博士論文

大腸菌シトクロム*bo*複合体の酸化還元反応中心の構造

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吉川　純

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Molecular Structure of the Redox Reaction Center of the Cytochrome *b o* Complex in *Escherichia coli*

(大腸菌シトクロム*bo*複合体の酸化還元反応中心の構造)

１９９２年１月　　皆川　純

MOLECULAR STRUCTURE OF THE REDOX REACTION CENTER OF THE CYTOCHROME BO COMPLEX IN ESCHERICHIA COLI

BY

JUN MINAGAWA

THESIS

Submitted in partial satisfaction of the requirements for the degree
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Dedicated To: my family for their love and support.

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Finally, I would like to thank Kanako, for her love and understanding. She has been my closest friend.

ABBREVIATIONS

kb	kilo base pairs
SDS	sodium dodecyl sulfate
MCD	magnetic circular dichroism
EXAFS	extended X-ray absorption fine structure
ENDOR	electron nuclear double resonance.
EC	*Escherichia coli*
HH	*Halobacterium halobium*
BS	*Bacillus subtilis*
PS	Thermophilic bacillus PS3
BJ	*Bradyrhizobium japonicum*
PD	*Paracoccus denitrificans*
SC	*Saccharomyces cerevisiae* (yeast)
SP	*Saccharomyces pombe* (fission yeast)
AN	*Aspergillus nidulans*
NC	*Neurospora crassa*
CR	*Chlamydomonas reinhardi*
WH	*Triticum aestivum* (wheat)
ZM	*Zea mays* (maise)
SO	*Sorghum bicolor milo*
OR	*Oriza* (rice)
SB	*Glycine max* (soy been)
OB	*Oenothera bercerius* (evening primrose)
TB	*Trypanosoma brucei*
LT	*Leishmania tarentolae* (*Trypanosoma plantydanctuli*)
TE	*Tetrahymena pyriformis*
PA	*Paramecium aurelia*
DM	*Drosophila melanogaster*
DY	*Drosophila yakuba*

SU	*Paracentrotus lividus* (sea urchin)
XL	*Xenopus laevis*
MS	*Mus culus* (mouse)
RA	*Rattus norvegicus* (rat)
BV	*Bos primigenius* (bovine)
HU	*Homo sapiens* (human)

ABSTRACT OF THE DISSERTATION

Molecular Structure of the Redox Reaction Center of the Cytochrome *bo* Complex in *Escherichia coli*

by

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Doctor of Philosophy in Pharmaceutical Science

the University of Tokyo, Tokyo, 1992

The cytochrome *bo* complex is a terminal ubiquinol oxidase in the aerobic respiratory chain of *Escherichia coli* and functions as a proton pump. It belongs to the heme-copper oxidase superfamily with the *aa*3-type cytochrome *c* oxidases in mitochondria and aerobic bacteria. To identify ligands of hemes and copper, eight conserved histidines in subunit I were replaced by alanine, in addition, and His-106, -284, -419 and -421 by glutamine and methionine. Alterations of invariant His residue at positions 106, 284, 333,

334, 419, and 421, of subunit I caused loss of the enzymatic activity *in vivo* and *in vitro*. Spectroscopic analysis revealed that replacements of His-106 and -421 specifically eliminated a 563.5 nm peak of the low-spin heme, and that replacements of His-106, -284, and -419 reduced the extent of CO-binding high-spin heme. Atomic absorption analysis showed that alterations of His-106, -333, -334, and -419 eliminated Cu_B almost completely. From these findings, His-106 and -421 are concluded to be the axial ligands of the-low spin heme and that His-284 to be a possible ligand of the high-spin heme. His-333, -334, and -419 are concluded to be the ligands of Cu_B.

To insure the assignment described above and to identify the residues which play important roles on the enzymatic function, such as electron transfer and involving the ligand binding pocket, twelve aromatic amino acid residues were altered by site-directed mutagenesis. Of twelve constructs ,five alterations, W280L, Y288L, F328L, W331L, and F348L were identified to cause loss of the enzymatic activity *in vivo*. All of these mutants except F328L also lost Cu_B. Spectroscopic analysis revealed that replacements of Phe-112 and -113 caused red-shift of a 563.5 nm peak of the low-spin heme, and that replacements of Trp-280 and -282 reduced the extent of CO-binding high-spin heme. These findings are consistent with the results from His-to-Ala mutations, and led me to present a helical wheel model of the redox reaction center in subunit I that consists of the transmembrane helices II, VI, VII, VIII and X.

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Chapter I. General Introduction

Background

Respiratory Chain

Life is a phenomenon in which there is a continuous flow of energy, originating from the sun. Although most of the solar energy that reaches Earth is dissipated into heat, a small portion of it is converted to chemical energy by the process of photosynthesis: water is split by a reaction energized by photon, the liberated electrons are then donated to CO_2 molecule. Here the energy from the sun is locked into stable organic molecule, such as glucose.

In heterotrophic organisms, the organic molecules are metabolized in the glycolytic pathway and the citric acid cycle. After the reaction, electrons are liberated and fed into a respiratory chain, only by which reaction non-photosynthetic organisms can obtain energy. Respiratory chain consists of a number of redox components which span mitochondrial inner membrane in eukaryotes or cytoplasmic membrane in prokaryotes The electric current through them is coupled to the translocation of protons across the membranes, where the free energy is temporarily stored in a form of electrochemical gradient. ATP, the immediate source of free energy in most endergonic reactions of living cells, as first suggested by Mitchell (1961) is synthsized using the energy in a form of electrochemical gradients.

Problems of the organization of respiratory components and the molecular mechanisms of acquisition of energy by the redox reactions have been studied extensively. Studies in the last 20 years have been concentrated on the biochemical properties of cytochromes in various organisms. A major development in the research on respiratory components in the late 80's was the elucidation of bacterial respiratory chains, while often have multiple branches (Anraku 1988). Molecular cloning of mitochondrial genomes has been accomplished, whereas the expression of those genes have not yet been succeeded. Then, more and more researchers have turned their eyes to the study on bacterial respiratory chains (Saraste 1990).

Terminal Oxidases

It is common to most living organisms on Earth, such as bacteria, fungi, plants and animals, that the last component of respiratory chain is a terminal oxidase, which catalyzes reduction of the terminal electron accepter and oxidation of the intermediate electron mediator, such as cytochrome *c*, ubiquinonol, and menaquinol. Most aerobe and facultative anaerobe have a terminal oxidase which uses molecular oxygen as a terminal electron accepter. In a narrow sense, such an enzyme is named to terminal oxidase.

All mitochondrial terminal oxidases use cytochrome *c* as a reductant and contain two *a*-type hemes (heme *a* and a_3) as well as two copper atoms (Cu_A and Cu_B). On the other hand, considerably more variety is found in bacterial respiratory chain, which have been classified based on

whether or not they can oxidize cytochrome *c* (Raitio *et al.* 1987). In the late 80's, it was established that the terminal oxidases in most bacteria can use quinol, such as ubiquinol and/or menaquinol as a reductant. The bacterial terminal oxidases has been accordingly classified (Anraku 1988). Recently, various combinations of heme species were found in the terminal oxidases of most bacteria—aa_3-type cytochrome *c* oxidase (*P. denitrificans*), ba_3-type cytochrome *c* oxidase (*T. thermophilus*), caa_3-type cytochrome *c* oxidase (thermophilic bacteria PS3), *cao*-type cytochrome *c* oxidase (Alkalophilic bacillus YN-2000), *bo*-type and *bd*-type quinol oxidase (*E. coli*), aa_3-type quinol oxidase (*S. acidocaldarius*), *b*-type quinol oxidase (thermophilic bacteria PS3), and ba_1-type quinol oxidase (*A. aceti*). Hence, we cannot say but more data are necessary to classify properly the enzymes indicated above.

Two terminal ubiquinol oxidases in E. coli

Keilin (1934) analyzed bacterial cytochromes spectroscopically and reported that *E. coli* has four cytochormes—cytochrome a_1, a_2, b_1, and d_1. Cytochromes a_1 and a_2 were later renamed cytochromes b_{595} and d, respectively (Green *et al.* 1986). Shipp (1972) measured fourth-order finite spectra of low-temperature difference spectra of cytochromes from *E. coli* and concluded that the α-band, previously attributed to cytochrome b_1, is a composite of the absorption bands of five or more different pigments and tentatively assigned these bands to at least three *b*-type cytochromes. Since then, several investigators have reported the spectral and potentiometric relations of the *b*-type cytochromes, and concluded that the major membrane-bound cytochrome components of the *E. coli*

aerobic respiratory chain are localized to the cytochrome *bo* ubiquinol oxidase, the cytochrome *bd* ubiquinol oxidase, and the succinate dehydrogenese, which have α-band at about 555 and 562, 558, and 556 nm, respectively.

