular energetic units of the cardiac cell

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The role of mitochondrial structure, arrangement and interconnections for the metabolic channeling and energy transfer within intracellular energetic units (ICEUs) were investigated by confocal imaging and enzyme kinetics/competition approaches.

The difference in regulation of mitochondrial respiration in vitro versus in situ was independent on the composition of the medium and cannot be explained by the changes in the matrix volume. Alternatively, the controlled proteolysis and hypo-osmotic treatment of in situ mitochondria resulted in decrease of apparent K_m(ADP), stimulatory effects of creatine and AMP and loss of ADP micro-compartmentalization revealed by PK/PEP entrapping. Confocal imaging of mitochondrial flavoproteins and NADH has demonstrated the disorganization of mitochondrial regular position and loss of protein-based interconnections after proteolysis. In contrast, the regular mitochondrial arrangement was preserved in hypoosmotic shock, despite the matrix swelling and severe rupture of mitochondrial outer membrane.

Therefore, both precise mitochondrial structural organization and integrity of membranes are crucial for the metabolic channeling within ICEU, based on the intracellular coupled CK and AK systems and heterogeneous ADP diffusion. The knowledge of metabolic consequences of mitochondrial functional complexes in normal and pathological conditions provides a basis for the understanding of generalized principles of mitochondrial regulation and mechanisms of ischemic and cardiomyopathic failures.

Keywords: Heart; Respiration; Energy transfer; Compartmentation; Channeling

K.34. Activity of carvedilol against oxidative damage in heart mitochondrial proteins

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Background: The electron transport chain of mitochondria is well known for its intrinsic production of reactive oxygen species (ROS) as toxic products during respiration. However, in several cardiopathologies (p.e. ischemia/reperfusion injury), reactive oxygen overwhelms the normal defence systems. Free radicals attack polyunsaturated fatty acids and proteins in the cell membrane. Transition metals, like iron ions, have an integral role in the generation of ROS which initiated lipid and protein oxidative damage via a Fenton-like reaction. We had already shown that lipid damage induced on heart mitochondria by iron is inhibited by carvedilol, a new vasodilating beta-adrenoceptor antagonist [1].

Methods and results: In the present study, we observed protein oxidative damage induced in isolated rat heart mitochondria in the presence of ADP-Fe^{2+} as oxidative inducer. Oxidative modifications of mitochondrial proteins indicated by thiol contents decrease, carbonyl group formation and changes on tryptophan spectra were found. In the same conditions, carvedilol present a concentration-dependent protective action against protein oxidation.

Conclusions: Our observations strongly suggest that carvedilol as antioxidant compound in addition to inhibition of lipid peroxidation on mitochondria also presents important activity against protein oxidation, both might contribute to the global antioxidant cardioprotective effects of carvedilol.

Keywords: Heart mitochondria; Reactive oxygen species; Oxidative stress; Protein oxidation; Carvedilol

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K.35. Toxicity of caffeine in heart mitochondria: enhancement of permeability transition and disturbance of mitochondrial bioenergetic processes

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Caffeine (1,3,7-trimethylxanthine), a compound present in beverages such as tea and coffee, is known to be toxic at high concentrations. Some of the observed clinical conditions include cardiovascular disease and reproductive disorders, among others. The possible toxic effects of caffeine on heart mitochondria are still poorly understood. The objective of this study was to investigate whether caffeine, at toxic concentrations, influenced mitochondrial function, including calcium loading capacity. Our results showed that caffeine reduced mitochondrial ability to accumulate calcium by increasing the susceptibility of heart mitochondria to the opening of the transition pore. Caffeine also showed a complex array of effects on heart mitochondrial bioenergetics, as evaluated by respiratory parameter measurements and the top-down approach. We observed an increase in state 4 respiration and a depression in state 3 respiration. The top-down analysis revealed caffeine affects the respiratory chain but not the membrane proton leak. Rate of ATP synthesis was also affected by caffeine. Nevertheless, the ADP/O of isolated heart mitochondria was unchanged.

