II-E Structure and Function of Respiratory Terminal Oxidases

In the aerobic respiratory chain of *Escherichia coli*, there are structurally unrelated two terminal oxidases. A heme-copper oxidase, cytochrome *bo* is predominantly expressed under highly aerated growth conditions while an alternative oxidase, a putative heme-heme oxidase, cytochrome *bd*, is predominant under microaerobic conditions. Both oxidases catalyze the two-electron reduction of ubiquinol-8 and the four-electron reduction of dioxygen, whereas only cytochrome *bo* exhibits vectorial proton transport. However, only a little structural information has been given for these ubiquinol oxidases. To clarify the molecular mechanism of electron transfer, chemical reaction of dioxygen, and proton pumping in the two respiratory terminal oxidases, we utilize various molecular spectroscopic techniques (*e.g.*, resonance Raman, EPR, FTIR) in conjunction with methods of molecular biology and biochemistry.

II-E-1 Fourier-Transform Infrared Studies on Azide Binding to the Binuclear Center of the *Escherichia coli bo*-Type Ubiquinol Oxidase

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[*FEBS Lett.* **449**, 191 (1999)]

Azide-binding to the heme-copper binuclear center of *bo*-type ubiquinol oxidase from *Escherichia coli* was investigated with Fourier-transform infrared spectroscopy. Deconvolution analyses of infrared spectra of the azide ($^{14}N_3$)-inhibited air-oxidized form showed a major infrared azide antisymmetric stretching band at 2041 cm^{-1}. An additional band developed at 2062.5 cm^{-1} during a longer incubation. Isotope substitutions with terminally ^{15}N-labeled azides did not show a splitting of the major band, indicating that the geometry of the bound azide is mainly in a bridging configuration between high-spin heme *o* and Cu_B.[1,2] The band at 2062.5 cm^{-1} showed clear splittings upon substitution with the terminally ^{15}N-labeled azides, indicating the Cu_B^{2+}–N=N=N structure. Partial reduction of the oxidase with β-NADH in the presence of azide ($^{14}N_3$) caused an appearance of new infrared bands at 2038.5 (major) and 2009 (minor) cm^{-1}. The former band also showed clear splittings in the presence of the terminally ^{15}N-labeled azides, indicating that reduction of low-spin heme *b* alters the structure of the binuclear center leading to the Fe_o^{3+}–N=N=N configuration.[3]

References

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II-E-2 Fluoride-Binding to the oxidized *Escherichia coli bd*-Type Ubiquinol Oxidase Studied by Visible Absorption and EPR Spectroscopies

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Cytochrome *bd*-type ubiquinol oxidase in the aerobic respiratory chain of *Escherichia coli* contains two hemes *b* (b_{558} and b_{595}) and one heme *d* as redox metal centers.[1] To clarify the structure of the reaction center, we analyzed the fully oxidized enzyme by visible and EPR spectroscopies using fluoride ion as a monitoring probe. The visible spectral changes upon fluoride-binding were typical of ferric iron-chlorine species, indicating heme *d* as a primary binding site. The negative peak at 645 nm in the difference spectrum indicates that heme b_{595} also provides the low-affinity fluoride-binding site. Fluoride-binding caused a complete disappearance from the EPR spectra of the low-spin signals ascribable to heme *d* and spectral changes in both rhombic and axial high-spin signals. After fluoride-binding, each component of the rhombic high-spin signal showed superhyperfine splitting arising from the interaction of the unpaired spin of the heme *d* iron with the nuclear magnetic moment of ^{19}F. The axial high-spin species was converted to a new rhombic high-spin species assignable to heme b_{595}-fluoride. The $g = 2$ component of this new species also gave ^{19}F-superhyperfine splitting. These results indicate that both heme *d* and heme b_{595} can coordinate with a fluoride ion with different affinities in the fully oxidized state.