The terminal respiratory chain of *E. coli* branches into two ubiquinol oxidases described above (Fig. I-1, see Anraku and Gennis 1987). The cytochrome *bo* complex is expressed predominantly under the conditions where oxygen tension is high (Minagawa *et al.* 1990), whereas the cytochrome *bd* complex is expressed at low oxygen tension (Georgiou *et al.* 1988b). Mutant lacking one of the two oxidases can grow normally, but a mutant lacking both oxidases cannot grow aerobically on nonfermentable carbon sources (Au *et al.* 1985). Electron-transfer from ubiquinol-8 through either of these oxidases results in reduction of oxygen to water (Minghetti and Gennis 1988). Although these two oxidases have functional similarities, they also possess many distinguishing features. These differences can be seen structurally in a comparison of the number and size of subunit and prosthetic group composition. Both of the enzymes have been purified to homogeneity, and the preparations indicate that the cytochrome *bd* complex contains 2 subunits (Miller and Gennis 1983) with 2 mol of *b*-type heme and 2 mol of *d*-type heme (Lorence *et al.* 1986). The cytochrome *bo* complex, on the other hand, has been purified in several laboratories and is reported to contain from two (Kita *et al.* 1984), four (Matsushita *et al.* 1984, Georgiou *et al.* 1988a),

to five subunits (Minghetti *et al.* 1992, Mogi *et al.* 1992). The preparations of four or five subunits contained 2 mol of heme and 1 mol of copper atom (Fig. I-2). The genes, *cyoABCDE* and *cydAB*, coding for the cytochrome *bo* and cytochrome *bd* complex, respectively, have been cloned and sequenced (Green *et al.* 1984, Green *et al.* 1988, Chepuri *et al.* 1990a, Nakamura *et al.* 1990). The primary sequences of these two oxidases were found to share no homology.

Recently, functional difference was also found: Proton pumping experiments performed on these complexes in sphaeroplasts indicated that only the cytochrome *bo* complex is able to function as a proton pump (Puustinen *et al.* 1989). Although the cytochrome *bo* complex is functionally similar to the cytochrome *bd* complex, and can actually replace the cytochrome *bd* complex *in vivo*, they are probably quite distinct.

Heme-copper oxidase superfamily

At the time I entered graduate school, 1986, a few researchers regarded the cytochrome *bo* complex as a model system for the study of terminal oxidases (Kita *et al.* 1984, Hata *et al.* 1985). The cytochrome *bo* complex appeared to be structurally and functionaly rather different from the eucaryotic cytochrome *c* oxidase: Purified enzyme contains at most five subunits while eucaryotic oxidase comprises of seven to thirteen subunits. As for heme species, *E. coli* enzyme contains protoheme IX (Kita *et al.* 1984, Matsushita *et al.* 1984), whereas eucaryotic enzyme has two *a*-type hemes. Until the primary structure of the cytochrome *bo* complex was

elucidated (Chepuri *et al.* 1990a), the aa_3-type cytochrome *c* oxidase from *P. denitrificans* had been regarded as a model system for the study of cytochrome *c* oxidase. The enzyme from this organism has the following advantages: Purified samples have only two subunits (Ludwig and Schatz 1980) and molecular genetical approach is able to apply.

The primary structure of *E. coli* enzyme unveiled that subunit I of this enzyme has strong homology to subunit I of the aa_3-type cytochrome *c* oxidase of mitochondria and aerobic bacteria, in that 40 % of the amino acids are conserved over a 550 amino acid overlap (Fig. I-3). This finding implies that the *E. coli* enzyme is structually similar at least on the level of primary structure. Moreover, the following 'key' properties (Table I-1) were identified in the *E. coli* enzyme, which had been found in the eucaryotic enzymes: 1) Both low-spin and CO-reactive high-spin hemes were identified in EPR spectra (Hata *et al.* 1985). 2) The low-spin and high-spin hemes were identified in both of its reduced and air-oxidized states (Uno *et al.* 1985). 3) Proton pumping activity was measured in spheroplasts ($H^+/e^-=2$) (Puustinen *et al.* 1989). 4) Spin coupling interaction between the high-spin heme and Cu_B was detected by the potentiometric analysis of the EPR spectrum (Salerno *et al.* 1989). 5) Redox interaction between the low-spin and high-spin heme was observed in EPR spectrum (Salerno *et al.* 1990). 6) The CO-binding high-spin heme localized to subunit I (Nakamura *et al.* 1990). 7) The carbon monooxide molecules which are photodisociated from the high-spin heme immediately bound to Cu_B (by FTIR, see Chepuri *et*

al. 1990b). These reports suggested that eucaryotic and *E. coli* enzyme function in a very similar fashion and have a common molecular architecture of the reaction center.

Therefore, it is reasonable that "heme-copper oxidase" superfamily includes all the mitochondrial and many bacterial terminal oxidases whether or not they can oxidase cytochrome *c* (Gennis 1991). Recently, Puustine and Wikström (1991) showed another important property that supported the existence of "heme-copper oxidase" superfamily. They found a novel heme, heme *O*, in the cytochrome *bo* complex in place of protoheme IX. This has similar structure to the basal structure of *a*-type heme, in which a hydroxyethylfarnesyl side chain are bound at position 2, but no formyl group are bound at position 8. Furthermore, very recent work has showed that caa_3-type cytochrome *c* oxidase in PS3 can bind heme *O* at the O_2-binding active site, when the cells were cultured under slightly air-limited conditions (Sone and Fujiwara 1991). The same phenomenon has been observed in the case of ba_1-type ubiquinol osidase in *A. aceti* (Matsushita *et al.* 1991). Although the conclusion of Puustinen and Wikström that both chromophores of the cytochrome *bo* complex are heme *O* is still controvertial, it is established that several oxidases including the cytochrome *bo* complex contain heme *a*-like heme at the O_2-binding active site (Mogi and Anraku 1992). This findings further substantiates the structural similarities between the cytochrome *bo* complex and the $\alpha\alpha_3$-type mitochondrial oxidase, and thus, "heme-copper oxidase superfamily" is generally received (Fig. I-2, see Saraste *et al.* 1991a, Haltia and Wikström 1992).

Scope of this thesis

The research presented in this thesis focuses on the structure-function studies on the cytochrome *bo* complex using site-directed mutagenesis. From the comparison of the primary structures of subunit I of the cytochrome *bo* complex and its counterparts of the aa_3-type cytochrome oxidase, six His residues are found to be invariable and two His residues, His-54 and His-411, are found to be highly conserved. First, I have carried out site-directed mutagenesis of these eight conserved histidines in subunit I in the hope that these residues must be involved in ligating the low- and high-spin hemes and Cu_B. Second, to detect the residues which locate in the vicinity of the prosthetic groups, and which involve the enzymatic activity, such as electron-transfer, twelve conserved aromatic amino acid residues were altered. At last, a helix-bundle model of the redox reaction center of the cytochrome *bo* complex is presented.

Table

Table I-1 Similarities between the terminal oxidases of *E. coli* and mitochondria

	E. coli Cytochrome *bo* complex	Mitochondrial Cytochrome *c* oxidase
Substrate	Ubiquinol-8 / O_2	Cytochrome *c* / O_2
Number of subunits	5	7—13 (9:yeast, 13:mammals)
Heme group	1 Protoheme IX 1 Heme *O*	2 Heme *a*
Copper atom	1 (Cu_B)	2 (Cu_A and Cu_B)
High-spin heme	Yes	Yes
Low-spin heme	Yes	Yes
High-spin heme/Cu_B binuclear center	Yes	Yes
Proton pumping	Yes	Yes

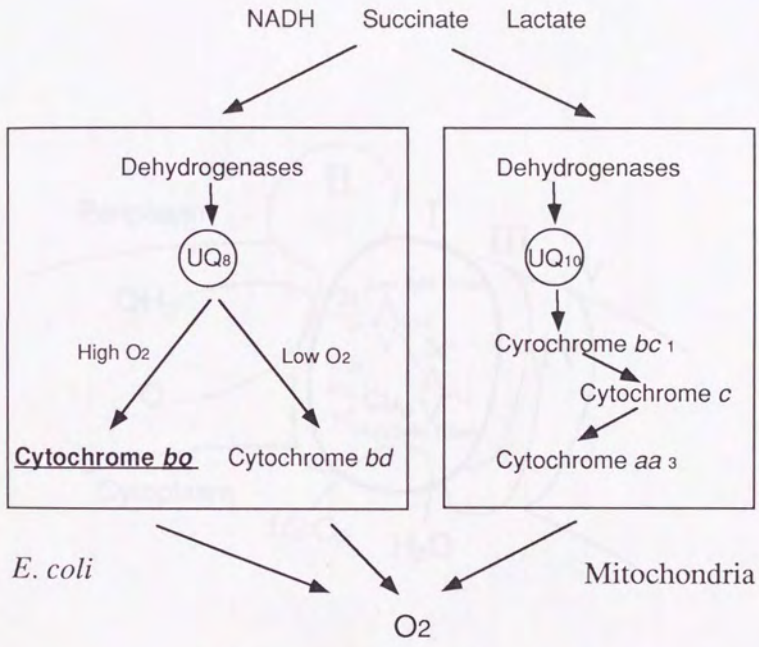

Figure I-1 Cytochrome *bo* complex is a terminal ubiquinol oxidase of aerobic respiratory chain in *E. coli*

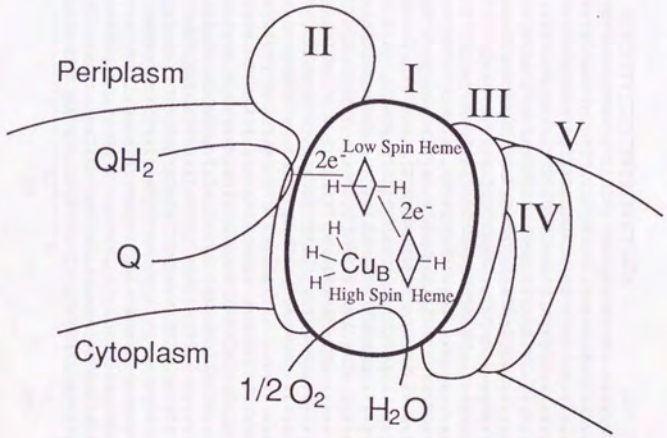

Figure I-2. Subunit I of the cytochrome *bo* complex acts as a redox reaction center.


