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.

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### 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.

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### 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.

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### 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.

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### 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

*E. coli* cell-free system supplemented with *E. coli* cell membrane fractions, heme A and $Cu_2SO_4$ (about 500 μg of the proteins/ml was produced). Blue native PAGE and SDS-PAGE of dodecylmaltoside-solubilized $^{35}S$-labeled proteins revealed a protein complex with a normal subunit stoichiometry. Synthesized proteins were purified by a MonoQ column chromatography, giving a fraction exhibiting the KCN sensitive ferrocytochrome *c* oxidation activity and the normal Soret/visible spectra. The elution volume of the fraction identical to that of the authentic enzyme also suggests the native conformation of the synthesized enzyme. These results indicate successful cell-free synthesis of the native enzyme. However, the amount of the functional enzyme was still about 1% of the synthesized proteins.

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**S11.24 Mutations of possible proton-transfer pathways of bovine heart cytochrome *c* oxidase**

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X-ray structures of the bovine enzyme at 1.8–1.9 Å resolution show a possible proton-pumping pathway (H-pathway) composed of a hydrogen bond network and a water channel. The former includes the peptide bond, which could facilitate unidirectional proton transfer. Mutant enzymes in which proline is introduced to block proton transfer through the peptide bond and in which bulkier amino acids are introduced to the water channel to block its water-transfer function showed complete abolishment of the proton pumping without affecting the dioxygen reduction activity. These results and the previous proton pumping site mutation (Asp51Asn) result strongly support the proposed proton pumping function of H-pathway. On the other hand, a mutation (Asn98Asp in bovine numbering) of bacterial enzymes in D-pathway, a possible proton-transfer pathway connecting the negative-side surface with the dioxygen reduction site, abolishes proton pumping without impairing the $O_2$ reduction activity, suggesting that D-pathway of bacterial enzymes convey pumping-protons also. However, the Asn98Asp mutant of the bovine D-pathway negligibly influenced the $O_2$ reduction and proton pumping ($H^+/e^-$, 0.64 ($n$=8) for wild type and 0.65 ($n$=7) for the mutant). The present results suggest that function of D-pathway is not conserved between bovine and bacterial enzymes.

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**S11.25 X-ray structure of carbon monoxide at copper site of the dinuclear site of cytochrome *c* oxidase**

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The copper site ($Cu_B$) in the $O_2$ reduction site of cytochrome *c* oxidase is silent to most of spectroscopic techniques and thus the role of $Cu_B$ in the $O_2$ reduction mechanism is poorly understood. The fully reduced carbon monoxide (CO) derivative of bovine heart cytochrome *c* oxidase photolyzed below 140 K shows C–O stretch band at 2062 $cm^{-1}$, assignable to CO bound at $Cu_B$. However, the infrared result provides no direct geometric information for the bound CO. Electron density map of the fully reduced-CO bound form of bovine heart cytochrome *c* oxidase under light conditions at 100 K at 1.8 Å resolution shows an electron density peak assignable to CO near $Cu_B$ atom. The $F_o$–$F_c$ map strongly suggests a side-on binding of CO to $Cu_B$, although the possibility of an end-on binding cannot be excluded at this resolution. The distances between $Cu_B$ and the two atoms of CO are 2.5 Å and 2.4 Å, suggesting a fairly weak metal/ligand interaction. The weak interaction is likely to contribute the stability of the oxygenated form of the enzyme ($Fe_{a3}$–$O_2$), which is prerequisite for the four electron reduction of $O_2$ at $Fe_{a3}$.

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**S11.26 Structural analyses for lipid/protein interactions in bovine heart cytochrome *c* oxidase**

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All 13 lipids, including two cardiolipins, one phosphatidylcholine, three phosphatidylethanolamines, four phosphatidylglycerols and three triglycerides were identified in a crystalline bovine heart cytochrome *c* oxidase (C*c*O) preparation. The chain lengths and unsaturated bond positions of the fatty acid moieties determined by mass spectrometry suggest that each lipid head group identifies its specific binding site within C*c*O. Binding of dicyclohexylcarbodiimide to the $O_2$-transfer pathway of C*c*O causes two palmitate tails of phosphatidylglycerols to block the pathway, suggesting that the palmitates control the $O_2$ transfer. The phosphatidylglycerol with vaccenate (*cis*-$\Delta^{11}$-octadecenoate) was found in C*c*O of *Paracoccus denitrificans*, a possible ancestor of mitochondrion. This indicates that the vaccenate is conserved in bovine C*c*O in spite of the abundance of oleate (*cis*-$\Delta^9$-octadecenoate). The X-ray structure indicates that the protein moiety selects *cis*-vaccenate against *trans*-vaccenate for the $O_2$-transfer pathway. These results suggest that vaccenate plays a critical role in the $O_2$-transfer mechanism and that the lipid binding specificity is determined by both the head group and the fatty acid tail.

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**S11.27 X-ray structural analysis of zinc/cadmium inhibitory site in bovine heart cytochrome *c* oxidase**

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