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11P.2 Oxygenated heme *d* in cytochrome *bd* from *Escherichia coli*

Dmitry A. Bloch[1], Vitaliy B. Borisov[2], Michael I. Verkhovsky[1]
[1]*University of Helsinki, Institute of Biotechnology, PB 65 (Viikinkaari 1), 00014, Helsinki, Finland*
[2]*Lomonosov Moscow State University, Belozersky Institute of Physical Chemical Biology, Moscow, Russia*
E-mail: dmitry.bloch@helsinki.fi

Cytochrome *bd* is typically expressed under low oxygen tension and has high affinity for O_2. The enzyme as isolated is a mixture of two stable or metastable forms: (i) ferrous heme *d* bound to molecular oxygen ("state **A**"), and (ii) ferryl oxene heme *d* ("state **F**"), with the latter form contribution of *ca* 20–50%. Both forms can be reversibly destroyed by (a) anaerobic (at $E_h > +380$ mV *vs NHE*, pH 7, 0.1% SML) or aerobic ($>+620$ mV) oxidation or (b) depletion of oxygen at redox potentials where the enzyme mostly remains in one-electron-reduced state; in both cases state "**O**" is formed. Binding of O_2 to heme *d* as a function of E_h was studied using the quasi-equilibrium OTTLE spectro-electrochemistry, where oxygen at varying concentration was allowed to equilibrate with the enzyme at a given redox potential. Under the anaerobic conditions heme *d* has the apparent midpoint potential E_m^{app} about +260 mV with both hemes *b* mostly oxidized [1]; at 1.2 mM O_2, the E_m^{app} value becomes $+495 \pm 5$ mV. The $[O_2]$-dependence of E_m^{app} is essentially linear at the concentrations above 10 μM with the slope -60 mV/pO_2 and the effective dissociation constant for the reduced heme *d*, $K_D^{(eff)}$ about 150 ± 20 nM O_2. The latter value differs from the earlier data, $K_D^{(direct)} \approx 280$ nM, where the heme *d* oxygen affinity was directly measured in one-electron-reduced isolated enzyme [2]. The difference is attributed to the fact that in the presence of oxygen the enzyme slowly turns over catalyzing a steady-state flux of electrons supplied by the working electrode. The turnover numbers of the enzyme at each redox potential can be determined *in situ* as the difference between the values of the cell current (I_{WE}) in the presence and in the absence of the enzyme. Kinetic modeling proves that in the steady-state, the $K_D^{(eff)}$ of about 280 nM can be reached when the (rate-limiting) rate constants for the **O** → **A** and **A** → (**F**) → **O** transitions are equal, which is indeed true, provided that both constants are limited by the electron delivery from the working electrode, and the **A** → **F** transition is much faster. The **O** ↔ **A** and **A** ↔ **F** transitions show similar E_m^{app} values during the redox titration over the broad $[O_2]$ range. We propose that in the state **F**, heme *d* of cytochrome *bd* has an unusually low redox potential comparable to that of the state **A**-heme *d*.

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11P.3 Characterization of the semiquinone radical stabilized by the cytochrome aa_3-600 menaquinol oxidase of *Bacillus subtilis*

Sophia M. Yi[1], Kuppala V. Narasimhulu[2], Rimma I. Samoilova[3], Robert B. Gennis[1], Sergei A. Dikanov[2]
[1]*University of Illinois, Department of Biochemistry, USA*
[2]*University of Illinois, Department of Veterinary Clinical Medicine, USA*
[3]*Institure of Chemical Kinetics and Combustion, Russian Academy of Sciences, Russian Federation*
E-mail: dikanov@illinois.edu