Reference

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II-E-3 Azide- and Cyanide-Bindings to the *Escherichia coli bd*-Type Ubiquinol Oxidase Studied by Visible Absorption, EPR and FTIR Spectroscopies

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Cytochrome *bd*-type ubiquinol oxidase contains two hemes *b* (b_{558} and b_{595}) and one heme *d* as the redox metal centers. To clarify the structure of the reaction center, we analyzed *Escherichia coli* cytochrome *bd* by visible absorption, EPR and FTIR spectroscopies using

azide and cyanide as monitoring probes for the exogenous ligand binding site. Azide-binding caused the appearance of a new EPR low-spin signal characteristic of ferric iron-chlorin-azide species and a new visible absorption band at 647 nm. However, the bound azide ($^{14}N_3$) anti-symmetric stretching infrared band (2,010.5 cm^{-1}) showed anomalies upon ^{15}N-substitutions, indicating interactions with surrounding protein residues or heme b_{595} in close proximity. The spectral changes upon cyanide-binding in the visible region were typical of those observed for ferric iron-chlorin species with diol substituents in macrocycles. However, we found no indication of a low-spin EPR signal corresponding to the ferric iron-chlorin-cyanide complexes. Instead, derivative-shaped signals at g = 3.19 and g = 7.15, which could arise from the heme $d(Fe^{3+})$-CN-heme $b_{595}(Fe^{3+})$ moiety,[1)] were observed. After the addition of cyanide, a part of ferric heme *d* showed the rhombic high-spin signal that coexisted with the g_z = 2.85 signal ascribed to the minor heme b_{595}-CN species. This indicates strong steric hindrance of cyanide-binding to ferric heme *d* with the bound cyanide at ferric heme b_{595}.

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II-F Structure and Function of Transmembrane Electron Transfer System in Neuroendocrine Secretory Vesicles

In neuroendocrine secretory vesicles of higher animals, intravesicular ascorbate (AsA^-) functions as the electron donor for copper-containing monooxygenases. Upon these monooxygenase reactions, monodehydroascorbate (MDA) radical is produced by oxidation of AsA^-. The MDA radical is reduced back to AsA^- by membrane-spanning cytochrome b_{561} and subsequently the oxidized cytochrome b_{561} is reduced by cytosolic AsA^-. To clarify the molecular mechanism of the electron transfer, we utilize various biophysical techniques (*e.g.* EPR, resonance Raman, and pulse radiolysis) in conjunction with methods of molecular biology and biochemistry. We found previously that purified cytochrome b_{561} from bovine adrenal medulla contains two hemes B per molecule, each exhibiting an independent EPR signal in oxidized state. The reaction of MDA radical with purified cytochrome b_{561} was investigated by the technique of pulse radiolysis. Radiolytically generated MDA radical oxidized rapidly reduced cytochrome b_{561} to yield the oxidized form. Subsequently, the oxidized form was re-reduced by AsA^- in the medium. At excess MDA radical, only half of the heme was oxidized, indicating that only one of the two heme centers can react with MDA radical.

II-F-1 Diethylpyrocarbonate-Modification Abolishes Fast Electron Accepting Ability of Cytochrome b_{561} from AsA^- but Does Not Influence on Electron Donation to MDA Radical: Identification of the Modification Sites by Mass Spectrometric Analyses

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Cytochrome b_{561} in bovine adrenal chromaffin vesicles contains two hemes B[1)] and transports electron equivalents across the vesicle membranes to convert intravesicular MDA radical to AsA^-. To elucidate the mechanism of the transmembrane electron transfer, effects of the treatment of purified cytochrome b_{561} with diethylpyrocarbonate, a reagent specific for histidyl residues, were examined. We found that, when AsA^- was added to the oxidized form of diethylpyrocarbonate-treated cytochrome b_{561}, less than half of the heme iron was reduced but with a very slow rate. In contrast, radiolytically-generated MDA radical was oxidized rapidly by the reduced form of diethylpyrocarbonate-modified cytochrome b_{561}, as observed for untreated cytochrome b_{561}.[2)] These results indicate that the heme center specific for the electron acceptance from AsA^- is perturbed by the modification of amino acid residues nearby. We identified the major modification sites by mass spectrometry at Lys85, His88, and His161, all of which are fully conserved and located at extravesicular side of cytochrome b_{561} in the membranes. We suggest that specific *N*-carbethoxylation of the histidyl ligands of the heme *b* at extravesicular side abolish the electron accepting ability from AsA^-.

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