1
20
40
60
80
100
A
F
A
EC MFGKLSLDAVPFHEPIVMVTIAGIILGGLALVGLITYFGKWTYLWKEWLTSVDHKRLGIMYIIVAIVMLLRGFADAIMMRSQQALASAGEAGFL**********PPHHYDQIFTAHGV
HH M**ATAASSITLTVLMGVLLVGVVAVLARLEDWRSYTPLSDVGGGLGERTGYTHEEKKKPGGIIRWFTTVDHKDIGILYGVYGTIAFAWGGVSVLLMRTELATSSETLISPSLYNGLLTSHGI
BS M*****************************LNALTEKRTRGSMLWDYLTSVDHKKIAILYLVAGGFFFLVGGIEAMFIIQLAKPENAFL*****************SAQAYNEVMTMHGT
PS ***********************************STIARKKGVGAVLWDYLTTVDHKKIAHLYLISGGFFFLLGGLEALFIRIQLAKPNNDFLV*****************GGLYNEVLTMHGT
BJ M******************ATSAAAHGDHAQDHGHDEHAHPTGWRRYVYSTNHKDIGTMYLIFAVIAGVIGAAMSIAIRAELMYPGVQIF*****************HETHTYNVFVTSHGL
PD M*****************************SAQISDSIEEKRGFFTRWFMSTNHKDIGVLYLFTAGLAGLISVTLTVYMRMELQHPGVQYM*CLEGMRLVADAAAECTPNAHLWNVVVTYHGI
SC M***VQRWLYSTNAKDIAVLYFMLAIFSGMAGTAMSLIIRLELAAPGSQYL*****************HGNSQLFNVLVVGHAV
SP M***NSWWTYVNRWMFSTNAKDIAMTYLLFGLVSGMIGSVFSFMIRMETSAPGSQF*****************TSGNGQLYNVAISAHGM
AN ***LIIDLNTNNVLGKKFSTSTKKENIKQIESSSFLTFKQPTEWQERWYLSSNAKDIGTLYLMFALFSGLLGTAFSVLIRLELSGPGVQYI*****************ADNQLYNSIITAHAI
NC LFIRYVTIIIKTIILFNQLNSEEFGLSLNSSKRSVGLMSSISIWTERWFLSTNAKDIGVLYLIFALFSGLLGTAFSVLIRMELSGPGVQYI*****************ADNQLYNAIITAHAI
CR M***RWLYSTSHKDIGLLYLVFAFFGGLLGTSLSMLIRYELALPGRGLL*****************DGNGQLYNVIITGHGI
WH M***TNMVRWLFSTNHKDIGTLYFIFGAIAGVMGTCFSVLIRMELARPGDQIL*****************GGNHQLYNVLITAHAF
ZM M***TNLVRWLFSTNHKDIGTLYFIFGAIAGVMGTCFSVLIRMELARPGDQIL*****************GGNHQLYNVLITAHAF
SO M***TNLVRWLFSTNHKDIGTLYFIFGAIAGVMGTCFSVLIRMELARPGDQIL*****************GGNHQLYNVLITAHAF
SB M***TNPVRWLFSTNHKDIGTLYFIFGAIAGVMGTCFSVLIRMELARPGDQIL*****************GGNHQLYNVLITGHAF
OB M***TNPVRWLFSTNHKDIGTLYFIFGAIAGVMGTCFSVLIRMELARPGDQIL*****************GGNHQLYNVLITAHAF
TB M***FFLCLVCLSVSHKMIGICYLLVAILCGFIGYIYSLFIRLELSLIGCGVL*****************FGDYQFYNVLITSHGL
LT M***FWLCLVCLSVSHKMIGLCYLLVAILSGFVGYVYSLFIRLELSLIGCGIL*****************FGDYQFYNVLITSHGL
TE *******DFIEQTKSFKVSVNNYFYYNKIKKLFTYLNDRLRKHILKKYVYTNNHKRIAINYLYFSMVTGLSGAALATMIRMELAHPESPFFK*****************GDSLRYLQVVTAHGL
PA **INHKRIALNYFYFSMWTGLSGAALATMIRLEMAYPGSPFFK*****************DSIKYLQVATAHGL
DM M***SRQWLFSTNHKDIGTLYFIFGAWAGMVGTSLSILIRAELGHPGALI*****************GDDQIYNVIVTAHAF
DY M***SRQWLFSTNHKDIGTLYFIFGAWAGMVGTSLSILIRAELGHPGALI*****************GDDQIYNVIVTAHAF
SU M***QLSRWLFSTNHKDIGTLYLIFGAWAGMVGTAMSVIIRAELAQPGSLL*****************NDDQIYNVVVTAHAL
XL M***AITRWLFSTNHKDIGTLYLVFGAWAGLVGTALSLLIRAELSQPGTLL*****************GDDQIYNVIVTAHAF
MS M***FINRWLFSTNHKDIGTLYLLFGAWAGMVGTALSILIRAELGQPGALL*****************GDDQIYNVIVTAHAF
RA M***FVNGWLFSTNPKDIGTLYLLFGAWAGMVGTALSILIRAELGQLGALL*****************GDDQIYNVLVTAHAF
BV M***FINRWLFSTNHKDIGTLYLLFGAWAGMVGTALSLLIRAELGQPGTLL*****************GDDQIYNVVVTAHAF
HU M***FADRWLFSTNHKDIGTLYLLFGAWAGVLGTALSLLIRAELGQPGNLL*****************GNDHIYNVIVTAHAF
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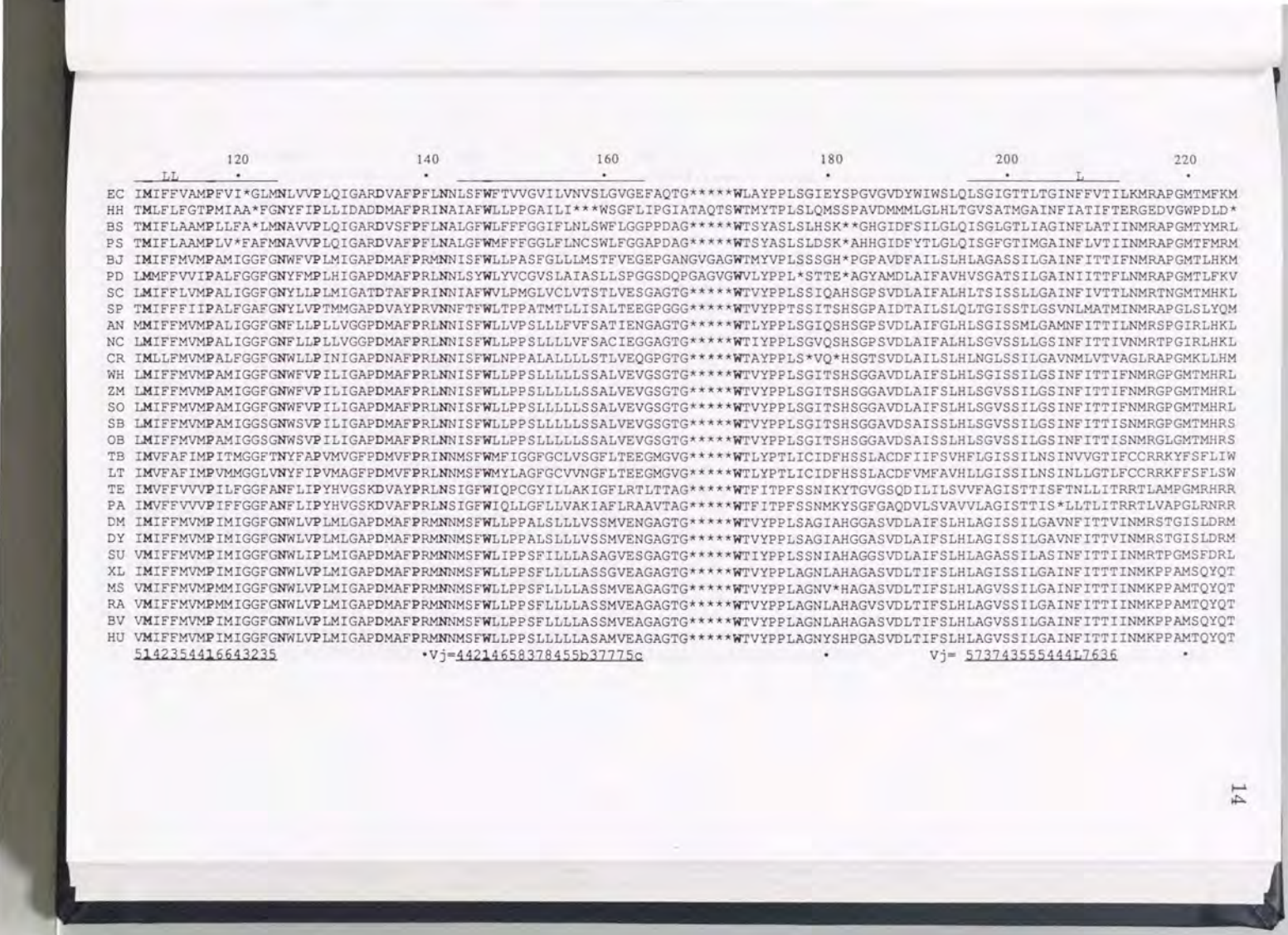
Vj= 65635125


