II-D 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-D-1 Probing Molecular Structure of Dioxygen Reduction Site of Bacterial Quinol Oxidases through Ligand Binding to the Redox Metal Centers

TSUBAKI, Motonari[1,2]; HORI, Hiroshi[2]; MOGI, Tatsushi[3]
([1]Himeji Inst. Tech.; [2]Osaka Univ.; [3]Univ. Tokyo)

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Cytochromes *bo* and *bd* are structurally unrelated terminal ubiquinol oxidases in the aerobic respiratory chain of *Escherichia coli*. The high-spin heme-Cu_B binuclear center serves as the dioxygen reduction site for cytochrome *bo*, and the heme b_{595}-heme *d* binuclear center for cytochrome *bd*. Cu_B coordinates three histidine ligands and serves as a transient ligand binding site en route to high-spin heme, one-electron donor to the oxy intermediate, and a binding site for bridging ligands like cyanide. In addition, it can protect the dioxygen reduction site through binding of a peroxide ion in the resting state, and connects directly or indirectly Tyr288 and Glu286 to carry out redox-driven proton pumping in the catalytic cycle. Contrary, heme b_{595} of cytochrome *bd* participate a similar role to Cu_B in ligand binding and dioxygen reduction but cannot perform such versatile roles because of its rigid structure.

II-D-2 Active Site Structure of SoxB-Type Cytochrome bo_3 Oxidase from Thermophilic *Bacillus*

UCHIDA, Takeshi[1]; TSUBAKI, Motonari[1,2,4]; KUROKAWA, Tatsunori[3]; HORI, Hiroshi[4]; SAKAMOTO, Junshi[3]; KITAGAWA, Teizo[1]; SONE, Nobuhito[3]
([1]IMS; [2]Himeji Inst. Tech.; [3]Kyushu Inst. Tech.; [4]Osaka Univ.)

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Two-subunit SoxB-type cytochrome *c* oxidase in *Bacillus stearothermophilus* was over-produced, purified, and examined for its active site structures by electron paramagnetic resonance (EPR) and resonance Raman (RR) spectroscopies. This is cytochrome bo_3 oxidase contained heme B at the low-spin heme site and heme O at the high-spin heme site of the binuclear center. EPR spectra of the enzyme in the oxidized form indicated that structures of the high-spin heme O and the low-spin heme B were similar to those of SoxM-type oxidases based on the signals at g = 6.1, and g = 3.04. However, the EPR signals from the Cu_A center and the integer spin system at the binuclear center showed slight differences. RR spectra of the oxidized form showed that heme O was in a 6-coordinated high-spin (ν_3 = 1472 cm^{-1}), and heme B was in a 6-coordinated low-spin (ν_3 = 1500 cm^{-1}) state. The Fe^{2+}–His stretching mode was observed at 211 cm^{-1}, indicating that the Fe^{2+}–His bond strength is not so much different from those of SoxM-type oxidases. On the contrary, both the Fe^{2+}–CO stretching and Fe^{2+}–C–O bending modes differed distinctly from those of SoxM-type enzymes, suggesting some differences in the coordination geometry and the protein structure in the proximity of bound CO in cytochrome bo_3 from those of SoxM-type enzymes.

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

In neuroendocrine secretory vesicles of animals, intravesiclular 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}. Subsequently, the oxidized cytochrome b_{561} is reduced by cytosolic AsA^-. 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. 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-E-1 Reduction of Heme Iron Suppresses the Carbethoxylation of Two Histidyl and One Tyrosyl Residues Indispensable for the Transmembrane Electron Transfer Reaction of Cytochrome b_{561}

TAKEUCHI, Fusako[1]; KOBAYASHI, Kazuo[2]; TAGAWA, Seiichi[2]; TSUBAKI, Motonari[1,3]
([1]Himeji Inst. Tech.; [2]Osaka Univ.; [3]IMS)

We found previously that treatment of oxidized cytochrome b_{561} with diethyl pyrocarbonate (DEPC) caused specific *N*-carbethoxylation of three fully conserved residues (His88, His161, and Lys85) located at the extravesicular side.[1)] The modification lead to a selective loss of the electron accepting ability from AsA^- without affecting the electron-donation to MDA radical. In the present study, we found that the *O*-carbethoxylation of one tyrosyl residue (Tyr218) locating at the extravesicular side was significantly enhanced in an alkaline condition, leading to a very slow reduction process of the oxidized heme with AsA^-. Presence of AsA^- during the reaction with DEPC was found to suppress the carbethoxylation of the heme-coordinating histidyl (His88 and His161) and the tyrosyl (Tyr218) residues, whereas the modification level of Lys85 was not affected. Concomitantly, the final reduction level of heme *b* with AsA^- was protected, although the fast reduction process was not fully restored. A similar protective effect was observed in the presence of sodium dithionite or isoascorbate. These results suggest that the modification of the histidine residues were suppressed in the reduced form of heme *b*. On the other hand, Tyr218, together with Lys85, has a role in the recognition/binding process for AsA^- and is indispensable for the fast electron transfer reaction from AsA^-.

Reference

1) Tsubaki, M., Kobayashi, K., Ichise, T., Takeuchi, F. and Tagawa, S., *Biochemistry* **39**, 3276 (2000).

II-E-2 Planarian Cytochrome b_{561}: A Transmembrane Electron Transfer Protein Unique to Neuroendocrine Secretory Vesicles

ASADA, Akikazu[1]; KUSAKAWA, Takashi[1]; ORII, Hidefumi[1]; AGATA, Kiyokazu[1]; WATANABE, Kenji[1]; TSUBAKI, Motonari[1,2,3]
([1]Himeji Inst. Tech.; [2]IMS; [3]Osaka Univ.)

