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

cases, the bovine C*c*O spectra are more complex than those of bacterial C*c*Os in that they involve more than one carboxyl group. For all C*c*Os, it is thought that the highly conserved Glu242 contributes an IR signature, consistent with its proposed involvement in an internal proton transfer that is crucial for interhaem electron transfer. In bovine C*c*O, crystallographic data suggest that Asp51 and Asp91, residues located in possible proton transfer pathways undergo redox-linked changes. Hence, they might be expected to provide additional carboxyl IR changes. To investigate this further, FTIR difference spectra of bovine C*c*O were recorded in the presence of Ca^{2+}/Na^{+} or Zn^{2+}/Cd^{2+}, since these metals have been shown to bind closely to Asp51 and Asp91, respectively, and might be expected to affect any ligand- or redox-linked structural changes that they might undergo. We will report the effects of these metals on CO photolysis- and redox-induced difference spectra, with particular attention to the 1800–1700 cm^{-1} carboxylic region. These results will be discussed in terms of carboxyl group functions in the proton pathway(s) of C*c*Os.

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S11.12 What can thermodynamics tells us about haem-copper oxygen reductases: A comparison between families

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Haem-copper oxygen reductases are enzymes that catalyze the reduction of oxygen to water coupled to the translocation of up to four additional protons across the membrane. Based on differences in the amino acid residue composition of their proton conducting channels and on the ligand proprieties of their binuclear centres (a high-spin haem and a copper ion, Cu_B) the existence of three families was proposed (one of them was further divided into 2 subfamilies). Despite these differences, the similarity between their overall structure fold, the redox metal centres composition of the catalytic subunit and the common function suggests the existence of a similar mechanism. In the pursuit of the existence of a common denominator, the study of the thermodynamic redox behaviour of the haems of enzymes representative of each family and subfamily of haem-copper oxygen reductases was performed at different pH values. By using the same methodology, a direct comparison between the obtained results could be done. It was observed that in solution, neither the haems redox pH dependency nor the overall order of their midpoint redox potentials is common to the members of the different subfamilies. This questions how the redox proprieties of these redox-driven pumps influence their general operation mode.

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S11.13 High resolution X-ray diffraction experiment of bovine cytochrome *c* oxidase

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Cytochrome *c* Oxidase (C*c*O) is a large membrane protein with a molecular weight of 200 kDa which couples proton pumping and oxidation-reduction reaction. It is essential to directly observe the protonation/deprotonation states of carboxyl groups participating in the proton pumping to make the mechanism clear at atomic level. We have determined the three dimensional structures in the oxidized state and the reduced state at 1.8 Å and 1.9 Å, respectively which were not sufficient to observe hydrogen atoms in the electron density. In general, higher than 1.2 Å resolution data is needed to observe hydrogen electron density in an (Fo-Fc) difference Fourier map, because atomic parameters should be accurately determined at higher ratio of number of Fo data to number of parameters. We have developed a new annealing method to improve the quality of crystals and have collected two datasets at 1.6 Å resolution on beamline BL44XU at SPring-8 and at 1.5 Å resolution on beamline X06SA at Swiss Light Source. Devising structural refinement, we are trying to determine protonation or deprotonation states of carboxyl groups with 1.5 Å and 1.6 Å resolution data.

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S11.14 Isolation and characterization of cytochrome *c* oxidase and its supercomplex from the hyperthermophilic eubacterium *Aquifex aeolicus*

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Cytochrome *c* oxidase was purified from native membranes of *Aquifex aeolicus*. Subunit I (coxA2) and subunit II (coxB2) were identified from current preparation by mass spectrometry. Interestingly, no transmembrane spanning segments predicted in subunit II by program TMHMM 2.0. The activity of cytochrome *c* oxidase was measured spectrometrically monitoring the oxidation of reduced horse-heart cytochrome *c* at 550 nm. The specific activity is 0.35 U/mg at 80° and it remains constant at 60° for 15 h. Meanwhile, a supercomplex containing at least cytochrome *c* oxidase and ubiquinol-cytochrome *c* oxidoreductase was also isolated in a highly stable form. This project focuses on investigating structural and functional relationships of cytochrome *c* oxidase and its related protein complexes.

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S11.15 Mutations within the k-channel of cytochrome *c* oxidase lead to rapid destabilization of the O_H state

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We have shown earlier that the freshly formed O_H state of *Paracoccus denitrificans* aa_3-type cytochrome *c* oxidase (C*c*O) is capable to pump protons, as opposed to the "relaxed" state O (Bloch et al., 2004, PNAS *101*, 529–533; Belevich et al., 2007, PNAS *104*, 2685–2690). The nature of the difference between these two oxidized states remains unclear. Recent data on mutants of C*c*O, where the T351 residue in the K-channel is replaced (T⇒S, T⇒A, T⇒N), show that (a) there is no appreciable effect on the oxidative phase, except slightly slower rates (as with the K354⇒M mutant); (b) flash-induced, $Ru(bpy)_3$-mediated