120
140
160
180
200
220
LL
L
EC IMIFFVAMPFVI*GLMNLVVPLQIGARDVAFPFLNNLSFWFTVVGVILVNVSLGVGEFAQTG*****WLAYPPLSGIEYSPGVGVDYWIWSLQLSGIGTTLTGINFFVTILKMRAPGMTMFKM
HH TMLFLFGTPMIAA*FGNYFIPLLIDADDMAFPRINAIAFWLLPPGAILI***WSGFLIPGIATAQTSWTMYTPLSLQMSSPAVDMMMLGLHLTGVSATMGAINFIATIFTERGEDVGWPDLD*
BS TMIFLAAMPLLFA*LMNAVVPLQIGARDVSFPFLNALGFWLFFFGGIFLNLSWFLGGPPDAG*****WTSYASLSLHSK**GHGIDFSILGLQISGLGTLIAGINFLATIINMRAPGMTYMRL
PS TMIFLAAMPLV*FAFMNAVVPLQIGARDVAFPFLNALGFWMFFFGGLFLNCSWLFGGAPDAG*****WTSYASLSLDSK*AHHGIDFYTLGLQISGFGTIMGAINFLVTIINMRAPGMTFMRM
BJ IMIFFMVMPAMIGGFGNWFVPLMIGAPDMAFPRMNNISFWLLPASFGLLLMSTFVEGEPGANGVGAGWTMYVPLSSSGH*PGPAVDFAILSLHLAGASSILGAINFITTIFNMRAPGMTLHKM
PD LMMFFVVIPALFGGFGNYFMPLHIGAPDMAFPRLNNLSYWLYVCGVSLAIASLLSPGGSDQPGAGVGWVLYPPL*STTE*AGYAMDLAIFAVHVSGATSILGAINIITTFLNMRAPGMTLFKV
SC LMIFFLVMPALIGGFGNYLLPLMIGATDTAFPRINNIAFWVLPMGLVCLVTSTLVESGAGTG*****WTVYPPLSSIQAHSGPSVDLAIFALHLTSISSLLGAINFIVTTLNMRTNGMTMHKL
SP TMIFFFIIPALFGAFGNYLVPTMMGAPDVAYPRVNNFTFWLTPPATMTLLISALTEEGPGGG*****WTVYPPTSSITSHSGPAIDTAILSLQLTGISSTLGSVNLMATMINMRAPGLSLYQM
AN MMIFFMVMPALIGGFGNFLLPLLVGGPDMAFPRLNNISFWLLVPSLLLFVFSATIENGAGTG*****WTLYPPLSGIQSHSGPSVDLAIFGLHLSGISSMLGAMNFITTILNMRSPGIRLHKL
NC LMIFFMVMPALIGGFGNFLLPLLVGGPDMAFPRLNNISFWLLPPSLLLLVFSACIEGGAGTG*****WTIYPPLSGVQSHSGPSVDLAIFALHLSGVSSLLGSINFITTIVNMRTPGIRLHKL
CR IMLLFMVMPALFGGFGNWLLPINIGAPDNAFPRLNNISFWLNPPALALLLLSTLVEQGPGTG*****WTAYPPLS*VQ*HSGTSVDLAILSLHLNGLSSILGAVNMLVTVAGLRAPGMKLLHM
WH LMIFFMVMPAMIGGFGNWFVPILIGAPDMAFPRLNNISFWLLPPSLLLLSSALVEVGSGTG*****WTVYPPLSGITSHSGGAVDLAIFSLHLSGISSILGSINFITTIFNMRGPGMTMHRL
ZM LMIFFMVMPAMIGGFGNWFVPILIGAPDMAFPRLNNISFWLLPPSLLLLSSALVEVGSGTG*****WTVYPPLSGITSHSGGAVDLAIFSLHLSGVSSILGSINFITTIFNMRGPGMTMHRL
SO LMIFFMVMPAMIGGFGNWFVPILIGAPDMAFPRLNNISFWLLPPSLLLLSSALVEVGSGTG*****WTVYPPLSGITSHSGGAVDLAIFSLHLSGVSSILGSINFITTIFNMRGPGMTMHRL
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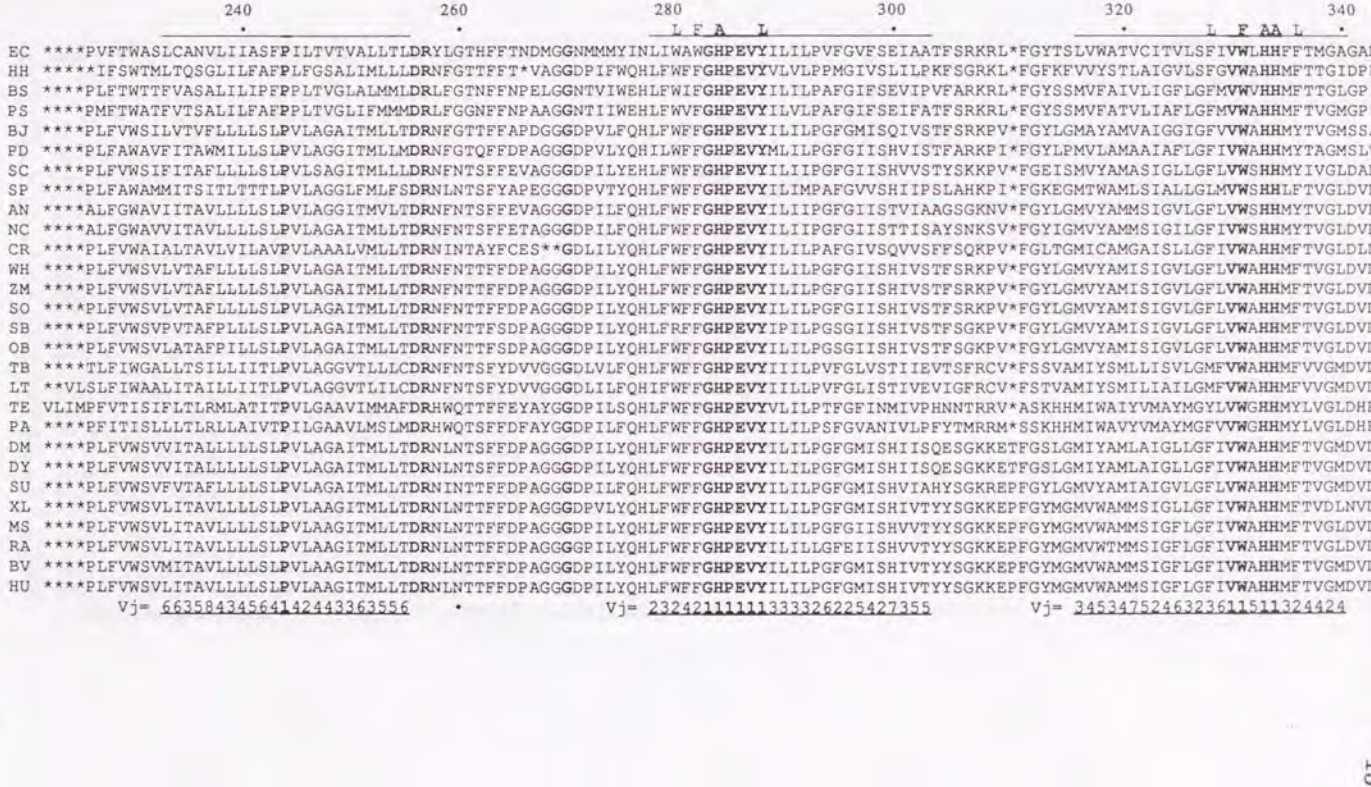
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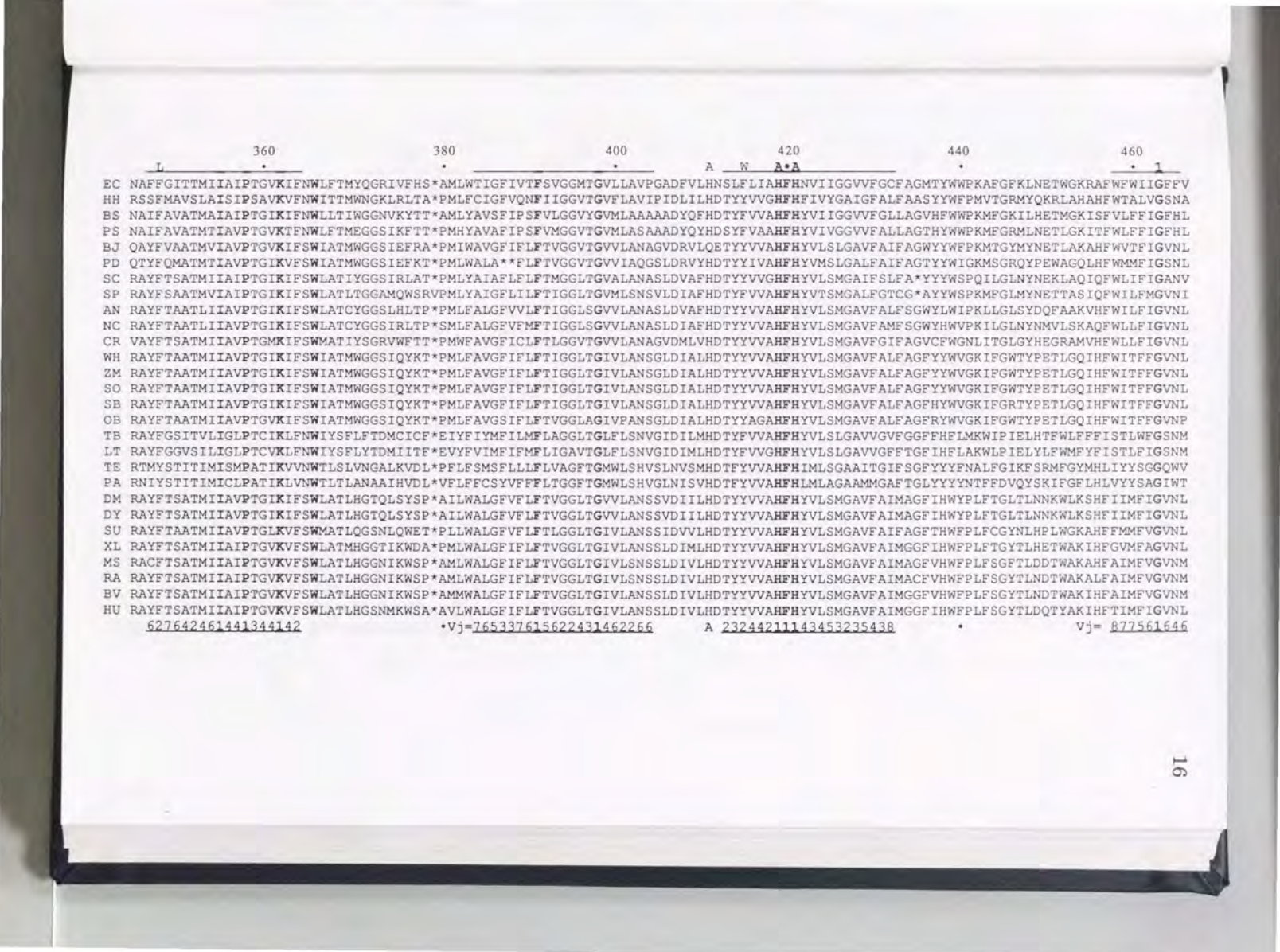
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Figure I-3. Sequence alignment of subunit I of the heme-copper oxidase superfamily. The amino acid sequences aligned are : *Escherichia coli (Chepuri,* et al. *1990a)*, Halobacterium halobium *(Denda,* et al. *1992)*, *Bacillus subtilis (Saraste,* et al. *1991b)*, thermophilic bacillus PS3 *(Ishizuka,* et al. *1990)*, *Bradyrhizobium japonicum (Bott,* et al. *1990)*, *Paracoccus denitrificans (Holm,* et al. *1987)*, *Saccharomyces cerevisiae (Bonitz,* et al. *1980)*, *Saccharomyces pombe*, *Aspergillus nidulans (Waring,* et al. *1984)*, *Neurospora crassa (Burger,* et al. *1982)*, *Chlamidomonas reinhardi (Boer,* et al. *1985)*, wheat, maize *(Isaac,* et al. *1985)*, *Sorghum bicolor milo*, Soy bean *(Grabau 1986)*, *Oenothera bercerius* (evening primrose) *(Hiesel,* et al. *1987)*, *Trypanosoma brucei (Hensgens,* et al. *1984)*, *Leishmania tarentolae*, Tetrahymena pyriformis, *Paramecium aurelia (Pritchard,* et al. *1986)*, *Drosophila melanogaster* (fruit fly) *(De Bruijn 1983)*, *Drosophila yakuba*, *Paracentrotus lividus* (sea urchin) *(Cantatore,* et al. *1989)*, *Xenopus laevis* (flog) *(Roe,* et al. *1985)*, mouse, rat, bovine *(Anderson,* et al. *1982)*, and human *(Anderson,* et al. *1981)*] sequences. The numbering refers to the *E. coli* sequence. Deletions are shown as *. The His residues and aromatic amino acid residues altered in this study are marked by 'A', and 'L', 'F',or'W'.

