

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

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Cytochrome c Oxidase (CcO) 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 a₃-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 CcO (**O** state). A **P** state can also be formed artificially by reaction of CcO 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 CcO can undergo. Here we show that the **F** state, classically generated by reaction with an excess of H₂O₂, 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 CcO is reversible upon lowering pH. Activity of CcO 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 Ca²⁺-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 Ca²⁺ or Na⁺. In the bacterial oxidase of wild type the site is occupied by tightly bound Ca²⁺. For a long period the role of Ca/Na site remained obscure. Recently we have found that Ca²⁺ 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 Ca²⁺ but not by Mg²⁺ ions. The effect is titrated with the apparent K_i value of 10⁻⁶ M close to that obtained from a Ca²⁺-induced red shift of heme a absorbance spectra. Similar Ca²⁺-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 Ca²⁺ ions appeared to be tissue-specific: liver COX isoform (liver, kidney) was blocked by Ca²⁺ for about 80% while inhibition of heart COX isoform (heart, skeletal muscle) was less than 60%. Titration of the Ca²⁺-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 Ca²⁺-induced red shift of heme a absorbance spectra in the same mitochondria. The physiological significance of this phenomenon is not completely understood. Ca²⁺-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 Ca²⁺ 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 a₃ 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 O₂ reduction and proton pumping gate mechanisms of bovine heart cytochrome c oxidase

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The O₂ reduction site of cytochrome c oxidase (CcO), composed of iron (Fe_{a3}) and copper (Cu_B) ions, is probed by X-ray structural