11L.6 Roles of amino acids and waters in the protonmotive mechanism of cytochrome oxidase

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Water molecules are integral to the mechanism of cytochrome *c* oxidase. They are ligands of the binuclear centre metals in several of the catalytic intermediates of oxygen reduction; water is the final product of oxygen reduction; the pathways for intraprotein proton transfer are likely to be formed in large part from structured water arrays. I will review unresolved aspects of these roles of water and recent progress in their investigation by FTIR spectroscopy with C*c*O from several sources. An accurate description of the ligand sate of the oxidised enzyme is needed in order to understand the subsequent reaction steps. Empirical observations established that stable charge changes within the binuclear centre have to be counterbalanced by electrostatically-linked protonation changes and that the oxidised state at physiological pH values has two protonatable sites that become reduced in the R state. UV/visible and FTIR data on their most likely nature, and the nature of different forms of oxidised C*c*O, will be reviewed. FTIR spectroscopy has been used to observe functional waters in bacteriorhodopsin [3] and reaction centres [2]. Redox FTIR spectra of C*c*O also reveal evidence of changes of weakly H-bonded water molecules. Such changes are much more extensive in spectra of CO photolysis from fully reduced C*c*O. Changes induced with D_2O or $H_2^{18}O$ exchange confirm that the signals arise from alterations of structured waters. These data will be related to suggested models [3] in which internal water molecules form transient ordered chains that protonically link a crucial glutamic acid (E242 in bovine subunit I) with either a 'trap' site for proton translocation or the oxygen-reducing binuclear centre.

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11L.7 Real time recording of the cytochrome oxidase proton pump

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Cytochrome *c* oxidase (C*c*O) is the terminal enzyme of the respiratory chain in mitochondria and many aerobic bacteria. This enzyme converts free energy released upon oxygen reduction to an electrochemical proton gradient by functioning as a redox-coupled proton pump. Although the 3D structure and functional studies revealed some proton-conducting pathways in the enzyme and depicted general topology, the location of proton donor and acceptor groups and their role in proton pumping and providing protons for catalysis are not well defined. One of the most direct ways to test the role of proton-conducting pathways and functionally important groups in the enzyme is specific replacement of presumably important amino acids for the nonfunctional ones. For example, the block of the entrance of the so-called D-channel by converting the protonatable D124 to the nonprotonatable analogue N completely abolishes proton pumping activity, and also strongly retards the catalytic cycle of C*c*O after formation of ferryl (**F**) intermediate. Time-resolved infrared spectroscopy shows that the mutant enzyme with such a replacement forms the **F** state, consuming the proton from another protonatable residue located at the bottom of the D-channel — E278. Subsequent replacement of E278 to glutamine blocks the catalytic cycle one step earlier. These examples show that the essential amino acids in the channel structure are important not only for the formation of the right configuration of water molecules for proton conductivity, but also can serve as a source of protons for pumping and redox chemistry. To elucidate the role of different amino acids in the D-channel in proton pumping and redox chemistry, we tried to block the D-channel at different depths. Combination of time-resolved optical and FTIR spectroscopies with time-resolved electrometry, applied to follow coupled electron and proton transfer in real time, shows that even the mutant enzymes incapable of pumping protons start their catalytic cycle from proton translocation to a "pump site", prior to the following chemistry. The efficiency of this reaction depends on the presence of proton donors. For example, for the *Paracoccus denitrificans* enzyme these donors are E278 at the bottom of the channel, and Y35, located in its middle. In the D-channel mutants the protons preloaded to the "pump site" return back, and are consumed in the reaction of water formation later on in the catalytic cycle.

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Posters

11P.1 Mutagenesis studies on D-pathway function of bovine heart cytochrome *c* oxidase

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X-ray analyses of bovine heart and bacterial cytochrome *c* oxidase revealed three putative proton transfer pathway designated as D-, K-, and H-pathways. It has been proposed from the studies primarily on the bacterial enzymes that D-pathway conveys water forming and pumping protons. D-pathway is structurally very similar between the bovine and bacterial enzymes. Furthermore, the amino acid residues essential to the bacterial D-pathway are completely conserved in the bovine enzyme. This conservation strongly suggests that the bovine and bacterial D-pathways have the same functions. However, the function of the bovine D-pathway has not been studied by the mutagenesis. Here we mutated Asn98 and Asn163 of the bovine D-pathway to Asp employing the HeLa cell's bovine/human hybrid enzyme expression system [1, 2]. Each mutation of the bacterial counterpart is known to abolish the proton pumping activity without impairing O_2 reduction activity, supporting the proton pumping D-pathway proposal. The Asn98Asp and Asn163Asp mutation of the bovine D-pathway did not change both O_2 reduction and proton pumping activities contradicting to the mutation results of the bacterial enzyme. The genes encoding the core subunits, bovine subunit I and human subunits II and III, were PCR-cloned from the genome DNA and the mitochondrial DNA and sequenced. Any mutation was not detected in the three genes except for the mutation inserted into the bovine subunit I gene, showing that no back mutation has been induced. The present mutation results indicate that the function of D-pathway is not conserved.

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

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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*

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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

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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