Chapter II. Experimental Procedures

Media

For preparation of cytoplasmic membranes, *E. coli* cells were grown in a rich medium [0.68 %(w/v) Bacto Yeast extract (DIFCO), 0.13 %(w/v) Bacto Casamino acids (DIFCO, technical), 1.3 mg/ml sodium citrate, 2.7 mg/ml $(NH_4)_2SO_4$, 12.0 mg/ml K_2HPO_4, 1 mM $MgSO_4$, 1 %(w/v) glycerol, 10 μg/ml $FeSO_4$, and 5 μg/ml $CuSO_4$]. For analysis of the copper content, $FeSO_4$ and $CuSO_4$ were omitted from the medium. For complementation tests during aerobic growth, DM minimal medium (Davis and Mingioli 1959) was used. Where indicated, 1% (w/v) glucose or glycerol was added as a carbon source. For large-scale preparation of plasmid DNA, Terrific Broth was used (Sambrook *et al.* 1989). When cells were cultured under anaerobic conditions, sodium nitrate was added at 40 mM as a terminal electron accepter. Ampicillin was added at 100 μg/ml (for multi-copy plasmids), or 15 μg/ml (for mini-F plasmids). The concentrations of other antibiotics were used as described (Sambrook *et al.* 1989)

Bacterial Strains

E. coli strain SCS1 (Hanahan 1983) was used for standard plasmid selection and propagation. Strain TG1 was obtained from Amersham Co. and used for production of single-strand DNAs of phagemid pCYOF2 and its derivatives, which were used as the templates for oligonucleotide-directed mutagenesis. The *cyo cyd* double deletion mutant ST2592 was constructed as follows: First,

the Δ*cyd*::Km^r locus was transduced into the wild-type strain W3110 (Bachmann 1987) by P1 phage grown on strain GO103 (Oden *et al.* 1990). Second, the Δ*cyo*::Cm^r locus of ST4674 (Nakamura *et al.* 1992) was transduced into the resultant strain, ST2590, to obtain strain ST2591. Acquisition of the double mutation was confirmed on a DM minimal plate containing 1 %(w/v) glucose or glycerol. Strain ST2591 did not grow aerobically on glycerol, but grew on glucose. Finally, the *recA* mutation was transduced from strain NK6659 (Hfr *srlA*::Tc^r *recA ilv thi thr relA spc*) (Murakami *et al.* 1984). Strain ST4700 (W3110 Δ*cyo*::Cm^r / pACT7-I^q) carrying the pCYOF2 derivatives was used for the expression of mutant genes that were under the control of the T7 promoter (Mogi *et al.* 1992).

Genetic Procedures and DNA Manipulations

Generalized transductions by P1$_{vir}$ phage were done as described (Miller 1972). Restriction enzyme digestion and agarose gel electrophoresis were carried out as described (Sambrook *et al.* 1989). DNA ligation was done using a Ligation kit (Takara Shuzo Co., Kyoto, Japan) according to the recommendations of the supplier. DNA fragments were purified on agarose gel with a GeneClean kit (Bio^{101}). Large and small scale preparations of plasmid DNAs were performed by the alkaline lysis method (Sambrook *et al.* 1989). Oligonucleotide primers for site-directed mutagenesis and for DNA sequencing were synthesized in a model 381A DNA synthesizer (Applied Biosystems Inc.). Oligonucleotides were purified by

denaturing polyacrylamide gel electrophoresis as described (Lo *et al.* 1984).

Introduction of additional restriction sites in the cyoB *gene*

(A) Constructions of pCYOF4 and pMFO4

Multi-copy phagemid pCYOF4, which contains the unique *Nhe*I site in the upstream region of the *cyoB* gene, has been constructed from a T7 expression vector carrying the f1 replication origin and the intact *cyo* gene, pCYOF2 (Mogi *et al.* 1992). It was constructed by site-directed mutagenesis using an In Vitro Mutagenesis system (Amersham Co.) and a primer P-NHE (Table II-1) corresponding to nucleotides 767-792 of the *cyoA* gene (Fig.II-1). The *Nhe*I site was introduced without any change in the amino acid sequence of subunit II and could be used for subcloning the whole *cyoB* gene from the phagemid into a mini-F plasmid. The nucleotide sequence between the *Sma*I and *Sal*I sites was confirmed not to have any unexpected change: Only the Lys-253—Leu-254 codons were changed (AAA—CTG to AAG—CTA). Then, the *Sma*I-*Sal*I fragment containing the *Nhe*I site was replaced by the counterpart from wild-type phagemid pCYOF2. The *Nsp*(7524)V-*Eco*RI fragment (2.6kb) of mini-F plasmid pMFO1 (Nakamura *et al.* 1992) that carries the F-prime-derived replication origin was then replaced by the corresponding region of pCYOF4. The resultant single copy plasmid, pMFO4, which contains the unique *Nhe*I site, was used as a wild-type control of the *cyo* operon in the study of histidine replacements.

