11P.11 Using EPR up close and from afar: Elucidating mechanisms in haem copper oxidases

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Haem copper oxidases constitute the terminal complex of the respiratory chain and catalyse the reduction of oxygen to water. This exergonic redox reaction is coupled to proton pumping across the inner mitochondrial or bacterial membrane. O_2 reduction occurs at the binuclear haem-Cu_B centre. Despite high resolution X-ray crystallographic structures, the properties of the catalytic redox states of the metal centres and their relation to protonation states within this class of enzyme remain still poorly understood. Modern EPR techniques (also in combination with magneto-optical studies) enable us to probe different catalytic intermediate states either directly or indirectly. From afar pulsed ELDOR spectroscopy, a technique for accurately measuring inter spin distances in the range 2–8 nm, is used to resolve subtle structural changes when applied to spin-labelled systems trapped in different intermediate states (e.g. P, R & F states) and which allows the study of local conformational changes in great detail. Using this technique conformational change within the proton uptake channels is discussed. Up close both EPR and magneto-optical techniques (Magnetic Circular Dichroism) are used to address the nature of the metal ligands in the binuclear centre as well as transiently formed radical species from different intermediate states as well as in oxidases from different species.

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11P.12 Fourier transform infrared spectroscopy reveals water molecules reorganization in cytochrome *c* oxidases

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The overall mechanism of electron transfer and the oxygen reduction chemistry in cytochrome *c* oxidases (CcO) are fairly well understood. However, the associated proton transfer pathways and the way protons are gated either to the binuclear center or to a site for translocation remains unclear. One feasible mechanism involves a network of water molecules that reorganize to protonically connect a conserved glutamic acid (E242 in bovine CcO) either to the binuclear center or to a trap site above the hemes on the proton translocation pathway (Wikström M *et al.,* 2003, *Biochim. Biophys. Acta* **1604**: 61–65). Water molecules have been resolved by X-ray crystallography both in the D channel that leads to E242 and also in the region above the hemes through which translocated protons might be expected to pass. However, none of these changed markedly between oxidized and reduced forms and water molecules that could connect E242 and the binuclear center or the proton trap site have never been observed. Such water networks are H-bonded chains that can have both strongly and weakly H-bonded –OH groups. The weakly H-bonded groups absorb in the infrared spectrum between 3500 and 3800 cm^{-1}. We used FTIR difference spectroscopy to detect such weakly H-bonded –OH groups that might change organization during catalysis in bovine CcO. Complex spectral changes between 3680 and 3560 cm^{-1} were observed on diatomic ligand binding to the reduced binuclear center, a reaction that mimics the catalytic oxygen binding step. Their sensitivities to D_2O and $H_2^{18}O$ media confirmed that they arose from water molecules. Redox difference spectra also exhibited simpler changes in this region at 3674, 3619 and 3607 cm^{-1}. These transitions can be correlated to changes in the environment of a protonated carboxyl group that has been assigned to E242. Similar patterns of water reorganization can be observed in other CcO homologues suggesting that they are caused by a common conserved mechanism. The data are discussed in relation to possible functional roles in proton/electron coupling.

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11P.13 Photo-dependent binding structures of CO and NO on the heme-copper site in bovine cytochrome *c* oxidase

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Cytochrome *c* oxidase (CcO) catalyzes the O_2 reduction on the heme a_3-Cu_B site. The binding of O_2 on the ferrous heme a_3 iron (Fe_{a3}) as the sixth ligand results in formation of the catalytic intermediate as O_2-bound form. The O_2 analogues, carbon monoxide (CO), nitric oxide (NO) are also bound as the sixth ligand. The cuprous Cu_B is coordinated by three histidine imidazoles and the fourth coordination position is empty. Previous infrared studies reported that CO photo-dissociated from Fe_{a3} is transiently bound to Cu_B before the rebinding to Fe_{a3} in a temperature dependent manner [1, 2]. As in the case of CO, NO can be photo-dissociated from Fe_{a3} and rebound to Fe_{a3}, though the rebinding rate of NO is faster than that of CO [3, 4]. Here, we report photo-dependent binding structures of CO and NO in bovine CcO analyzed by absorption spectra and X-ray structures under low temperatures [5, 6]. The observed absorption spectral changes of the CcO crystals indicate that CO and NO are irreversibly photodissociated from Fe_{a3} at the temperatures of 100 K and 50 K, respectively. X-ray structures determined at above temperatures under light illumination revealed that both CO and NO were similarly bound at the fourth coordination position of Cu_B by the side-on manner. The CO- and NO-bound Fe_{a3} structures have been also determined at the temperatures of 280 K and 100 K, respectively. These results suggest that the photo-dissociated forms of CO and NO are stabilized nearby Cu_B under low temperatures. To directly demonstrate this explanation, we determined the NO-binding geometry at 50 K in the dark. The X-ray structure showed that NO remained bound to Fe_{a3} in the dark.

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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