Our work may be relevant to cardiovascular problems linked to caffeine toxicity and also to in vitro experiences involving caffeine-induced calcium release from the sarcoplasmic reticulum and uptake by mitochondria.

Keywords: Caffeine; Mitochondria; Cardiotoxicity; Permeability transition

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K.36. On the mechanisms of the reciprocal control between nitric oxide and cytochrome *c* oxidase in cultured tumor cells

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The reaction mechanism involved in the interaction between NO and cytochrome *c* oxidase (CcOX) has been investigated in situ using cultured human SY5Y neuroblastoma cells, whose respiratory chain is reversibly inhibited by NO, either exogenously or endogenously provided [1]. The experiments were carried out based on the hypothesis, verified using purified CcOX in detergent solution, that in turnover, and in the presence of NO, either the nitrosyl- or the nitrite-bound adduct of CcOX may accumulate, depending on experimental conditions [2].

Here we report oxygraphic measurements carried out using neuroblastoma cells, and showing that: (i) respiration is promptly and reversibly inhibited by exogenous NO, (ii) either the nitrosyl- or the nitrite-bound inhibited adduct is formed depending on the concentration of reductants at the CcOX site (reduced cytochrome *c*) (iii) after inhibition, in the absence of external reductants and after NO scavenging (by hemoglobin) the respiratory efficiency is restored, with a kinetics characteristic of the nitrite displacement from the active site, showing that CcOX in neuroblastoma cells is able to efficiently catalyse the oxidation of toxic NO to nitrite.

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Keywords: Nitric oxide; Respiratory chain; Mitochondria; Reaction mechanism; Control of respiration

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K.37. Release of Mg^{2+} from mitochondria by long-chain fatty acids in alkaline saline media

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We have previously found that myristic acid initiates release of endogenous Mg^{2+} from rat liver mitochondria suspended in alkaline KCl medium [1]. Here we report on studies on the mechanism of this fatty acid-linked Mg^{2+} release. The following results were obtained: Rapid release of Mg^{2+} in alkaline medium occurs only with fatty acids that have protonophoric activity. These fatty acids induce Mg^{2+} release in saline but not in sucrose media. Mg^{2+} release is not due to a damage of the membrane integrity or the formation of unspecific pores. The rate of Mg^{2+} release drastically decreases with decreasing medium pH. The rate of Mg^{2+} release is doubled by energization of mitochondria with respiratory substrates at pH 7.2–7.6 but not at 8.0. Mg^{2+} release is accompanied by cyclosporin A-insensitive large-amplitude swelling of mitochondria. In energized mitochondria, both swelling and Mg^{2+} release are blocked by the exogenous K^+/H^+ exchanger nigericin. These observations suggest that long-chain fatty acids under alkaline conditions activate latent Mg^{2+}-sensitive ion-conducting pathways, which mediate swelling and Mg^{2+} release. We hypothesize that fatty acids activate an intrinsic Mg^{2+}/H^+ exchanger that is related to, or identical with, the K^+/H^+ exchanger.

Keywords: Fatty acid; Mg^{2+} transport; K^+/H^+ exchange; Anion channel; Swelling; Mitochondria

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K.38. Activity of an inner mitochondrial membrane channel reduced by methyl pyruvate

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An ATP-sensitive K-channel, mK(ATP), was previously described in the inner mitochondrial membrane. It might be important for NO signaling, insulin secretion, intracellular Ca^{2+} regulation, or ischemia–reperfusion injury. Only one paper described it at the single channel level [1]. We tried both, demonstrating mK(ATP) by patch clamp methods and testing if methyl pyruvate (MPyr), thought to be substrate, could block K(ATP) channels in plasma (pK(ATP)) and mitochondrial membranes.

Mitochondria were prepared from cultured Jurkat T-lymphocytes as described by Borecky et al. [2] and single channel recordings were made from mitoplasts (inner membrane). Whole-cell recordings were performed at mouse pancreatic B-cells.