(B) Constructions of pCYOF9 and pMFO9

To facilitate site-specific mutagenesis and sequencing at any desired segment of the *cyoB* gene, "cassette mutagenesis" was performed. To construct system for cassette mutagenesis, several unique restriction sites were introduced in the *cyoB* gene without any codon change (Table II-1). Phegemid pCYOF5, pCYOF6, pCYOF7, and pCYOF8 were made to carry additional *Apa*I, *Mlu*I, *Xho*I, and *Eco*81I sites by similar method used for the introduction of *Nhe* I site into pCYOF2 (Table II-1), following which one of two *Hin*dIII sites was excluded. The resultant phagemid pCYOF9 were confirmed not to carry any codon change (Fig. II-1). *Afl*II-*Spl*I fragment of pCYOF9 was then introduced in the corresponding region of pMFO4. The resultant single copy plasmid, pMFO9, contains six unique restriction sites, *Nhe*I, *Apa*I, *Mlu*I, *Xho*I, *Eco*81I, and *Hin*dIII, which was used as a wild-type control in the study of replacements of the conserved aromatic amino acids.

Site-directed Mutagenesis

(A) Replacements of Conserved His Residues

The cassette mutagenesis procudure performed in this study was as follows: Single strand DNA template for mutagenesis was prepared from phagemid pCYOF4. Site-directed mutagenesis was done by the method of Taylor *et al.* (Taylor *et al.* 1985). The reagents and enzymes for the reaction were obtained from Amersham Co. The DNA

sequences of the *Sal*I-*Pst*I fragment (for the I-H54A[2] and I-H106A mutants) and the *Afl*II-*Spl*I fragment (for all other mutants) of pCYOF4 from candidate clones were confirmed to contain the desired codon change by direct plasmid sequencing (Zhang *et al.* 1987) via the dideoxy method (Sanger *et al.* 1977). The mutagenized fragments were then introduced into their counterparts of the wild-type gene, and mutations in the resulting constructs were further confirmed by DNA sequencing.

(B) Replacements of conserved aromatic amino acid residues

Site-directed mutations at the conserved aromatic amino acid residues were introduced using the same procudure as used for the His-mutagenesis. Nucleotide sequences of the *Sal*I-*Pst*I fragment (for the I-Y61F, F112L, and F113L mutants), the *Afl*II-*Apa*I fragment (for the F208L mutant), the *Apa*I-*Xho*I fragment (for the W280L, W282F, and Y288L mutants), the *Xho*I-*Mlu*I fragment (for the F328L, W331L, and F336L mutants), or the *Mlu*I-*Eco*81I fragment (for the F348L and F415W

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[2]Designations of mutants are based on standard one-letter abbreviations for amino acids. Thus, "I-H54A" signifies the mutant in which the histidine at position 54 in subunit I has been replaced by alanine: in some figures this mutant is simply expressed as "H54A".

mutants) of the candidate clones was confirmed to contain the desired codon change by direct plasmid sequencing. Then, the mutant fragments were introduced into their counterparts of the wild-type gene, and mutations on the resulting constructs were further confirmed by DNA sequencing.

Preparation of Cytoplasmic Membranes and Purification of Mutant Enzymes

Mutant enzymes encoded by pCYOF4 derivatives were expressed in ST4700 in the T7 polymerase / T7 promoter system (Mogi *et al.* 1992, see Fig. II-2). Cytoplasmic membranes were prepared by the method of Yamato *et al.* (Yamato *et al.* 1975) with slight modifications. Spheroplasts were disrupted by two passages through a French press (1000 kg/cm^2). Total membrane vesicles were precipitated by centrifugation (140,000 x *g*, 1 h). Then, the membrane vesicles suspended in 3 mM sodium EDTA (pH 8.0) were subjected to isopycnic sucrose density gradient centrifugation. The dialysis step was omitted. Mutant enzymes were solubilized with sucrose monolaurate (Mitsubishi-Kasei Food Co., Tokyo), and separated from all other cytochromes present in the cytoplasmic membrane by HPLC on DEAE-5PW (Tosoh Co., Tokyo, see Mogi *et al.* 1992).

Complementation Test for Aerobic Growth

Since the phagemid vector pCYOF4 has many copies in a cell, it is required to exclude multi-copy

suppression effect to examine the ability of the mutant enzyme to complement for aerobic growth. The mutagenized regions on pCYOF4 were excised by *Nhe*I and *Sph*I enzymes, and introduced in the corresponding region of pMFO4. The mini-F plasmids carrying these mutant regions were transfected into the terminal oxidase-deficient strain ST2592 (W3110 Δ*cyo*::Cm^r Δ*cyo*::Km^r *recA*). Transformants were obtained on LB-ampicillin plates under anaerobic conditions in sealed jars (Gas-Pack Anaerobic System; BBL Microbiology Systems, Cockeysville, MD) and then grown aerobically on minimal-glycerol and minimal-glucose plates at 37°C for 2 days (Fig. II-3).

Optical Spectroscopy

Measurements of the dithionite-reduced *minus* air-oxidized difference spectra of cytochromes at 77K, and the CO plus reduced *minus* reduced difference spectra (CO-binding difference spectra) at room temperature were performed with a UV-3000 dual wavelength spectrophotometer (Shimadzu Co., Kyoto) as described previously (Kita *et al.* 1984). Digital outputs were recorded in a PC-286VS computer (Epson Co., Tokyo) using a program written by the author using a subroutine kindly provided by Dr. Matsuura (Tokyo Metropolitan University), and transferred to a Macintosh IIcx computer (Apple Computer Inc., Cupertino, CA). The digital data were processed and analyzed in the Macintosh computer using a software Igor (WaveMetrics, Lake Oswego, OR). The amount of cytochrome *o* was calculated from the CO-binding difference spectra at a wavelength

pair of 416-430 nm using a value for the molar extinction coefficient of 254,000 cm^{-1} (Mogi *et al.* 1992).

EPR Spectroscopy

The cytoplasmic membranes used for EPR analysis were prepared as for the optical spectroscopy. EPR samples were prepared just prior to use. First, membranes were suspended in 50 mM TES buffer (pH7.0). In order to oxidize samples, then, 5 μL of 5 mM TMPD and 1 μL of 100 mM ammonium persulfate (freshly prepared) were added to the suspension. This sample was vortexed briefly and transferred to liquid nitrogen after freezing in a mixture of 5:1 isopentane:methylcyclohexane in a liquid nitrogen bath.

The EPR spectrum was recorded using Bruker ESP300 ESR spectrometer equiped at University of Illinois at Urbana-Champaign, U.S.A. Spectra of oxidized samples were taken under the following conditions: Modulation frequency, 100kHz; modulation amplitude, 10.238G; time constant, 163.84 ms; sweep time, 335.544 s; center field, 2100 G; sweep width, 4000 G; frequency, 9.45 GHz; and power, 5.02 mW.

Other methods

Copper content was determined by atomic absorption analysis using a Perkin-Elmer 370 or Shimadzu AA-640 atomic absorption spectrophotometer. The output signals were calibrated by running standards of Cu (ranging from 0.02 to 0.4 ppm). Ubiquinol-1 oxidase activities were assayed as described (Kita *et al.* 1984). Protein concentration was determined by the BCA method

(Smith *et al.* 1985) with bovine serum albumin as a standard. SDS-Polyacrylamide gel electrophoresis was done essentially by the method of Laemmli (1970). Western immunoblotting was performed by the method of Towbin *et al.* (1979) with the following modifications. Filters were blocked with Tris-buffered saline (10 mM Tris-HCl, pH 8.0, and 150 mM NaCl) containing 0.05% Tween 20 and 1 % bovine serum albumin for 1 h at room temperature. Primary antibodies on the filter were detected by incubation with alkaline phosphatase-conjugated goat anti-rabbit IgG (Jackson Immunoresearch Laboratory), and staining with 330 μg/ml nitro blue tetrazolium and 165 μg/ml 5-bromo-4-chloro-3-indolyl phosphate in 20 mM Tris-HCl, pH 9.5, 100 mM NaCl, and 5 mM $MgCl_2$. Anti-subunit I and anti-subunit II sera were kindly provided by Dr. Kita (University of Tokyo).

Materials

Restriction endonucleases and other enzymes for DNA manipulation were purchased from Takara Shuzo or New England BioLabs. Modified T7 DNA polymerase and sequencing reagents were from United States Biochemical Co. Isopropyl thio-β-D-galactopyranoside was from Nova Chemicals. [α-^{32}P]dCTP (111 TBq/mmol) was from ICN Radiochemicals. Sep-Pak C18 cartridges were from Millipore Co. Triethylammonium bicarbonate was purchased from Wako Chemicals, Kyoto. Other chemicals were commercial products of analytical grade.

