S11/3 Carboxyl group functions in the heme-copper oxidases: Information from mid-ir vibrational spectroscopy

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Carboxyl groups of possible functional importance in bovine and bacterial cytochrome *c* oxidases (CcO) are reviewed and assessed. A critical analysis is presented of available mid-infrared vibrational data that pertain to these functional carboxyl groups. These data and their interpretations are discussed in relation to current models of the mechanism of proton and electron coupling in the protonmotive CcO superfamily.

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S11/4 Fast ligand and electron transfer dynamics in oxidases and cytochrome *c*

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The active site of heme-copper oxidases contains two cofactors, heme a_3 and Cu_B, which can both bind external ligands as the substrate O_2 and signalling molecules NO and CO, and which are both involved in electron transfer processes. Over the last few years we have exploited the fact that the heme-ligand bond can be dissociated by a short light pulse to explore the dynamics of CO and NO in the active site and the interaction between the two cofactors using ultrafast spectroscopic techniques. For example, we have time-resolved the CO transfer from heme a_3 to Cu_B and shown that it occurs in a ballistic way in ~500 fs, which presumably reflects rigidity of the active site. Heme *a* is located close to heme a_3 (~7 Å edge-to-edge) and acts as electron donor for the active site. Using mixed valence oxidases we have extended the 'reverse electron flow' technique to the ultrafast regime and demonstrated that this electron transfer process occurs in only 1.2 ns. The process is activationless and associated with a very low reorganization energy (<200 meV), in contrast to common assumptions but in general agreement with the hydrophobic environment of the reactants. Finally, ligand dynamics in native and modified cytochrome *c* reflects the rigidity required for optimal electron transfer properties.

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S11/5 The role of the conserved tryptophan272 of the *Paracoccus denitrificans* cytochrome *c* oxidase in proton pumping

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The superfamily of heme-copper oxidases comprises the cytochrome oxidases and the NO reductases, the former catalyzing the reduction of molecular oxygen to water, the latter the reduction of NO to N_2O. Cytochrome oxidases are the final electron acceptors in the respiratory chains of bacteria, archaea and mitochondria. Based on the structure of its D- and K-proton pathways cytochrome aa_3 from *Paracoccus denitrificans*, has been classified as a Type A oxidase. The reduction of oxygen generates a proton electrochemical gradient across the cytoplasmic membrane. Four protons are used for the formation of water (chemical protons) and four are pumped across the membrane. The chemical protons join with electrons and O_2 at the heme a_3-Cu_B binuclear center yielding water. The thermodynamics of the chemical protons can be understood on basis of the chemical, redox and acid-base properties of the metallo-binuclear center. However, what constitutes the driving force for the pumped protons? Our recent microsecond freeze-hyperquenching experiments revealed the formation of a transient tryptophan radical derived from the strictly conserved W272 in the transition $F \rightarrow F_{W^*} \rightarrow O_H$. We propose that the redoxchemistry and acid/base properties of W272 provide the thermodynamic and directional force for proton pumping by the cytochrome oxidases.

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S11/6 Looking for the minimum common denominator in haem-copper oxygen reductases: Towards a unified catalytic mechanism

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Haem-copper oxygen reductases are transmembrane protein complexes that reduce oxygen to water and pump protons across the mitochondrial or periplasmatic membrane, contributing to the transmembrane electrochemical potential. Seven years ago we proposed a classification of these enzymes into three different families (A, B and C), based on the constituents of their proton channels, later supported by the so far identified characteristics of the catalytic centre of each family members. The members of the three families have in common the same general structural fold, the same or analogous prosthetic groups and the existence of proton channels, in the catalytic subunit. These observations raise the hypothesis that the mechanisms for oxygen reduction, proton pumping and coupling may be the same for all haem-copper oxygen reductases. Under this hypothesis they should be performed and controlled by the same or equivalent elements/events. The identification of retained elements in all families will reveal their importance and may prompt the definition of the enzyme operating mode. Thus the search for a minimum common denominator may have a crucial importance. In this article we highlight what is already established for the haem-copper oxygen reductases and emphasize the main questions still unanswered in a comprehensive basis.