Cytochrome (cyt) aa_3-600 is one of the principle respiratory oxidases from *Bacillus subtilis* and is a member of the heme-copper superfamily of oxygen reductases. This enzyme catalyzes the 2-electron oxidation of menaquinol and the 4-electron reduction of O_2 to $2H_2O$. Cyt aa_3-600 is of interest because it is a very close homologue of the cyt bo_3 ubiquinol oxidase from *E. coli*, except that it uses menaquinol instead of ubiquinol as a substrate. One question of interest is how the proteins differ in response to the differences in structure and electrochemical properties between ubiquinol and menaquinol. Cyt bo_3 has a high affinity binding site for ubiquinol that stabilizes an ubisemiquinone (SQ). This has permitted the use of pulsed EPR techniques to investigate the protein interaction with the ubiquinone. Pulsed EPR methods have revealed several salient features of the interactions between the residues at the Q_H-site of cyt bo_3 and the SQ: 1) The hydrogen bonding to the SQ is highly asymmetric, with strong hydrogen bonds to carbonyl O-1 and weaker interactions at carbonyl O-4 side; 2) There is one strong hydrogen bond between the ε-nitrogen of R71 and carbonyl O-1 of the SQ, resulting in substantial transfer of unpaired electron spin to this nitrogen; 3) There is a strong hydrogen bond between D75 and carbonyl O-1 of the SQ; 4) There is a weak interaction between H98 and carbonyl O-4 of the SQ with a small amount of spin density found on the nitrogens of H98; 5) There is a very weak interaction between carbonyl O-4 of the SQ and the side chain of Q101; 6) The SQ in cyt bo_3 is in the neutral, protonated state at pH 7.5. The current work initiates studies to characterize the equivalent site in cyt aa_3-600. Cyt aa_3-600 has been cloned and expressed in a his-tagged form in *B. subtilis*. Following isolation of the enzyme in dodecylmaltoside, it is shown that the pure enzyme contains one equivalent of menaquinone-7, and that the enzyme stabilizes a mena-semiquinone. Pulsed EPR studies have shown that there are both similarities as well as significant differences in the interactions of the mena-semiquinone with cyt aa_3-600 in comparison with the ubi-semiquinone in cyt bo_3. Our data indicate weaker hydrogen bonds of the menaquinone in cyt aa_3-600 in comparison with ubiquinone in cytochrome bo_3. In addition, the electronic structure of the SQ cyt aa_3-600 is more shifted towards the anionic form from the neutral state in cyt bo_3.

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11P.4 Circular dichroism of cytochrome *c* oxidase: Modeling spectra using classical polarizability theory

Artem V. Dyuba[1,2], Tatiana V. Vygodina[1], Natalia V. Azarkina[1], Alexander M. Arutyunyan[1], Yury A. Sharonov[1], Anastasia V. Kalinovich[1], Alexander A. Konstantinov[1]
[1]*A. N. Belozersky Institute of Physico-Chemical Biology, Department of Bioenergetics, M. V. Lomonosov Moscow State University, Moscow 119992, Russia*
[2]*M. V. Lomonosov Moscow State University, School of Bioengineering and Bioinformatics, Moscow, Russia*
E-mail: dyubon@gmail.com

Circular dichroism (CD) spectroscopy in the heme absorption bands provides a sensitive tool for monitoring functional state of cytochrome *c* oxidase (CcO) redox centers. Although CD spectra of mitochondrial CcO were described in the past (Tiesjema RH & Van Gelder BF, 1974, *Biochim. Biophys. Acta* **347:** *202–214;* Myer YP (1985) In *Curr Topics Bioenerg*/ Edited by C. P. Lee. — Orlando, San Diego, New York, London, Toronto, Montreal, Sydney, Tokyo, Academic Press, Inc.*)*, the origin of the observed optical activity was not established. In this work we compare CD and absorption spectra of bovine heart CcO in the fully reduced ($a^{2+}a_3^{2+}$), fully oxidized ($a^{3+}a_3^{3+}$) and various ligand-bound states ($a^{2+}a_3^{2+}$-CO, $a^{3+}a_3^{3+}$-CN, $a^{3+}a_3^{2+}$-CO, $a^{2+}a_3^{3+}$-CN). In addition, we

have measured CD and absorption spectra of ba_3-type cytochrome *c* oxidase in the fully reduced ($b^{2+}a_3^{2+}$), fully oxidized ($b^{3+}a_3^{3+}$), reduced CO-bound ($b^{2+}a_3^{2+}$-CO) and mixed-valence, CN-bound ($b^{3+}a_3^{2+}$-CN) states. CD spectra in the Soret absorption band of both enzymes are very non-conservative, with the area under positive lobe of the spectra strongly prevailing over the area under the negative part. Knowing the crystal structure of the enzyme, we attempted at modeling the CD spectra of the fully reduced and fully oxidized forms of bovine CcO using classical polarizability theory. With this approach, we can explain the non-conservative character of the CD spectra in the Soret band and reproduce reasonably well the magnitudes and lineshapes of the CD signals. The analysis suggests that optical activity of the enzyme in the Soret band originates in the interaction of the hemes with each other as well as with the aromatic amino-acid residues around.

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11P.5 Role of membrane potential on the control of cytochrome *c* oxidase over respiration in intact hepatoma HepG2 cells

Elena Forte[1], Maria Elena Dalmonte[1,3], Maria Luisa Genova[3], Marzia Arese[1], Daniela Mastronicola[1], Alessandro Giuffrè[2], Giorgio Lenaz[3], Paolo Sarti[1]
[1]*Department of Biochemical Sciences, Sapienza University of Rome, Italy*
[2]*CNR Institute of Molecular Biology and Pathology, Sapienza University of Rome, Italy*
[3]*Department of Biochemistry, G. Moruzzi, University of Bologna, Italy*
E-mail: elena.forte@uniroma1.it