Tables

Table II-1 Oligonucleotides used for the introduction of unique restriction sites in the *cyoB* gene.

	Mutagenic Primer	Restriction Site
P-NHE	3'- 792 GCAAGCTTTTCGATCGGCGCGGATCG-5'	insert *Nhe*I
P-HND	3'- 1437 TGTTGAATTCAAAGACCAAAT-5'	delete *Hind*III
P-APA	3'- 1650 ACTTCTACGCCCGGGCCCGTACT-5'	insert *Apa*I
P-XHO	3'- 1921 TTGGAAGAGCTCCTTTGCAGACA-5'	insert *Xho*I
P-MLU	3'- 2040 TGCATTTGCGCAAGAAACCAT-5'	insert *Mlu*I
P-E81	3'- 2328 CCACCGGATTCCGCAAGCCAA-5'	insert *Eco*81I

Table II-2 Oligonucleotides used for site-directed mutagenesis of conserved His residues in subunit I

Mutant	Mutagenic oligonucleotide[a]	Codon change
I-H54A	3'-AGGCAGCTG**CG**ATTTGCGGAG-5'	CAT→GCT
I-H106A	3'-AAATGGCGC**CG**GCCGCACTAA-5'	CAC→GCC
I-H106Q	3'-AATGGCGCGT**C**CCGCACTAAT-5'	CAC→CAG
I-H106M	3'-GAAATGGCGC**TAC**CCGCACTAAT-5'	CAC→ATG
I-H284A	3'-CGGACCCCG**CGA**GGCCTTCAA-5'	CAC→GCT
I-H284Q	3'-GGACCCCGGT**C**GGCCTTCAAA-5'	CAC→CAG
I-H284M	3'-CCGGACCCCG**TAC**GGCCTTCAAA-5'	CAC→ATG
I-H333A	3'-GCAAACCGAC**CG**GGTGAAGAAA-5'	CAC→GCC
I-H334A	3'-AACCGACGTG**CG**GAAGAAATGC-5'	CAC→GCC
I-H411A	3'-AAGCAAGAC**CG**ATTGTCGGAC-5'	CAT→GCT
I-H419A	3'-GGACTAACGC**CG**GAAGGTATTG-5'	CAC→GCC
I-H421A	3'-ACGCGTGAAG**CG**ATTGCACTAG-5'	CAT→GCT
I-H421Q	3'-GCGTGAAGGT**C**TTGCACTAGT-5'	CAT→CAG
I-H421M	3'-ACGCGTGAAG**TAC**TTGCACTAGT-5'	CAT→ATG

[a]Sequences of mutagenic primers complementary to the *cyoB* sense strand used to replace His codons at given positions. In each case, the mutagenized codon is underlined, and the nucleotides changed are shown in boldface type.

Table II-3 Oligonucleotides used for site-directed mutagenesis of conserved aromatic amino acids in subunit I

Mutant	Mutagenic oligonucleotide[a]	Codon change
I-Y61F	3'-CCATAGTACA**A**ATAGTAGCAC-5'	TAT→TTT
I-F112L	3'-AATACTAGAA**C**AAGCACCGCT-5'	TTC→TTG
I-F113L	3'-TACTAGAAGAA**C**CATCGCTAC-5'	TTC→TTG
I-F208L	3'-CCATAGTTGAA**C**AAGCAATGG-5'	TTC→TTG
I-W280L	3'-TTGGACTAAA**A**CCGGACCCCG-5'	TGG→TTG
I-W282F	3'-AAACCCGGA**AA**CCGACTAACGCG-5'	TGG→TTT
I-Y288L	3'-CTTCAAA**AC**TAGGACTAGG-5'	TAC→TTG
I-F328L	3'-CACGACAGCAA**C**TAGCAAACC-5'	TTC→TTG
I-W331L	3'-AAGTAGCAAA**A**CGACGTGGTG-5'	TGG→TTG
I-F336L	3'-GTGGTGAAGAA**C**TGCTACCCA-5'	TTT→TTG
I-F348L	3'-TGCGCAAGAA**C**CCATAGTGGT-5'	TTT→TTG
I-F415W	3'-TTGTCGGACA**CC**GACTAACGCG-5'	TTC→TGG

[a]Sequences of mutagenic primers complementary to the *cyoB* sense strand used to replace aromatic amino acids at given positions. In each case, the mutagenized codon is underlined, and the nucleotides changed are shown in boldface type.

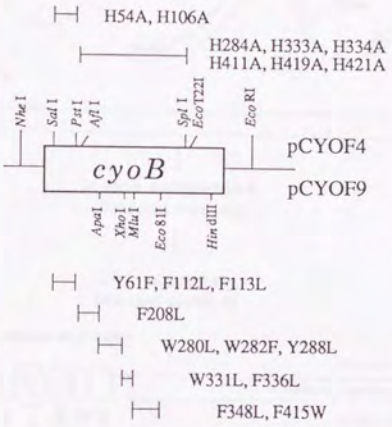

Figure II-1 Physical map of the *cyo* operon in vector pCYOF4 and pCYOF9. The restriction sites artificially introduced are marked by asterisks. The *Sac*I site was introduced by subcloning of the operon. The *Nhe*I, *Apa*I, *Xho*I, *Mlu*I, and *Eco*81I sites, designed for subcloning, were introduced via site-directed mutagenesis without any amino acid change. One of the *Hind*III sites locating between the *Pst*I and *Afl*II sites was deleted via site-directed mutagenesis (pCYOF9). The approximate coding regions of the *cyoABCDE* genes are shown by open rectangles.

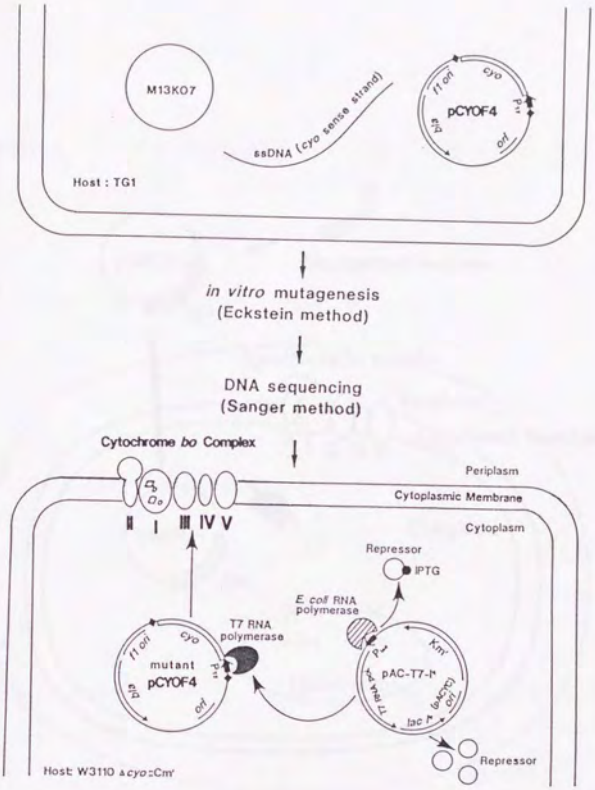

Figure II-2 Mutagenesis and expression of the mutants. Mutant enzymes encoded by pCYOF4 derivatives were expressed in ST4700 in the T7 polymerase / T7 promoter system.

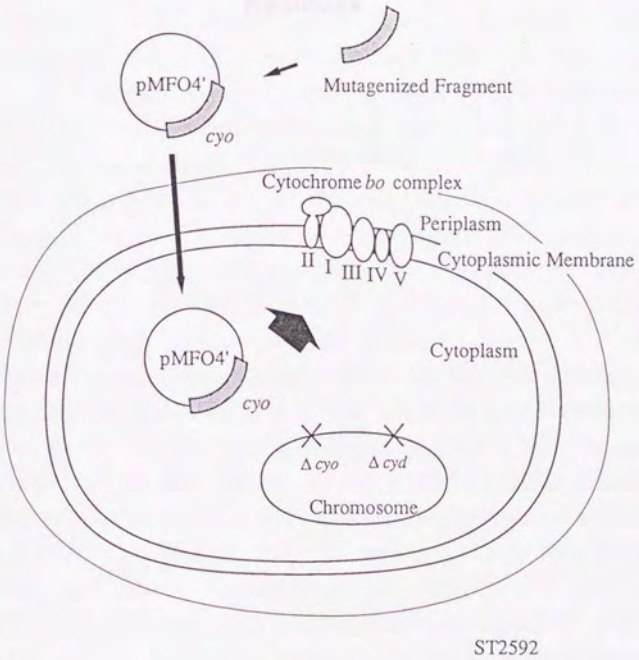

Figure II-3 Strategy for the complementation test for aerobic growth.

