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11P.14 Yeast cytochrome *c* oxidase: A model system for determining the specific role of bound phospholipids

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Cytochrome *c* oxidase (EC 1.9.3.1; CcO) catalyzes the transfer of electrons from ferrocytochrome *c* to oxygen, a reaction coupled to proton translocation across the inner mitochondrial membrane. One feature that most directly impacts upon the structural and functional integrity of CcO is cardiolipin (CL) tightly bound to the enzyme. Hypotheses have been put forward that (i) CL acts as "glue" to stabilize the multi-subunit complexes; and (ii) CL functions as a "proton antenna" to facilitate proton entry into the active site of enzyme. One of the best ways to test these hypotheses is site-directed mutagenesis of *Saccharomyces cerevisiae* CcO. The enzyme is structurally similar to mammalian CcO and is amenable to genetic manipulation of its structure. Towards this goal, CcO was isolated from baker's yeast. The enzyme was extracted from mitochondria using dodecyl maltoside and purified by high performance Q-Sepharose column chromatography. The resulting enzyme has the expected oxidized and reduced visible absorption spectrum, and a molecular activity of about 120 s^{-1} when assayed spectrophotometrically using ferrocytochrome *c* as a substrate. The subunit composition of yeast CcO was analyzed by a combination of RP-HPLC and mass spectrometry. Seven major HPLC elution peaks were detected using absorption at 214 nm. The identity of the 8 nuclearly encoded subunits that eluted from the RP-HPLC column was determined by electrospray ionization mass spectrometry. Normal phase silicic acid HPLC analysis of the extracted from isolated CcO phospholipids confirmed that a small number of phospholipids including CL, phosphatidylcholine, and phosphatidylethanolamine co-purified with CcO. Treatment of CcO with phospholipase A2 resulted in partial inactivation of the enzyme indicating the important functional role of at least some of these bound phospholipids.

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11P.15 Membrane-facilitated proton transfer to the surface of a membrane-spanning proton transporter

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A key step in energy metabolism of a living organism is translocation of protons across a membrane, conducted by membrane-spanning proton transporters of the respiratory chain. The electrochemical gradient maintained by these transporters is utilized, for example, for synthesis of ATP. In the present study we used Fluorescence Correlation Spectroscopy (FCS) to investigate the interplay between the components of the respiratory chain and the membrane, and the effect of the membrane on the proton transfer in the energy-conservation machinery. The FCS technique was used earlier in our laboratories to study membrane-facilitated proton transfer, by determining the protonation kinetics of a fluorescein molecule anchored to the surfaces of membranes of different composition. The results from these studies showed that the protonation rate of the fluorescein molecule increased upon incorporation of the probe into a membrane. This acceleration in the rate was interpreted in terms of a proton-collecting antenna, composed of the lipid molecules, that acts to facilitate protonation of the surface-bound probe [1]. Here we have used the FCS technique to investigate the interplay between the membrane surface and the protein surface of one of the proton transporters of the respiratory chain. A fluorescein molecule was covalently linked to the surface of cytochrome *c* oxidase from *Rhodobacter sphaeroides*. The protonation kinetics was determined for the fluorescein molecule linked to the detergent-solubilized protein as well as to the protein incorporated into di-oleyl-phosphatidylglycerol vesicles. The results show that the protonation rate increased by a factor of about 400, from about 7×10^{10} s^{-1} M^{-1} for the detergent-solubilized oxidase to about 3×10^{13} s^{-1} M^{-1} upon incorporation into vesicles. Collectively, these results indicate that there is proton transfer to the protein surface facilitated by the membrane surface [2].

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11P.16 Characterisation and flash photolysis of carbon monoxide adducts of heme-copper binuclear model compounds

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In studies of heme proteins including cytochrome *c* oxidase, carbon monoxide has often been used as a surrogate for the physiological reactant dioxygen. Investigations employing CO flash photolysis have been useful in probing the dynamics and coordination chemistry of the heme-Cu_B binding pocket after CO photolysis [1, 2]. Therefore, we have carried out systematic studies of CO coordination and photodissociation, on a series of biomimetic models of the binuclear Fe/Cu (heme/trismidazole) active site of cytochrome *c* oxidase. Based upon a porphyrin core, all these models have a covalently linked pyridine in the proximal site of the porphyrin but they differ strongly by the environment around the copper and their rigidity. In order to explore the influence of copper (I) on the heme-bound CO, we have used cryogenic difference FTIR spectroscopy and a Nd-Yag laser for photodissociation. In the absence of copper bound in the distal picket, the CO stretching frequency of (L)Fe^{II}-CO is observed at 1978–1982 cm^{-1} for all complexes. Interestingly, the more rigid model compound showed two Fe-CO stretching frequencies upon addition of copper (I), one in the same region as the model without copper and one strongly downshifted by about 33 cm^{-1}. Upon photolysis, the CO was transferred from the heme to the copper (I) ion. This was not the case for

the less rigid models. Thus, the large frequency shift ascribable to the presence of copper (I) suggests that the copper ion resides in close proximity to the bound CO. The existence of two conformers is discussed. Additional IR studies of oxygen reactivity with these model compounds were also investigated.

