11P.26 Interconversions of P and F intermediates of cytochrome *c* oxidase from *Paracoccus denitrificans*

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Cytochrome *c* Oxidase (C*c*O) is the terminal enzyme of the respiratory chain. The redox driven proton pump catalyses the four electron reduction of molecular oxygen to water. Electrons are delivered by cytochrome *c* to the bimetallic Cu_A centre and transferred *via* haem *a* into the binuclear haem a_3-Cu_B centre where the reduction of oxygen takes place. Elucidation of the intermediate structures in the catalytic cycle is crucial for understanding the mechanism of oxygen reduction. **P** and **F** states are doubly and triply reduced catalytic intermediates, respectively, when starting from oxidised C*c*O (**O** state). A **P** state can also be formed artificially by reaction of C*c*O with carbon monoxide or upon addition of equimolar amounts of hydrogen peroxide. Artificial intermediates are not necessarily the same as physiological intermediates but nevertheless one can learn what kind of reactions C*c*O can undergo. Here we show that the **F** state, classically generated by reaction with an excess of H_2O_2, can be converted into a new **P** state by addition of ammonia at pH 9. This new **P** state has a difference absorption maximum at 612 nm. Electron paramagnetic resonance experiments show that this new **P** state possesses an amino acid radical. Binding of ammonia to C*c*O is reversible upon lowering pH. Activity of C*c*O is fully maintained in the presence of ammonia. Assuming that ammonia coordinates to Cu_B, these results suggest that spectroscopic differences between **P** and **F** states are caused by different Cu_B ligands.

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11P.27 Ca^{2+}-induced inhibition of the mammalian cytochrome *c* oxidase

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Subunit I of cytochrome *c* oxidase (COX) from mitochondria and many bacteria contains a cation binding site located near heme *a* and facing the P outer aqueous phase. Mitochondrial COX binds reversibly Ca^{2+} or Na^+. In the bacterial oxidase of wild type the site is occupied by tightly bound Ca^{2+}. For a long period the role of Ca/Na site remained obscure. Recently we have found that Ca^{2+} binding to COX stabilizes the reduced state of heme *a* by increasing its midpoint potential by approximately 20 mV. Under the same conditions ferrocyanide-induced respiration of bovine COX is reversibly inhibited by Ca^{2+} but not by Mg^{2+} ions. The effect is titrated with the apparent *K*i value of 10^{-6} M close to that obtained from a Ca^{2+}-induced red shift of heme *a* absorbance spectra. Similar Ca^{2+}-induced inhibition was observed with a natural electron donor, cytochrome *c*, when COX was turning over not too fast (less than 10 s^{-1}) and reproduced on mitochondria isolated from different tissues of rat (liver, kidney, heart and skeletal muscle). The inhibition of mitochondrial respiration by Ca^{2+} ions appeared to be tissue-specific: liver COX isoform (liver, kidney) was blocked by Ca^{2+} for about 80% while inhibition of heart COX isoform (heart, skeletal muscle) was less than 60%. Titration of the Ca^{2+}-induced inhibition of rat liver mitochondria carried out in Ca-buffer HEDTA in the presence of uncoupler (CCCP) gave the apparent *K*i (0.76×10^{-6}) M which was found to be very close to the apparent *K*d value of (0.5×10^{-6}) M obtained upon a titration of the Ca^{2+}-induced red shift of heme *a* absorbance spectra in the same mitochondria. The physiological significance of this phenomenon is not completely understood. Ca^{2+}-induced inhibition of the mammalian COX resulted in a decrease of the membrane potential at least in case of the COX liver isoform might prevent mitochondria from overloading by Ca^{2+} after its accidental emission from sarcoplasmic reticulum.

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11P.28 Studies on the proton-pathway of cytochrome *c* oxidase from *Paracoccus denitrificans*

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Cytochrome *c* oxidase (CcO) couples the reduction of dioxygen to water to a proton pumping process across the membrane to generate an electrochemical proton potential. Two proton pathways (K and D), identified by X-ray crystallography of *Paracoccus denitrificans* CcO and site-directed mutagenesis analysis, are involved in the uptake of protons *via* hydrogen bond chains to the active site and to the heme propionate groups. However, the accurate mechanism of this coupling remains to be determined. Above the hemes a water cluster is observed in the crystal structure of the two-subunit CcO at 2.25 Å resolution [1], but the details of proton exit pathway have been not identified. Previous work has shown that one aspartate residue, D399, may regulate the proton translocation by changing the protonation state within the heme a_3 propionate A and itself. In order to characterize the exact role of this conserved D399 in proton/electron coupling we combined X-ray crystallography and biochemical analysis to investigate D399-variants. Because of the low yield and poor stability of the variants the crystals diffracted only to 4–8 Å anisotropic resolution. We are also using site-directed mutagenesis to characterize the possible residues of the potential proton exit pathway by spectrophotometric and polarographic techniques. Four variants were created and the characterization of their proton pumping behaviour will be presented.

Reference

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11P.29 The O_2 reduction and proton pumping gate mechanisms of bovine heart cytochrome *c* oxidase

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The O_2 reduction site of cytochrome *c* oxidase (CcO), composed of iron (Fe_{a3}) and copper (Cu_B) ions, is probed by X-ray structural

analyses of CO, NO and CN^- derivatives to investigate the mechanism of the complete reduction of O_2. CO and NO derivatives revealed that the trigonal planar coordination of Cu_B^{1+} and the locations of Tyr244 and Cu_B^{1+}, relative to Fe_{a3} stabilize the oxygenated Fe_{a3} detectable as the initial intermediate. Formation of the Fe_{a3}^{2+}-CN^- derivative forms the trigonal planar coordination of Cu_B^{1+}, displacing one of its three coordinated imidazole groups while a water molecule bridges the CN^- ligand with the hydroxyl group of Tyr244 by forming two hydrogen bonds. When O_2 is bound to Fe_{a3}^{2+}, it is negatively polarized (O_2^-), and likely to induce the same structural change induced by CN^-. This allows O_2^- to receive three electron equivalents non-sequentially from Cu_B^{1+}, Fe_{a3}^{3+} and Tyr-OH, providing complete reduction of O_2^- with minimization of production of active oxygen species [1]. The proton pumping pathway of bovine CcO is composed of a hydrogen bond network and a water channel. Protons transferred through the water channel are pumped through the hydrogen-bond network electrostatically with positive charge created at the Fe_a center by electron donation to the O_2 reduction site. Binding of CO or NO to Fe_{a3}^{2+} induces significant narrowing of a section of the water channel near the hydrogen-bond network junction, which prevents access of water molecules to the network. In a similar manner, O_2 binding to Fe_{a3}^{2+} is expected to prevent access of water molecules to the hydrogen-bond network. This blocks proton back-leak from the network and provides an efficient gate for proton pumping [1].

Reference

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