Cytochrome b_{561} is a major transmembrane protein of catecholamine and neuropeptide secretory vesicles of central and peripheral nervous system in higher animals. We succeeded in the cloning of a full-length cDNA encoding planarian cytochrome b_{561}. The deduced amino acid sequence showed a very similar transmembrane topology to those of higher vertebrate and contained both putative AsA^-- and MDA radical-binding sites.[1)] Among the six totally-conserved His residues in higher vertebrate, one His residue was substituted with Asn residue indicating that His88 and His161 of bovine cytochrome b_{561} play roles as the heme *b* ligands at the extravesicular side. Northern- and Western-blot analyses confirmed the expression of the mRNA and the protein in planarian with the expected sizes, respectively. Distributions of the mRNA and the apoprotein were analyzed with in situ hybridization and immunocytochemical staining, respectively, which showed two morphologically distinct structures, a pair of the ventral nerve cords and the cephalic ganglion cluster in the head region. Present results suggest that the usage of AsA^- for the supply of electron equivalents to the neuroendocrine-specific copper-containing monooxygenases could be originated from organisms having a very simple nervous system.

Reference

1) Okuyama, E., Yamamoto, R., Ichikawa, Y. and Tsubaki, M., *Biochim. Biophys. Acta* **1383**, 269 (1998).

II-F Structure and Function of Steroidogenic Cytochrome P450 System

In adrenal cortex of higher animals, various cytochromes P450 perform steroid hormone biosynthesis. In the mitochondrial inner membranes, cytochromes P450scc and P45011β receive electron equivalents from a 2Fe-2S type ferredoxin, adrenodoxin, to perform the oxygen activation and the site-specific hydroxylations. On the other hand, in the endoplasmic reticulum membranes, there are two microsomal type cytochromes P450; namely P450c21 and P45017α. These cytochromes receive electron equivalents from flavin-containing cytochrome P450 reductase. The former catalyzes the C21 hydroxylation essential for the production of corticosteroid hormones (glucocorticoids and mineralcorticoids). We are currently investigating these steroidogenic cytochrome P450 systems utilizing various biochemical and biophysical techniques.

II-F-1 Direct Heme-Steroid Interaction in Cytochrome P450c21 Studied by FTIR Spectroscopy

TSUBAKI, Motonari[1,2]; TAKEUCHI, Kohji[1]
([1]Himeji Inst. Tech.; [2]IMS)

We showed previously that combination of 20β-hydroxysteroids (17α,20β-dihydroxyprogesterone and 20β-hydroxyprogesterone) with oxidized cytochrome P450c21 purified from bovine adrenocortical microsomes induced a type I difference spectrum and exhibited a concomitant development of a new low-spin

signal at g_z = 2.42, g_y = 2.21, and g_x = 1.966 and an increase in intensity of the *g*8 high-spin signal in EPR spectra.[1)] Being consistent with these substrate-like properties, we confirmed that cytochrome P450c21 have a 20β-oxidase activity for the 20β-hydroxysteroids in an enzyme-reconstituted system. In the present study, the heme-steroid interaction in reduced state was investigated by analyzing heme-bound C–O stretching vibration with FTIR spectroscopy, to clarify the mechanism of the site- and stereo-selective 20β-oxidase activity. In a substrate-free state, a C–O band was observed at 1949 cm^{-1}. Addition of 17α-hydroxyprogesterone or progesterone caused a peak-shift to 1952 and 1942.5 cm^{-1}, respectively. Additions of 17α,20β-dihydroxyprogesterone and 20β-hydroxyprogesterone caused a shift of main band to 1950 and 1955 cm^{-1}, respectively. Concomitantly, peculiar C–O bands were observed around 1998 cm^{-1} for these 20β-hydroxysteroid complexes. These results suggest a specific interaction between steroid hydroxy group(s) and heme prosthetic group, both in oxidized and reduced states.[2)]

References

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II-F-2 Adrenodoxin-Cytochrome P450scc Interaction as Revealed by EPR Spectroscopy: Comparison with Putidaredoxin-Cytochrome P450cam System

TAKEUCHI, Kohji[1]; TSUBAKI, Motonari[1,2,3]; HORI, Hiroshi[3]
([1]Himeji inst. Tech.; [2]IMS; [3]Osaka Univ.)

Cholesterol side-chain cleavage reaction catalyzed by cytochrome P450scc constitutes of three consecutive monooxygenase reactions (22R-hydroxylation, 20S-hydroxylation, and C20-C22 bond scission) to produce pregnenolone.[1,2)] The electron equivalents necessary for the oxygen activation were supplied from a 2Fe2S-type ferredoxin, adrenodoxin. We found that binding of oxidized adrenodoxin to ferric P450scc complexed with cholesterol or 25-hydroxycholesterol caused a shift of the g = 8 and g = 3.5 high-spin signal of the heme moiety but not for the low-spin signals at 15 K. On the other hand, ligation of CO or NO to the ferrous heme of P450scc complexed with reduced adrenodoxin and various steroid substrates did not show any change in a trough (at 346.8 mT) of the axial EPR spectrum of the reduced adrenodoxin (at 77 K). These results showed a remarkable contrast to those found for the cytochrome P450cam-putidaredoxin-substrate ternary complex suggesting that mode of the cross talk between adrenodoxin and P450scc is different from the *Pseudomonas system*.

References

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