Metabolic control analysis (MCA) [1] has been largely applied to the analysis of oxidative phosphorylation, in order to investigate the control exerted by each individual reaction step on the whole pathway [2]. Using this approach, the control exerted by the electron transport chain complexes was shown to be higher in experimental systems closer to *in vivo* conditions than in mitochondria. To study the effect of the mitochondrial transmembrane proton electrochemical gradient ($\Delta\mu_{H+}$) on the control of respiration by cytochrome *c* oxidase (CcOX) in intact cells, we applied MCA to mitochondrial respiration of HepG2 cells. Both the overall O_2 consumption and specific CcOX activity of actively phosphorylating cells were progressively inhibited by cyanide titration under conditions in which the electrical ($\Delta\psi$) and/or the chemical (ΔpH) component of $\Delta\mu_{H+}$ was selectively modulated by addition of ionophores. Under endogenous conditions, i.e., in the absence of ionophores, CcOX displayed a high control coefficient value, thus representing an important site of regulation of mitochondrial oxidative phosphorylation. A high CcOX control coefficient value was also measured in the presence of nigericin, when $\Delta\psi$ is maximal, and in the presence of nigericin and valinomycin, when $\Delta\mu_{H+}$ is abolished. On the contrary and interestingly CcOX displayed a markedly lower control coefficient in the presence valinomycin converting $\Delta\psi$ into ΔpH. These results show that CcOX activity and its control over oxidative phosphorylation critically depend on $\Delta\psi$ in actively phosphorylating cells [3].

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11P.6 Comparison of the recombinant wildtype with the ATCC wildtype of cytochrome *c* oxidase from *Paracoccus denitrificans*

Florian Hilbers[1], Bernd Ludwig[2], Hartmut Michel[1]
[1]*Molecular Membrane Biology, Max Planck Institute of Biophysics, D-60438 Frankfurt/Main, Germany*
[2]*Institute of Biochemistry, Molecular Genetics, Johann Wolfgang Goethe-University, D-60438 Frankfurt/Main, Germany*
E-mail: Florian.Hilbers@biophys.mpg.de

The cytochrome *c* oxidase from the soil bacterium *Paracoccus denitrificans* consists of four subunits and is the terminal member of the respiratory chain. It pumps four protons from the negative (N) to the positive (P) side of the membrane and catalyses the reduction of molecular oxygen to water using four more protons.

$$4\ \text{cyt}\ c^{+\,\text{II}} + O_2 + 8\ H^+_{(N)} \rightarrow 4\ \text{cyt}\ c^{+\,\text{III}} + 2\ H_2O + 4\ H^+_{(P)}$$

This process takes place in the protein's active binuclear center, housing a haem a_3 and a copper ion (Cu_B). In order to compare variants and the wild type (WT) in a more controlled way a recombinant WT was constructed carrying the SU I gene on a low copy number plasmid. Comparing the rec WT to the ATCC WT no differences were expected, yet a significant increase in the reactivity towards hydrogen peroxide was found for the rec WT. This observation raises the question: Why do these two differently expressed wild type forms of the enzyme vary in this reactivity? Presumably a somehow misplaced Cu_B leads to an increase in hydrogen peroxide reactivity. To test this hypothesis we used a His-tagged variant in order to complex copper during assembly of the protein. Therefore the His-tag could be used as a copper source for copper inserting chaperones during assembly. Additionally extra copies of both bacterial chaperones *ctaG* and *surf1c* required for metal insertion into SU I were cloned on the same plasmid along with the SU I gene to compensate the slight overproduction of SU I from the plasmid. We constructed variants H276R and H276K, and tested them for their activity. These variants presumably lack Cu_B but maintain the overall electrostatic properties. Additionally the variants H276D and H276E were expressed to potentially incorporate a Fe(III) ion instead of the Cu (II) ion. The negative charge for the side chain was chosen to maintain the overall electrostatic properties in this site when incorporating a ferric iron. The results of metal content measurements as well as the results of activity assays will be presented.

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11P.7 Structural studies of catalytic intermediates in cytochrome *c* oxidase

Ann-Louise Johansson, Peter Brzezinski, Martin Högbom
Center for Biomembrane Research, Department of Biochemistry and Biophysics, Stockholm University, Stockholm, Sweden
E-mail: annlouise@dbb.su.se

Cytochrome *c* oxidase (CytcO) is the final electron acceptor in the electron-transport chain in the inner membrane of mitochondria and bacteria. This enzyme catalyzes the reduction of molecular oxygen to water and energetically couples this reaction to proton transfer across the biological membrane. In this project we use X-ray crystallography together with time-resolved spectroscopic techniques to study the specific intermediates of cytochrome *c* oxidase formed during catalysis. By solving the three dimensional crystal structures of these short-lived intermediates we aim to obtain a better understanding for how CytcO accomplishes the coupling between electron- and proton transfers during the reduction of molecular oxygen to water. In our attempts to trap these intermediates we use structural variants of the enzyme that have been shown to stop the catalytic reaction at specific steps in the reaction cycle. A second approach we use is soaking