A blocking effect of 20 mM MPyr on the pK(ATP) was proven by whole cell experiments. It could be partially reversed by diazoxide. Recording from mitochondrial membranes, we occasionally observed current events of about 55 pS. These events appeared regularly after pretreatment with 50 uM diazoxide. After adding 20 mM MPyr, Po was reversibly reduced. Also 8 mM ATP blocks the channel. We conclude that a channel exists in the inner mitochondrial membrane that can be activated by diazoxide and is partially blocked by MPyr and ATP. Therefore, it is likely to be the mK(ATP) channel.

Keywords: Methyl pyruvate; ATP; K-channel; Potassium; Mitochondria

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K.39. The protective effect of isosorbide dinitrate on cardiac mitochondria during ischemia is not mediated by nitric oxide

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Several authors have emphasized the role of nitric oxide in the isosorbide dinitrite anti-ischemic (IDN) effect, although it is not known if nitric oxide mediates its possible protective effect on mitochondrial bioenergetics.

We used an animal model of ex vivo global myocardial ischemia, with the use of a Lagendorff perfusion system and four experimental groups—A (control), B (ischemia), C (ischemia + IDN) and D (ischemia + IDN + L-NAME). Mitochondria were isolated and used to evaluate the activities (EA) of complexes I, II–III and IV of the mitochondrial respiratory chain (MRC), as well as the RCR, state 3 respiration (S3), mitochondrial membrane electrical potential (delta psi), phosphorylative lag phase (PLP) and energetic charges (EC).

IDN significantly decreased S3 and was able to reduce the PLP; regarding delta psi, the result was globally positive, but only statistically significant when glutamate/malate was used. The EA of Complexes II–III was statistically superior to that of the ischemic group. In the group treated with L-NAME, the results were similar to those of the IDN group.

The results allowed us, for the first time, to hypothesize that the cytoprotective effects of IDN regarding cardiac mitochondrial metabolism are not mediated by the release of nitric oxide.

Keywords: Isosorbide dinitritel; Ischemia; Mitochondria; Heart

K.40. Correlation between altered ATPase 6 gene and dysfunction of mitochondria from cells of patients affected by different neuropathies

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The F0 sector of the mitochondrial ATP synthase (ATPase) contains two essential polypeptides, ATPase 6 and ATPase 8, encoded by mtDNA. ATPase 6 corresponds to subunit a of *E. coli*, and it is involved in proton translocation, whereas ATPase 8 seems to play an important structural role [1,2]. In the last decade, several point mutations in the ATPase 6 gene have been reported, including the T8993G and the T8993C, that change Leu-156 with Arg and Pro, respectively [3]. If present in high proportions in the patient tissues, the two mutations are pathogenic for several syndromes including neuropathy, ataxia, retinitis pigmentosa (NARP) and the fatal Leigh syndrome [3]. It has been suggested that the T → G mutation impairs proton transport through F0 [4], but we have recently shown that ATP-driven proton transport results are in fact unaffected [5]. This implies either a different effect of the mutations on the F1F0 function or an unidirectional impairment of proton flow only when the enzyme works physiologically (i.e. cytosol to matrix flow). We have investigated the physiological proton translocation with a novel fluorescent assay method and here we discuss the preliminary data found in relation to previous results reported in the literature.

Keywords: F1F0-ATPase; Mitochondria; mtDNA mutation; Proton translocation

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K.41. Modulation of the mitochondrial unspecific permeability by calcium

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In yeast, mitochondria may undergo a large increase in permeability to ions, due to the opening an unspecific pore, the yeast mitochondrial unspecific channel (YMUC) [1]. In regard to regulation, the YMUC is opened by ATP and is closed by Pi [2]. Other anions, similar to Pi such as SO_4 and AsO_4 also close the YMUC, probably by interacting with a specific site in the internal face of the mitochondrial inner membrane [3]. By contrast, it has been reported that Ca^{2+}, which evokes the permeability transition in mammalian mitochondria, does not open the YMUC [4]. Here, we report that Ca^{2+} addition results in closure of the YMUC inhibiting the uptake of K^+. By contrast, once mitochondria have taken up K^+, the exit of the monovalent cation is not inhibited by Ca^{2+}. Thus, Ca^{2+} is probably interacting only with the external surface of mitochondria, shielding negative charges. This was tested using hydrophobic cations which were capable of inhibiting both the uptake and the release of K^+. It is proposed that these cations interact directly with the YMUC and their hydrophobic nature allows them to remain attached under conditions where Ca^{2+} is released.

