Cytochrome *c* oxidase (CcO) catalyzes the oxygen reduction coupled to the electron and proton transfer. Previous biochemical studies indicated that Zinc (Zn) inhibited proton transfer by binding either inside or outside surface of CcO. Similar inhibition is also caused by (Cadmium) Cd. To identify the Zn/Cd inhibitory sites, we have carried out the X-ray structural analyses of bovine heart CcO–Zn/Cd complex. We found several Zn/Cd-binding sites by using the crystal of dimeric CcO. The highest affinity Zn/Cd-binding site (Zn2/Cd1 site) is located at the inside surface of the subunit III. The second highest affinity site (Zn3/Cd2 site) on the inside surface is located at the D-pathway entrance. The zinc binding affinity for the second site suggests that the zinc site is tightly coupled with the proton-pumping site. Recently, we analyzed Zn/Cd-binding to monomeric CcO which gives crystal packing different from that in the dimeric CcO crystal. The X-ray structural analysis showed Zn-binding to the Zn2, Zn3 and additional sites including the site near the K-pathway entrance. Several Zn-binding sites have been found on the outside surface. However none of them is located on the subunit I surface from which pumping protons exit.

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S11.28 The finding of the CBB_3-oxidase gene and the evidence for enzyme expression in extremely alkaliphilic bacteria

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The aim of this study was to determine whether the *cbb*$_3$-oxidase is expressed in new representatives of extremely alkaliphilic bacteria *Thioalkalivibrio* with the optimum of growth at pH = 10. For this purpose we used the synthetic oligopeptides identical to the C-terminal parts of the catalytic subunit and then worked out the specific antibodies against the catalytic subunit. A *cbb*$_3$-type oxidase was shown to be expressed in membranes of extremely alkaliphilic bacterium *Thioalkalivibrio versutus* using polyclonal rabbit anti-ccoN antibodies. The expressed oxidase is composed of 48, 34 and 29 kDa subunits, the two smaller of them being presented by cytochromes *c*. The 48 kDa subunit cross reacting with anti-ccoN antibodies was detected as a catalytic one. Sequence of the 5′-end terminal fragment of the catalytic subunit gene produced significant alignment with sequences of *Methylobacillus flagellatus* KT and *Thiobacillus denitrificans cbb*$_3$-type oxidase *ccoN*, displaying only distant phylogenetic relationship to them. Acknowledgments: authors thanks N. Pozdnyakova for assistance in work with animals.

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S11.29 Possible proton transfer mechanism through peptide groups in the H-pathway of the bovine cytochrome *c* oxidase

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A recently proposed proton transfer path (H-path) in bovine cytochrome *c* oxidase involves the peptide group connecting Tyr440 and Ser441 and interrupts the continuous hydrogen-bond network across which protons are expected to propagate. Our first-principles calculations show that the porpagation is not hindered, but occurs via a multi-step process. A proton is initially transferred to the carbonyl oxygen of a keto form of the Tyr440-Ser441 peptide group [–CO–NH–], producing an imidic acid [–C(OH)–NH–] as a metastable state. The amide proton of the imidic acid is then transferred in a barrierless way to the deprotonated carboxyl group of the Asp51 side chain, leading to the formation of an enol form [–C(OH)=N–]. Eventually, an enol-to-keto tautomerization is realized via a double proton transfer in the two adjacent Tyr440-Ser441 and Ser441-Asp442 peptide groups. An analysis of the pathway shows that each elementary process occurs through the shortest distance, thus preserving the X-ray structure, and the path is characterized by a reasonable activation barrier.

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S11.30 Oxygen reaction in the *cbb*$_3$-type cytochrome *c* oxidase from *Rhodobacter Sphaeroides*

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The *cbb*$_3$-type cytochrome *c* oxidase is a proton-pumping terminal oxidase present solely in bacteria. The enzyme is expressed mainly under very low oxygen tension and it has been shown to have a high apparent affinity for oxygen. Therefore it is of interest to investigate the properties of the enzyme that allow it to function under such conditions. In this work we have studied directly the reaction of cytochrome *cbb*$_3$ with molecular oxygen using fast kinetic approaches. The flow-flash method, where a reduced carbon monoxide inhibited enzyme is mixed with oxygenated buffer and the reaction is started by a laser flash, allowed us to follow the optical changes during the catalytic reaction at different oxygen concentrations as well as the potential generation across the membrane that takes place as a result of the charge transfer. The initial reaction with oxygen was found to be relatively slow ($k_{on} \sim 2 \times 10^7$ 10^7 M^{-1} s^{-1}) and the consequent steps of reduction were coupled to potential generation. Therefore the efficiency in reducing molecular oxygen at low oxygen concentration is not based on a particularly fast binding of O_2, but rather on the irreversibility of the oxygen binding to the enzyme.

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S11.31 Nitric oxide reductase from *Paracoccus Denitrificans* —what are the five conserved glutamates in NorB good for?

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The *c*-type nitric oxide reductase (NOR) from *Paracoccus denitrificans* is an odd member of the heme copper oxidase superfamily. It catalyses NO reduction; $2NO + 2e^- + 2H^+ \rightarrow N_2O$ $N_2O + H_2O$ and also oxygen reduction as a side reaction. All known *c*-type NORs have been shown to have five conserved glutamates (E) in the catalytic subunit (here NorB). These are, by *Paracoccus denitrificans* numbering, the E122, E125, E198, E202 and E267. The E122 and E125 are presumed to face the periplasm and the E198, E202 and E267 are thought to be located in the interior of the membrane, not far from the catalytic site.

