re-reduction of the freshly oxidized C*c*O is not coupled to proton pumping in anyone of the three mutants; (c) fast re-reduction of the freshly oxidized C*c*O 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 $F \rightarrow O_H$ transition helps stabilizing the latter state, and enables proton pumping at the following reductive phase of the catalytic cycle.

doi:10.1016/j.bbabio.2008.05.270

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(C*c*O), 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 C*c*O. 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 C*c*O was not removal by repeat dialysis. The occupancy of cyanide at the O_2 reduction site in the C*c*O 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.

doi:10.1016/j.bbabio.2008.05.271

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.

doi:10.1016/j.bbabio.2008.05.272

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.

doi:10.1016/j.bbabio.2008.05.273

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 reduction of NO and O_2. Previous studies showed that NOR is taking both electrons and protons from the periplasm during both NO and O_2 reduction and is thus electrically silent. Our data suggest that for the *cbb*$_3$s proton pumping and transfer of protons against an electrochemical gradient varies with substrate. The *cbb*$_3$s, despite their presumed ability to pump protons during O_2 reduction, are taking electrons as well as protons from the same side of the membrane, the periplasm, when reducing NO. We take this surprising finding as an indication of the importance of the formation of distinct chemical intermediates in order to be able to perform endergonic vectorial proton transfer.

doi:10.1016/j.bbabio.2008.05.274

S11.20 Oxidase assembly factor surf1 in *Paracoccus denitrificans*: Two copies specifically act on two different terminal oxidases

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Biogenesis of mitochondrial cytochrome *c* oxidase (COX) is a complex mechanism and involves a large number of assembly proteins, one of them being Surf1. The particular function of Surf1 is not yet understood, but a role in heme *a* insertion in COX subunit I and the stabilization of the nascent complex has been proposed. In humans, a fatal neurodegenerative disorder, the Leigh syndrome, is associated with the loss of Surf1 function. Still, Surf1 is not essential for COX assembly since mutant cells have residuals of fully assembled and active oxidase. Alignments revealed Surf1 homologues not only in eukaryotes, but also in prokaryotes. In the bacterium *Paracoccus denitrificans*, two homologous genes for Surf1 proteins have been identified: *surf1q* is the last gene of the *qox* operon coding for a *ba*$_3$-type ubiquinol oxidase (QOX), and *surf1c* is found at the end of the *cta* operon encoding subunits of the *aa*$_3$-type COX. We introduced chromosomal single and double deletions for both *surf1* genes, leading to significantly reduced oxidase activities in membrane. Our experiments show that both Surf1c and Surf1q are functional and act independently for the *aa*$_3$-type COX and the *ba*$_3$-type QOX, respectively. This is the first direct experimental evidence for the involvement of a Surf1 protein in the assembly of a quinol oxidase.

doi:10.1016/j.bbabio.2008.05.275

S11.21 The *BA*$_3$ cytochrome *c* oxidase from *Thermus thermophilus*

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Terminal oxidases catalyse the energy-transducing reduction of molecular oxygen to water, coupling the redox energy to proton translocation through the cytoplasmic (or mitochondrial) membrane. Three main families of oxygen reductases can be identified on the basis of common features of their core subunits. Most of the residues which are critical for proton pumping in other cytochrome *c* oxidases ("signature") are not present in the sox B-like *ba*$_3$ oxidase. Even though the structure of this three-subunits enzyme was solved in 2000, still very little is known about in what way it deviates from canonical oxidases. To shed light on this, an internal his-tag was placed into subunit I to facilitate the purification of this enzyme and its variant forms. The spectral properties and enzymatic activities of the recombinant *ba*$_3$ oxidase are comparable to the wild-type enzyme. Mutants in critical residues involved in possible proton channels or in radical formation, were produced and compared to the native protein complex.To provide a genetically "clean" background for homologous expression of mutant enzyme(s), a chromosomal deletion of the *ba*$_3$-encoding *cba* operon was introduced in *T. thermophilus* via double-homologous recombination.

doi:10.1016/j.bbabio.2008.05.276

S11.22 Biogenesis of cytochrome *c* oxidase subunit I: A cell-free expression approach

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Aim of the project is the development of a cell-free expression system suitable for biogenesis studies on cytochrome *c* oxidase (COX). Crucial steps during COX-biogenesis are the cofactor incorporation reactions into subunits I and II which generate the redox active centres. A transient mode of interaction has been proposed for the heme and copper insertion factors. *In vitro* translation, as a promising technique for the high-level production of membrane proteins, is used to address the biogenesis of COX subunit I. Unlike the *in vivo* situation the cell-free system offers the great advantage that immature biogenesis intermediates are not degraded and therefore accumulate. Moreover, samples can easily be manipulated by the addition of purified cofactors, chaperones and intact membrane vesicles deleted in specific biogenesis factors. In an *E. coli* extract expression of the full-length subunit I was achieved either as a precipitate or in a soluble form by the addition of suitable detergents. On a preparative scale subunit I was produced at a concentration of 500 µg per ml reaction mixture and purified by Immobilized Metal Affinity Chromatography. This approach should allow future expression and biogenesis studies in the presence of various maturation factors.

doi:10.1016/j.bbabio.2008.05.277

S11.23 Synthesis of functional *Paracoccus Denitrificans* cytochrome *c* oxidase by *Escherichia coli* cell-free coupled transcription/translation system

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Time-resolved infrared spectroscopy could provide direct information about changes in protonation state and polarity of amino acid functional groups driving the proton pumping process catalyzed by cytochrome *c* oxidase. For assignment of the infrared signals, it is prerequisite to site-specifically label the amino acid residue with stable isotopes by the cell-free synthesis of the enzyme. Subunits I, II and III of the *Paraccocus denitrificans* enzyme were synthesized in the