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Keywords: Unspecific permeability; Calcium; YMUC

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K.42. Movement of the metabolites within the skinned fiber: quantifying intracellular restrictions in silico

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According to recent measurements of the mitochondrial respiration kinetics in skinned cardiac muscle fibers [1,2], there is a large difference in the regulation of respiration depending on the source of ADP. When ADP was produced by intracellular ATPases from added MgATP, the ADP concentration in the medium was about 40 μM when about 70% of maximal rate of respiration was achieved. In contrast, the apparent K_m for exogenous ADP in regulation of respiration was equal to 300–400 μM. Exogenous ADP trapping system consisting of pyruvate kinase (PK, 20–40 IU/ml) and phosphoenolpyruvate (PEP, 5 mM) totally suppressed respiration activated by exogenous ADP, but the respiration maintained by endogenous ADP was suppressed by no more than 20–40%. This data can be explained by mathematical reaction-diffusion type model [3] assuming that movements of the metabolites within the fiber are influenced by two different restrictions—mitochondrial outer membrane and overall restriction by intracellular structural complexes of mitochondria with sarcoplasmic reticulum and cytoskeletal elements in myofibrils. On the basis of our simulations, we conclude that the high apparent K_m(ADP) value and respiration rates maintained even in the presence of PK + PEP system can be explained by heterogeneity of intracellular diffusion of ADP.

Keywords: ATPase; Compartmentation; Mitochondria; Myocyte; Mathematical model

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K.43. Mitochondrial permeability transition induced by chemically generated singlet oxygen

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Pure singlet molecular oxygen, ($^1O_2$), generated by thermal decomposition of the 3,3′-(1,4-naphthylidene) dipropionate endoperoxide ($NDPO_2$), inhibited respiration of isolated rat liver mitochondria supported by NADH-linked substrates or succinate, but not by *N*,*N*,*N*,*N*-tetramethyl-*p*-phenylene-diamine (TMPD)/ascorbate. Under the latter conditions, mitochondria treated with 2.7 mM $NDPO_2$ exhibited a decrease in transmembrane potential ($\Delta\Psi$) in a manner dependent on $NDPO_2$ exposure time. This process was sensitive to the mitochondrial permeability transition inhibitors EGTA, dithiothreitol, ADP, and cyclosporin A. The presence of deuterium oxide (D_2O), which increases $^1O_2$ lifetime, significantly enhanced $NDPO_2$-promoted mitochondrial permeabilization. In addition, $NDPO_2$-induced mitochondrial permeabilization was accompanied by DTT or ADP-sensitive membrane protein thiol oxidation. Taken together, these results provide evidence that mitochondrial permeability transition induced by chemically generated singlet oxygen is mediated by the oxidation of membrane protein thiols.

Keywords: Rat liver mitochondria; Permeability transition pore; Singlet oxygen

K.44. Effects of *N*-acylethanolamines on isolated mitochondria and cells

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N-acylethanolamines (NAEs) are derivatives of fatty acids in which the carboxylic group of the fatty acid is bound to the amino group of ethanolamine by an amide linkage. NAEs are present in various mammalian tissues and their content greatly increases in ischemic heart and brain [1]. The purpose of the present investigation is to get a broader insight into the action of different NAEs on isolated mitochondria and intact cells. It was found that both *N*-arachidonylethanolamine and *N*-oleoylethanolamine produced a release of accumulated Ca^{2+} from rat heart mitochondria and opening of a cyclosporin A-sensitive pore; *N*-palmitoylethanolamine was far less active. In nominally calcium-free media, NAEs exhibited slight protonophoric activity that was manifested by a decrease of the membrane potential and increase of resting state respiration. This protonophoric activity was, however, much lower than that of the corresponding free fatty acids.

