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

re-reduction of the freshly oxidized CcO is not coupled to proton pumping in anyone of the three mutants; (c) fast re-reduction of the freshly oxidized CcO by an excess of exogenous reductant ($Ru(NH_3)_6$) is linked to pumping in T⇒S, but not in T⇒A or T⇒N. The data suggest that the T351 mutations dramatically shorten the life-time of the O_H state. It appears that the availability of a proton from the K-channel during (or immediately after) the F→O_H transition helps stabilizing the latter state, and enables proton pumping at the following reductive phase of the catalytic cycle.

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S11.16 X-ray crystal structural analysis of cyanide binding cytochrome *c* oxidase

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For elucidation of the reaction mechanism of the cyotochrome *c* oxidase(CcO), it is desirable to determine the binding mechanism of cyanide to the oxygen reduction site of the enzyme. Here, we analyzed the structure of the cyanide derivative of the fully oxidized CcO. Cyanide induces extremely small change in the α-band spectrum of the enzyme. Thus, it is impossible to trace cyanide-binding to the enzyme in the crystals by measuring the absorption spectrum of the crystals, because accurate measurement of Soret-band of the enzyme in crystals is impossible. However, we found that cyanide once bound to CcO was not removal by repeat dialysis. The occupancy of cyanide at the O_2 reduction site in the CcO crystal was estimated by the Soret-band spectrum of the enzyme solution prepared by dissolving the cyanide-treated crystals after washing the cyanide-treated crystals with the cyanide-free medium.

The enzyme in crystals was saturated with cyanide in one week by exchanging the freshly prepared medium of the crystals including cyanide every day. Cyanide-bound enzyme crystals, prepared by this method, gave X-ray diffraction up to 1.8 Å resolution under 100 K. The result of the data analysis suggested two possibilities of the cyanide-binding geometry that cyanide is slightly bending(N–Fe–C=90°, Fe–C–N=150°) or tilting(N–Fe–C=80°, Fe–C–*N*=180°) versus heme plane.

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S11.17 A peroxide bridge between the two metals in the dinuclear center of the fully oxidized cytochrome *c* oxidase

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Three types of the fully oxidized forms of bovine heart cytochrome *c* oxidase ("slow", "fast" and "open") have been classified according to the cyanide-sensitivity. However, the roles and chemical structures of these forms have not been established. Here we report X-ray structural analyses of the dinuclear center of the "fast" form of bovine heart cytochrome *c* oxidase, at 100 K. The metal site reduction induced by the strong X-ray irradiation was monitored by an absorption spectrophotometer designed for determining the spectrum of the crystal under X-ray irradiation. The spectral change suggesting heme *a* reduction increased linearly to get the maximal change at 20 s. X-ray data set consists of 281 images was collected with 0.6° oscillation angle and a net average exposure time of 1/3 s per image in which the X-ray irradiation effect is negligible. The refined model for an elliptical residual electron density detectable in the dinuclear center indicates that a peroxide ligand bridges the two metals (Fe_{a3} and Cu_B). The O–O bond length (1.7 Å) is within the range of those of peroxide compounds reported thus far. The conclusion is consistent to the reductive titration results for the "fast" form.

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S11.18 Oxygen reduction in the terminal *cbb*$_3$-type oxidases

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The objective of our work is to elucidate the detailed mechanism of O_2 reduction in the *cbb*$_3$-type oxidases. The *cbb*$_3$-type oxidases catalyse the final step in the respiratory chain of many bacteria, the exergonic reduction of oxygen to water, and are thus terminal oxidases. Most terminal oxidases are members of the heme-copper oxidase (HCuO) family to which the well-known mitochondrial *aa*$_3$-type oxidase also belongs. The HCuOs have been shown to use the energy from oxygen reduction to pump protons across the membrane creating a gradient that can be used by the organism for energy-requiring processes. The *aa*$_3$-type oxidases have been structurally and functionally well characterized as a result of their important role in energy metabolism. Despite the similarities in function between the *aa*$_3$- and *cbb*$_3$-type oxidases, very little is known about the mechanistic details of how the *cbb*$_3$-type oxidases reduce oxygen and pump protons. Sequence alignments of the catalytic subunits of members of the HCuO family have revealed that the *cbb*$_3$-type oxidases lack most of the protonatable groups shown to be of importance for proton pumping in the *aa*$_3$-type oxidases *even though* they are able to pump protons with nearly the same efficiency as the *aa*$_3$-type oxidases. We will present results from the application of time-resolved laser-induced optical spectroscopy with the aim of elucidating this mechanism.

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S11.19 Substrate dictates the direction of vectorial proton transfer in heme-copper oxidases

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The aim of this study was to investigate charge transfer reactions in Nitric Oxide Reductase (NOR) from *Paracoccus denitrificans* and *cbb*$_3$ type oxidase from *Rhodobacter sphaeroides* during the reductions of either O_2 or NO. These two integral membrane proteins are distinct members of the heme copper oxidase superfamily in that they both can catalyze the reduction of O_2 and NO, though to different extents (low and high O_2 reduction activity for NOR and *cbb*$_3$, respectively and vice versa for NO reduction). We used time-resolved optical spectroscopy and electrometric flow-flash on the fully reduced enzymes to investigate electron and proton transfer events related to the