Earlier data has shown that the E122, E125, E198 and E267 are essential for activity, but not assembly, whereas the E202 is not essential for activity. We present single turn-over data (on fully reduced NOR and oxygen) on alanine mutants of all five conserved glutamates. Our data show that, except the E202, they are all crucial for the oxidative phase of the reaction which is limited by proton uptake to the active site. Together with a model of the NorB, we propose that the E122 and E125 sit at the entrance of the proton pathway which also contains E267 and E198, but not the E202.

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S11.32 Single-turnover of *ba*$_3$ oxidase from *Thermus thermophilus*

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Cytochrome *ba*$_3$ from *Thermus thermophilus* belongs to the large family of structurally related heme-copper terminal oxidases. It catalyses the process of oxygen reduction to water and couples it with creation of an electrochemical transmembrane gradient of protons, which is subsequently used for ATP synthesis. The kinetics of the oxidation of fully-reduced *ba*$_3$ oxidase by oxygen were followed by time-resolved optical spectroscopy and electrometry. Four catalytic intermediates were resolved during this reaction. The chemical nature and the spectral properties of three intermediates (A, P, O) reproduce the general features of *aa*$_3$-type oxidases. However the F intermediate in *ba*$_3$ oxidase has a spectrum identical to the P state. This indicates that the proton taken up during the P→F transition does not reside in the binuclear site but is rather transferred to the covalently cross-linked tyrosine near that site. The total charge translocation associated with the F→O transition in *ba*$_3$ oxidase is close to that observed during the F→O transition in the *aa*$_3$ oxidases. However, P_R→F is characterized by significantly lower charge translocation, which probably reflects the overall lower measured pumping efficiency during multiple turnovers.

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S11.33 Characterization of the membrane-bound tri-heme *c* quinol peroxidase functionally connected to the respiratory chain

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Recently, we discovered quinol peroxidase (QPO) activity, the reduction of hydrogen peroxide by ubiquinol-1 as an electron donor, from the membrane fraction of the bacteria *Aggregatibacter actinomycetemcomitans* that is closely related to *Haemophilus* and has been associated with localized aggressive periodontitis. The aim of this study is to biochemically characterize QPO. QPO was purified to >90% purity from the membrane fraction. Using the N-terminal amino acid sequence of the QPO, we identified the *qpo* gene. The amino acid sequence of QPO shared 46~54% sequence identity with gene homologues in *Escherichia coli*, *Bacteroides fragilis*, etc. QPO also has a high sequence homology to bacterial di-heme cytochrome *c* peroxidase (BCCP), but QPO did not catalyze peroxidation in the presence of horse heart cytochrome *c*. MALDI-TOF MS analysis showed that QPO is a 53.6-kDa protein that contains 3 heme *c* molecules. The Km value for ubiquinol-1 was 107 μM and the optimum pH was 7.5. The Kcat value was 582 s^{-1}, comparable to that of *Paracoccus pantotrophus* BCCP. Moreover, the membrane fraction of *A. actinomycetemcomitans* had an apparent QPO-dependent peroxidase activity in the presence of NADH and succinate. Based on these findings, we present a new mechanism for the scavenging of reactive oxygen species in which quinol in the respiratory chain is consumed.

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S11.34 The semiquinone at the Q_H site of the cytochrome *bo*$_3$ from *Escherichia coli*

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The aim of this study was advanced pulsed EPR characterization of the semiquinone (SQ) in the high-affinity Q_H-site of the cytochrome *bo*$_3$ ubiquinol oxidase. Our studies have shown that a SQ at the Q_H site is a *neutral* species in the wild-type protein, with two strong H-bonds to Asp-75 and either Arg-71 or Gln-101. Selective ^{15}N labeling of the side chain nitrogens was performed to distinguish between these two residues. Pulsed EPR studies have been extended to two mutants at the Q_H site. The D75E mutation has little influence on the catalytic activity, and the pattern of H-bonding is similar to the wild type. In contrast, the D75H mutant is virtually inactive. Pulsed EPR revealed significant structural changes in this mutant. The H-bond to Arg-71 or Gln-101 that is present in both the wild type and D75E mutant oxidases is missing in the D75H mutant. Instead, the D75H has a single, strong H-bond to a histidine, likely His-75. The D75H mutant stabilizes an *anionic* semiquinone as a result of the altered H-bond network. Either the redistribution of charge density in the semiquinone species, or the altered H-bonding network may be responsible for the loss of catalytic function.

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S11.35 Resolution of a novel catalytic intermediate in cytochrome *bd* terminal oxidase in real time: A true peroxy species?

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Cytochrome *bd* is a terminal quinol oxidase of bacterial respiratory chains containing three hemes: b_{558}, b_{595} and *d*. Transient formation of catalytic intermediates in reaction of cytochrome *bd* terminal oxidases from *Escherichia coli* and *Azotobacter vinelandii* with oxygen was monitored by microsecond-resolved absorption spectroscopy and electrometry. Initial binding of O_2 by three-electron-reduced enzyme is followed by conversion of oxy-complex (A) to previously unresolved oxygen species,