In Ehrlich ascites tumour cell, *N*-arachidonylethanolamine increased the production of oxygen free radicals, whereas *N*-oleoylethanolamine decreased it slightly.

Keywords: Anandamide; Uncoupling; Oxygen free radical; Permeability transition pore

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K.45. Hydrogen peroxide production by mitochondrial glycerophosphate dehydrogenase and its activation by ferricyanide

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Reactive oxygen species (ROS) are proposed as mediators in acceleration of degenerative processes in aging and in initiation of apoptotic and necrotic processes. The leak of electrons from the mitochondrial respiratory chain responsible for ROS generation was localized in Complex I and III. Our data also showed that significant glycerophosphate (GP)-dependent ROS production can be detected in mammalian mitochondria and that one electron acceptor, ferricyanide, highly stimulated GP-dependent, KCN-insensitive oxygen uptake indicative of hydrogen peroxide generation. This reaction was prevented by inhibitors of mGPDH, by coenzyme Q or by cytochrome *c*. Ferricyanide-induced hydrogen peroxide production was negligible when succinate or NADH were used as a substrate. Using luminometry, GP-dependent hydrogen peroxide production was detected in isolated mGPDH, devoid of Complex I and III. These results demonstrated that GP-dependent hydrogen peroxide production is directly connected with the function of mGPDH and that mGPDH represents an additional site of ROS generation in mammalian respiratory chain. Our data also reflect differences in the transport of reducing equivalents to CoQ pool between mGPDH and succinate or NADH dehydrogenase and suggest that more intensive production of ROS may be present in mammalian cells with active mGPDH.

Keywords: Mitochondrial glycerophosphate dehydrogenase; Ferricyanide; Hydrogen peroxide

K.46. Butyrolactone I affects cellular distribution and function of mitochondria

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Alzheimer's disease is characterized by accumulation of insoluble fibrous material in nervous tissue, both extra- and intracellularly. Highly phosphorylated forms of microtubule-associated tau protein represent the major component of intracellular deposits [1]. There is evidence that cyclin-dependent kinase 5 (Cdk5) is involved in tau hyperphosphorylation and changes in its regulation may be implicated in disturbances such as those occurring in Alzheimer's disease [2].

In this study, we used butyrolactone I (BL-I), a selective inhibitor of Cdk1 and Cdk5 kinases [3], to study the potential link between Cdk5–tau pathway and distribution and function of mitochondria in cultured human skin fibroblasts.

By fluorescence microscopy using MitoTracker™ Red, a fluorescent probe specific for mitochondria, we demonstrated that cell treatment with BL-I leads to progressive accumulation of mitochondria around the nucleus accompanied by change in general cell shape. Furthermore, cytofluorometric studies using TMRM showed that redistribution of mitochondria is accompanied by a decrease in mitochondrial membrane potential. In addition, reduced ADP activation of mitochondrial respiration in BL-I-treated cells was detected by polarographic analysis.

Our results indicate that BL-I strongly affects the distribution and energetic state of mitochondria. This is in agreement with the hypothetical role of tau protein as a regulator of vesicle and organelle transport [4].

Keywords: Butyrolactone I; Tau protein; Cdk5; Mitochondria; Alzheimer's disease

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K.47. Glycine can decrease the amplitude of the calcium-induced swelling in brain mitochondria

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It is well known that psychotropic drugs have different effects on metabolic processes in mitochondria [1,2]. Moreover, it was shown elsewhere that even a simple neuromediator (glycine) could protect human cell bioenergetics against damage [3]. This is why it is very important to know whether glycine can affect mitochondrial bioenergetic directly. The effect of glycine on some metabolic processes in mitochondria has been investigated at the present study. The organelles have been separated from brain and liver of Wistar rat males (200–220 g). It was shown that incubation of rat brain mitochondria with glycine could lead to a decrease of more than 50% of the amplitude of calcium-induced mitochondrial swelling. However, this effect was not discovered in rat liver mitochondria. At the same time, the incubation with glycine increase respiration control index and ADP/O ratio both in liver and brain mitochondria. We suppose that this effect can be a part of the whole neuroprotective effect of glycine on brain during increasing damage and neurotoxicity.