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11P.17 Cyanide inhibition and pyruvate-induced recovery of cytochrome *c* oxidase

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The mechanism of cyanide inhibition of the mitochondrial cytochrome *c* oxidase (COX) as well as the conditions for its reversal is not yet fully explained. With regard to the inhibition by KCN and its reversal by pyruvate, we investigated three parameters of COX function, namely the transport of electrons in the terms of oxygen consumption, the proton pumping evaluated as mitochondrial membrane potential ($\Delta\psi_m$) and the enzyme affinity to oxygen by means of p_{50} value calculation. We analyzed the function of COX in intact rat liver mitochondria, either within the respiratory chain or as a sole enzyme, using succinate or ascorbate + TMPD to fuel respiration. We found that 250 μM KCN completely inhibited both electron and proton transport function of COX, and this inhibition was reversible after washing of mitochondria. The addition of 60 mM pyruvate induced the maximal recovery of both parameters to 60–80% of original values. Using KCN in the low concentration range up to 5 μM, we observed a profound (30-fold) decrease of COX affinity to oxygen. Again, this decrease was completely reversed by washing of the mitochondria while pyruvate induced only a partial yet still significant recovery of oxygen affinity. Our results demonstrate the reversible nature of inhibition of COX by cyanide and reveal the limited potential of pyruvate to act as a cyanide poisoning antidote. Importantly, we also show that the COX affinity to oxygen is the most sensitive indicator for the detection of toxic effect of cyanide.

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11P.18 Evaluation of the mitochondrial metabolism of two invertebrates' species using permeabilized fibres in high-resolution respirometry

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The use of permeabilized fibres instead of mitochondrial isolations allows the estimation of mitochondrial metabolism in an in situ approach. This approach has several advantages compared to the *in vitro* approach, notably being closer to the real physiological environment (review in Kuznetsov et al., 2008) and has never been used to assess mitochondrial functions in invertebrates. Measurement of O_2 consumption using permeabilized fibers from high energetic muscles flight of *Drosophila* simulans were used for classic assessment of mitochondrial performances at several steps of the ETS. In another example on the whole body musculature of the polychaete *Nereis virens*, we evaluated the normal and the alternative oxidative pathways in order to understand the conditions of maximum efficiency of ETS and the intervention of alternative oxidase as terminal electron acceptor in some invertebrate species. In flies, results showed very good RCR for complex I with high state 3 respiration. The assessment of complex II showed significant contribution of succinate on the electron transport system. It is the first time that respiration from supplying complex II has been quantify in *Drosophila*. When ubiquinol pool was supplied through complex I, complex II and glycerol-3-phosphate dehydrogenase, the activity of the electron transport system reached a maximum state 3 and further uncoupling showed that the OXPHOS capacity was not overwhelmed suggesting that ATP synthase can support the maximum electron flux measured in the electron transport system. In *Nereis*, RCR for complexes I and II showed low values but consistent with previous studies on mitochondrial isolations from *Nereis pelagica* (Tschischka et al., 2000). When inhibiting complex III, O_2 consumptions measurements showed that 28.97% of state 3 respiration are dedicated to supply alternative oxidase in electrons as well as to the backflux of electrons to complex I and/or complex II. SHAM was used to further inhibit alternative oxidase and allowed us to corroborate the significant contribution of the alternative pathway. We demonstrated here that high-resolution respirometry with permeabilized fibres in invertebrates can be use as an accurate tool to evaluate the mitochondrial metabolism at each steps of the ETS and may insure better understanding of the regulation of several processes not detected in vertebrates like the alternative oxidase.

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11P.19 Computer simulations of proton transfer in cytochrome *c* oxidase and nitric oxide reductase

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Simulation of proton transfer (PT) in proteins through bridging water molecules and ionizable amino acid residues is a challenging task: classical MD simulations cannot, in principle, describe individual PT steps; on the other hand, ab initio QM/MM simulations of biosystems are still limited by many factors (e.g., sampling, simulation time, convergence). One of the most efficient approaches is the empirical valence bond (EVB) method. Recently, we have adopted an EVB-based multi-level modeling strategy for simulations of the coupled ET/PT events in proteins [1, 2]. (1) We will present the results of our recent computational study [1] of cytochrome *c* oxidase (CcO), a system that has long presented a conceptual challenge in bioenergetics [3]. After its structure has been solved more than a decade ago, CcO was the focus of numerous works, including a number of computational studies with different methods [2, 4]. Although these studies have shed light on many aspects of CcO functioning, the detailed molecular mechanism of proton pumping