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S11/7 The mechanism of haem copper oxidases studied by EPR spectroscopy

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Haem copper oxidases constitute the terminal complex of the respiratory chain and catalyses the four electron reduction of dioxygen to water. This is an extremely exergonic redox reaction which is coupled to proton pumping across the inner mitochondrial or bacterial

membrane. Here EPR spectroscopy is applied to address several issues relating to the structure and function of haem copper oxidases;

(i) Multi-frequency EPR together with site-directed mutagenesis and isotope labelling is used to identify obligate paramagnetic intermediates within the catalytic cycle of cytochrome *c* oxidase (C*c*O), providing impetus for a discussion of the natural catalytic cycle.
(ii) 2D pulsed EPR and quantum chemical (DFT) calculations are used to identify and characterise the putative site of switching from a 2 electron donor to sequential electron transfer, as yet not observed in crystallographic models.
(iii) PELDOR spectroscopy is used to probe the recent suggestion of coupling of electron input to proton pumping that may involve conformational changes within the protein.

Here we demonstrate the use of various EPR techniques and especially the correlation with modern DFT calculations to address several of these issues. The findings are discussed in relation to the function of this important class of enzymes.

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(S11) Terminal oxidase symposium abstracts (poster and raised abstracts)

S11.8 The coupling of electron and proton transfer in haem copper oxidases as studied by peldor spectroscopy

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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. Using a cysteine-free strain of quinol oxidase from *E. coli* (*bo*$_3$), cysteines were introduced at positions R134 and R309 and labelled with a spin label probe (MTSL). Using EPR these positions were probed as a function of different catalytic intermediate states. Pulsed ELDOR spectroscopy was used to resolve potentially subtle distance changes on the doubly labelled system (R134/309). Conditions for trapping intermediate states of the enzyme while maintaining the spin labels in their paramagnetic states were successfully developed permitting the study of local conformational changes in great detail. This allows us to probe the recent suggestions for electron/proton-transfer coupling, which may involve a conformational change within the proton uptake channels.

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S11.9 Characterisation of ubiquinol oxidase activity in a native-like membrane using voltammetry

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The aim of this study was to characterise the activity of the *E. coli* terminal oxidase, cytochrome *bo*$_3$ (*cbo*$_3$) with its lipophilic substrate ubiquinol in a native-like lipid membrane. Inner membrane vesicles extracted from an *E. coli* strain which overexpresses *cbo*$_3$, were mixed with *E. coli* phospholipid extract containing ubiquinol-10 (UQ-10). These mixed vesicles were added to electrodes functionalised with cholesterol tether molecules to form planar membranes on the surface. C*bo*$_3$ activity was monitored using cyclic voltammetry with electron transfer to *cbo*$_3$ mediated by UQ-10. The apparent K_M for oxygen was determined as 1.06(±0.38) μM which is in line with apparent K_M values determined using other assay methods and experimental conditions. The UQ-10 concentration in the membrane was varied to investigate the activity of *cbo*$_3$ with its lipophilic co-substrate. Increasing the concentration of UQ-10 in the membrane above physiological levels leads to a further increase in *cbo*$_3$ activity. A K_M cannot be determined due to the limit to how much UQ-10 can be incorporated into the bilayer and this limit appears to be lower than the concentration required for maximum *cbo*$_3$ activity. This native-like membrane model provides new insights into the interaction of transmembrane enzymes with hydrophobic substrates which contrasts with studies using hydrophilic UQ analogues.

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S11. 10 Steady state redox interactions of cytochrome *c* oxidase

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The aim of this study was to characterise the behaviour of cytochrome *c* oxidase during steady state, at different levels of reductive pressure. Firstly, we addressed whether the partially oxidised intermediates 'P' and 'F' were significantly populated at low reductive pressure as previously proposed. Secondly we determined whether the two initial electron-accepting centres of cytochrome *c* oxidase, CuA and heme *a*, are in redox equilibrium with their electron-donating partner cytochrome *c*. Steady state visible and NIR spectra of pure cytochrome *c* oxidase and cytochrome *c* were acquired at different levels of reduction by adjusting the concentrations of ascorbate and TMPD; samples were oxygenated by addition of H_2O_2 plus catalase. Although our system was able to detect low levels (5–10%) of the intermediates 'P' and 'F' none could be detected above these limits, at any level of reduction. We found that cytochrome *c*, CuA and heme *a* are in rapid redox equilibrium, with CuA at 234 mV, 16 mV more negative than that of cytochrome *c*. Heme *a* equilibrates in a biphasic manner with redox potentials of 262 mV and 322 mV. This effect is probably due to modulation of heme *a* by the redox state of other centres within the enzyme.

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S11.11 FTIR detection of carboxyl groups in bovine heart cytochrome *c* oxidase

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FTIR spectroscopic studies of bovine heart cytochrome *c* oxidase (C*c*O) have revealed carboxyl group changes linked both to CO dissociation from heme *a*$_3$ and to redox changes of heme *a*. In both