Keywords: Mitochondria; Glycine; Swelling; Calcium

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K.48. 3,3′,5-Triiodo-L-thyronine induces mitochondrial membrane depolarization and permeability transition pore opening following anoxia and reoxygenation

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Triiodothyronine (T_3) has been shown to improve cardiac function in heart failure [1] and to preserve function in animal models of ischemia/reperfusion injury [2]. In order to determine a possible mechanism for the putative cardioprotective role for T_3, we examined the effect of the thyroid hormone on the maintenance of membrane potential ($\Delta\psi_m$) and opening of the mitochondrial permeability transition pore (MPT) in isolated rat heart mitochondria under conditions that mimic ischemia/reperfusion. Under aerobic conditions up to 10 μM, T_3 failed to dissipate $\Delta\psi_m$ or to initiate MTP. Addition of 100 μM of the mitochondrial ATP-sensitive K (mitoK_{ATP}) channel opener diazoxide, either in the presence or absence of T_3, caused only a 2–3 mV decrease in $\Delta\psi_m$. Following 10 min of anoxia and then reoxygenation, however, T_3 caused a complete dissipation of $\Delta\psi_m$ that was not preventable by diazoxide or the mitoK_{ATP} channel antagonist, 5-hydroxydecanoate but was inhibited by EGTA. Under these conditions T_3 also promoted MTP. Moreover, diazoxide alone caused a 20 mV decrease in $\Delta\psi_m$ that was not inhibited by EGTA. Diazoxide promoted MTP opening but not to the same extent as T_3. These results suggest that T_3 may play a role in bioenergetic events associated with mitochondrial injury.

Keywords: Mitochondria; Triiodothyronine; Ischemia; Membrane potential

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K.49. Retroactive pathway involving mitochondria in electro-loaded cytochrome *c*-induced apoptosis and protective properties of Bcl-2 and Bcl-XL

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Square-wave pulse electropermeabilisation was used to load exogenous cytochrome *c* into Jurkat cells. Determination of optimal electro-loading conditions appeared to be a critical step considering the ability of electrical treatment to induce a low but significant level of cell death (apoptosis and/or necrosis).

Nevertheless, 3 h after cytochrome *c* uptake into the cells, characteristic phenotypes of apoptosis were specifically induced as revealed by aberrant phosphatidylserine exposure on the outer plasma membrane leaflet associated with viable cells, followed by hypoploid DNA content appearance. Full activation of caspase-9 and -3 were correlated with the induction of this apoptotic phenotype. However, a significant drop in the mitochondrial membrane potential (DYm) was also observed, while cytochrome *c* was generally considered to act downstream from the mitochondria. Parallel to the DYm drop, a NAD(P)H fluorescence decrease was detected and was followed by potassium efflux from the cell. Cardiolipid oxidation and release of pro-apoptotic proteins AIF and Smac from the mitochondria were also observed. Permeability transition pore inhibitors did not inhibit the disruption of mitochondrial homeostasis. Bcl-2 and Bcl-XL overexpression or use of *N*-acetyl-DEVD-aldehyde protected mitochondria from the membrane potential decrease, but also protected cells from apoptosis and secondary necrosis induced by exogenous cytochrome *c*.

Our results showed that the caspase-3 activation that followed exogenous cytochrome *c* uptake retroactively altered the mitochondrial homeostasis via a Bcl-2 or Bcl-XL inhibitable pathway. Such an event is totally inhibited in the absence of caspase-3 as well as all the cytochrome *c*-linked cell damages.

K.50. A bioenergetic approach of the destabilization of mitochondrial functions by tcBid and regulation by BH-3 only domains or by Bcl-2 and Bcl-XL

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Recent evidence supports the theory that mitochondria homeostasis is a key regulatory step in the execution of apoptosis through the intervention of members of the Bcl-2 protein family. Pro-apoptotic members of the family, such as Bax, Bad and Bid, can induce the loss of mitochondrial homeostasis. Here, we examine what mediates the specific targeting of tcBid to the mitochondrial membranes, as well as the bioenergetic consequences of tcBid binding to the membranes. Taking into account that (i) mitochondria exhibit many contact sites between outer and inner membranes and that (ii) cardiolipin and phophatidylethanolamine are lipids that favor the non-bilayer hexagonal H11 phase in isolation, one can expect that the contact site would have a special structure as a mixture of the two membranes. At the bionergetic level, tcBid is able to disturb the mitochondrial homeostasis in a lapse of time where it has been demonstrated that it also induces reorganization of the cristae membranes [2]. TcBid appeared to be able to act early on the mitochondrial physiology without Bax [1] The bionergetic support for this perturbation of mitochondrial homeostasis was studied with mitochondria extracted from transgenic mice liver (which overexpressed Bcl-2 and Bcl-XL) and also in the presence or absence of BH-3 peptides.

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K.51. Flux control of cytochrome-c-oxidase in intact human muscle cells and in cybrids with common deletion

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The effect of mutations of mt-DNA on enzyme pattern and functional properties of mitochondria is not well understood. Flux control coefficients (C0) of cytochrome-c-oxidase (COX) on mitochondrial respiration were measured in intact human myoblasts (MB), human vascular smooth muscle cells (SMC) and in cybrids with common deletion using high resolution respirometry and inhibitor titrations (azide, cyanide). From the inhibitor titration curves Co were calculated using a nonlinear regression programme [1].

C0 of COX on mitochondrial oxygen consumption was in MB (0.32) some higher than in SMC (0.24). FCCP strongly increased Co in all type of cells (0.83 - 0.88). Endogenous respiration of wild type cybrids decreased after addition of glucose from Jo = 25 pmol O2/s/106 cells to Jo = 18 pmol O2/s/106 cells whereas Co = 0.28 increased to Co = 0.42. In cybrids with common deletion (hetereoplasmy = 46%) at similar rates of respiration increased flux control (endogenous: Co = 0.59; glucose: Co = 0.72) was detected.

Data show that flux control coefficients in intact cells strongly depend on metabolic conditions, on functional state and on deletion-caused changes in enzyme pattern. Results demonstrate the applicability of metabolic control analysis for quantification of metabolic consequences of mitochondrial impairments in intact cell cultures.

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K.52. Respiratory complex activities in a mutant strain of *Drosophila subobscura*

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Most (80%) mitochondrial genomes in the studied mutant strain of *Drosophila subobscura* have undergone a large scale deletion (5 kb) [in the coding region. Complex 1 (ND1, ND4 ND4l ND6 and ND5 subunits and complex III (cytoB) are concerned by the mutation. Activities of these complexes are reduced by 50 and 30% respectively in this mutant strain mitochondria. Nevertheless, ATP synthesis capacity is identical to that observed in the wild-type strain except for specific substrate of complex II as alpha glycerophosphate which increase ATP synthesis capacity in the mutant mitochondria[1–4]. Rotenone, antimycin and KCN were used to inhibit the activities of complex I, III and IV, respectively, and threshold curves were generated. It was found that in the wild-type strain complex I activity could be decreased by 70% before major changes in mitochondrial respiration and ATP synthesis took place. In the mutant strain, rotenone inhibition by 20% affect the ATP synthesis, so the mutation decrease the threshold of this complex activity. The complex III activity threshold is below 20% in both strains, and we observed some difference in antimycin sensitivity, suggesting a modification of the complex enzymatic properties in the mutant. In contrast, threshold values of 70% were measured for complex IV inhibition.

Measures of the mitochondrial membrane electrochemical gradient including membrane potential (Δ□) and ΔpH are developed in order to study if some change in the membrane properties could explain the maintain of ATP synthesis capacity and the lack of pathological phenotype in the mutant.

Keywords: mitochondrial genome, complex I, complex III, electrochemical gradient

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K.53. The mitochondrial mechanisms of cell killing by the BCL-2 ligand HA14-1

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The Bcl-2 protein is overexpressed in a variety of tumors. It largely localizes to the outer mitochondrial membrane, where it suppresses release of cytochrome *c* and of other proapoptotic proteins. Following the literature report that the Bcl-2 ligand HA14-1 causes apoptosis via the mitochondrial pathway [1], we have investigated the mitochondrial and cellular effects of HA14-1. We show that HA14-1 has a dual effect on mitochondria: (i) it sensitizes opening of the permeability transition pore (PTP); this is a potentially useful effect because PTP opening in Bcl-2 overexpressing cells could lead to the selective killing of tumor cells; (ii) it both inhibits and uncouples respiration, which represent toxic side effects that may hamper the development and use of this drug in anticancer therapy. Because sensitization to PTP opening could be demonstrated in a concentration range that does not cause respiratory inhibition (up to 10 μM), while the toxic effect became prominent between 20 and 50 μM, we tested the mechanisms underlying the cytotoxic effects of HA14-1 at various concentrations. We found that relatively low concentrations of HA14-1 cause PTP opening *in situ*; and that slightly higher concentrations cause PTP-independent depolarization and cell death.

Keywords: mitochondria, BCL-2, cell death, permeability transition

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K.54. Mitochondrial dysfunction and cell death: Relationship with intracellular calcium

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We have investigated the relationship between cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$), mitochondrial permeability transition (PT) *in situ* and cell death by using the Me^{2+} ionophore A23187 (A23). Addition of A23 induced a modest and transient rise of $[Ca^{2+}]_c$ without mitochondrial depolarization and with no effects on cell viability. We show that addition of A23 in the presence of vanadate, which inhibits the plasma membrane Ca^{2+} pumps, caused instead a dramatic increase of $[Ca^{2+}]_c$ followed by cell death, which was inhibited by Cyclosporin A. To study the possible contribution of the PTP to mitochondrial depolarization and the downstream events we have measured the mitochondrial membrane potential *in situ* with TMRM. Since the plasma membrane potential may be affected by vanadate, which would in turn affect intracellular TMRM distribution, these experiments were performed in KCl-based media. Addition of A23 plus vanadate caused rapid mitochondrial depolarization and PTP opening. Thus, large increases of $[Ca^{2+}]_c$, which may not be achieved by the addition of A23 alone, are required for long-lasting PTP openings and mitochondrial depolarization.

Keywords: Mitochondria, cell death, calcium, permeability transition

K.55. How could mitochondria contribute to aging of Yeast *Saccharomyces cerevisiae*?

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Mutated RAS proteins have been first detected 20 years ago in human tumors. When mammalian cells are transformed with H-RAS^{val12}, they cease to proliferate. RAS2^{val19} is the yeast homologue of the human oncogenic allele. We report here that its expression greatly affects yeast replicative aging. Interestingly, this mutant produces abnormally high levels of Reactive Oxygen Species (ROS) and shows a high level of oxidative damage. Using the mitochondrial ATPase inhibitor, Tri-Ethyl-Tin (TET) in combination with the protonophore CCCP we could see that RAS2^{val19} mitochondria are constitutively respiring under non-phosphorylating conditions in glucose repressed environment. We have previously shown that such a respiratory state correlates with high level of oxidative damage *in vivo*. To test whether this phenotype is linked to the cAMP-PKA pathway, we introduced a plasmid carrying the PDE2 gene encoding a high affinity phophosdiesterase. PDE2 overexpression restored typical PKA-dependent phenotypes, but neither ROS production nor respiratory states were affected. This result suggests that Ras2p can probably regulate at least two pathways. Moreover, both of these pathways seem to be relevant for replicative life span.

Keywords: Replicative aging, Oxidative stress, RAS2

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