Chapter III. Replacements of the Conserved His Residues

Introduction

Biochemical Properties

The cytochrome *bo* complex in *E. coli* has been extensively studied biochemically, biophysically, and genetically (Anraku and Gennis 1987). Several purification protocols of the enzyme have been reported (Kita *et al.* 1984, Matsushita *et al.* 1984, Puustinen *et al.* 1991, Minghetti *et al.* 1992, Mogi *et al.* 1992). The preparation of Kita *et al.* (1984) is reported to have two subunits which contains two protoheme IXs and two equivalents of copper. Matsushita *et al.* (1984) described a four-subunit enzyme which contained two protoheme IXs. Puustine and Wikströme (1991) also purified a four-subunit oxidase, which has only one equivalent of copper, presumably CuB, and in that they identified a novel heme, heme *O*, in the place where formerly protoheme IXs are believed to be locate. Minghetti *et al.* (1992) and Mogi *et al.* (1992) each described a five-subunit preparation as well as those of four-subunit, both of which contain two heme groups and one equivalent of copper. Mogi *et al.* (1992) as well as Puustine and Wikstöme detected the heme *O* in the purified enzyme, but the former also detected protoheme IX at equal stoichiometry to heme *O*. Hence, despite the the existence of heme *O* is established, the heme composition of this enzyme is still controversial.

Genetical Aspects

Mutant deficient in the cytochrome *bo* complex has been isolated (Au *et al.* 1985), which have lesions in a locus that mapped at 10.2 minutes on the *E. coli* genetic linkage map (Bachmann 1987). The genes encoding the enzyme were cloned (Chepuri *et al.* 1990a, Nakamura *et al.* 1990), and sequenced (Chepuri *et al.* 1990a), and consequently five open reading frames, *cyoABCDE*, have been identified. The products of the *cyoA* and *cyoB* genes were assigned immunologically to be subunit II and I, respectively (Nakamura *et al.* 1990). Minghetti *et al.* (1992) confirmed the identities of subunits I, II and III as the *cyoB*, *cyoA*, and *cyoC* gene products, respectively, by partial amino acid sequence data.

The deduced amino acid sequences of the *cyo* genes indicatesthat there is a significant relationship between the subunit I, II, and III of *E. coli* and three of the subunits present in aa_3-type cytochrome *c* oxidases of mitochondria and aerobic bacteria, in that 40 % of the amino acids are identical over a 550 amino acid overlap (Chepuri *et al.* 1990a). Furthermore, there is clearly a relationship between the *cyoD* and *cyoE* gene products and several putative subunits of bacterial cytochrome *c* oxidases including *P. denitrificans* (Raitio *et al.* 1987) and *B. subtitilis* (Saraste *et al.* 1991b). This relationship could translate into the conservation of prosthetic group ligands, the existence of conserved active sites, and a common mechanism for electron-transfer, oxygen reduction, and proton pumping and electron transfer.

Localization of binding sites of prosthetic groups

In a complicated multi-subunit protein such as cytochrome *c* oxidase, the localization of the binding sites for the prosthetic groups would appear hopeless. However, the problem is simplified by two facts. First, the isolation of the enzyme from bacterial sources localized the redox centers in the two largest subunits, subunit I and subunit II. It has recently been experimentally determined that subunit I of the cytochrome *c* oxidases from *P. denitrificans* contains two α-type hemes and Cu_B (Müller *et al.* 1988a and 1988b). If the ligands of these prosthetic groups are conserved between *E. coli* and the *aa*3-type oxidases, we expect that subunit I of the cytochrome *bo* complex will contain all three of its prosthetic groups—two *b*-type hemes and Cu_B. Subunit II of the *aa*3-type oxidases has been proposed to contain the binding site for the Cu_A group associated with these oxidases (Stevens *et al.* 1982). However, since Cu_A is absent in the cytochrome *bo* complex, we anticipate that subunit II does not ligate any of the prosthetic groups found in the cytochrome *bo* complex.

Second, the identification of the highly conserved residues in subunit I and II may offer a simple way to try to identify heme and copper binding sites. Because of the extensive similarity between the cytochrome *bo* complex and the cytochrome *c* oxidase, we anticipate that these enzymes share a sequence-based catalytic core. Since conserved Cys residues are not found in subunit I but are present in subunit II, the latter subunit appears as an obvious candidate for binding Cu_A. On the other hand, only two strictly invariable histidines are present on subunit II,

which are sufficient for the coordination of Cu_A only; the remaining copper and the hemes must therefore be placed in subunit I where six conserved histidines are present.

Ligands of Prosthetic groups

Cytochrome *c* oxidases have been extensively studied from both plant and animal mitochondria, as well as from many bacterial sources. The oxidases most intensively studied to date are the beef heart oxidase and the bacterial aa_3-type oxidase from *P. denitrificans*. The prosthetic group contained in these oxidases are two heme *a* groups and at least two copper atoms referred to as Cu_A and Cu_B, which are considered to be responsible for its catalytic and spectroscopic properties. The different nomenclature is due to the different chemical environments where they are located and to the different properties which, consequently, they exhibit. The heme a_3-Cu_B couple, the binuclear center, is the site of reduction of oxygen. Spectroscopic data indicate an antiferromagnetic interaction between the heme a_3-Cu_B couple in the oxidized enzyme, which determines the unusual EPR-silent state of Cu_B; for these reasons they are normally considered as a pair rather than independent units. In addition, redox interaction between the low-spin heme and high-spin heme has been detected, possibly through conformational changes, a change in the redox state of one center can perturb the electronic structure of the others as reflected, for example, by modifications in midpoint potentials and subtle alterations in spectral properties.

Spectroscopic studies, such as MCD, EXAFS, and ENDOR studies of the aa_3-type cytochrome *c* oxidase indicate that heme *a* is bound through two His residues (Babcock *et al.* 1981, Eglinton *et al.* 1984), heme a_3 by one His residue (Blokzijl-Homan and Gelder 1971, Stevens and Chan 1981), and Cu_B by three His residues (Cline *et al.* 1983, Li *et al.* 1987, Scott *et al.* 1988). The fourth ligand of Cu_B is thought to form a bridge to heme a_3. The identity of the fourth ligand is still controversial (Capaldi 1990). Since the *E.coli* cytochrome *bo* complex belongs to the heme-copper oxidase superfamily together with the aa_3-type cytochrome *c* oxidase (Gennis 1991), at least three His residues probably function as heme ligands and the other three His residues are involved in binding of Cu_B, which is electronically coupled to the high-spin heme. To test these possibilities, I carried out oligonucleotide-directed site-specific mutageneses of the eight conserved histidines and examined spectroscopic properties and copper contents of the mutant oxidases. Resonance Raman spectroscopy (Uno *et al.* 1985) and EPR studies (Hata *et al.* 1985, Salerno *et al.* 1990) have suggested that the high-spin heme has at least one axial ligand (proximal ligand) and that the low-spin heme has two axial ligands. The crystal field parameters of the low spin heme from EPR spectra showed *bis*-histidine ligation of the low spin heme (Salerno *et al.* 1990, Haltia and Wikström 1992). The identity of the ligand of the high-spin heme remains uncertain, although a His residue has been found in most other heme proteins as the proximal ligand.

The aim of this study

I have carried out site-directed mutagenesis of eight conserved histidines in subunit I in the hope that these residues must be involved in ligating the low- and high-spin hemes and Cu_B. I found that His-106 and -421 function as the axial ligands of the low-spin heme and His-284 is a possible ligand of the high-spin heme. His-333, -334, and -419 residues are attributed to the ligands of Cu_B.

Results

Mutagenesis of conserved His residues in subunit I

Of twenty His residues in subunit I of the cytochrome *bo* complex, six His residues, His-106, -284, -333, -334, -419, and -421, were found to be invariable in aa_3-type cytochrome *c* oxidases of mitochondria and aerobic bacteria (Table III-1) and to be located in the transmembrane regions II, VI, VII, and X (Fig. III-1). Two other histidines, His-54 and -411 are conserved except in fungal oxidases (Bonitz *et al.* 1980, Burger *et al.* 1982, Waring *et al.* 1984) and Bradyrhizobium japonicum oxidase (Bott *et al.* 1990), respectively, and located in the putative hydrophilic loops 0-I and IX-X, respectively. Of possible heme-ligand residues other than histidine (Met, Tyr, Lys, and Cys), only Lys-55, Tyr-61, Met-110, Tyr-288, and Lys-362 are conserved (Fig. III-1).

The possible roles of these histidines as ligands of the low-spin and high-spin hemes and copper atom were tested by amino acid substitutions. Using oligonucleotide-directed site-specific mutagenesis, I have introduced single codon changes for replacement of these eight histidines by an Ala residue. His-106, -284, and -421 residues were also changed to glutamine and methionine. Residues with a small neutral side-chain such as alanine can be buried in a bundle of membrane-spanning helices. The Gln residue is similar in side-chain volume and hydrophobicity to the His residue, and the Met residue may act as an alternative

ligand for the. For instance, The Met residue acts as an axial ligand of soluble cytochrome b_{562} in *E. coli* (Lederer *et al.* 1981) and mitochondrial cytochrome *c* (Bushnell *et al.* 1990).

The nucleotide sequences of restriction fragments containing His codon changes were confirmed by direct plasmid sequencing, and the fragments were then introduced in place of their wild-type counterparts in the *cyo* operon. Directional cloning of the fragments was accomplished by choosing the unique *Sal*I-*Pst*I fragment (0.2 kb) for His-54 and His-106 mutagenesis and the *Afl*II-*Spl*I fragment (1.2 kb) for all other His-to-Ala mutations. Thus, I could eliminate the possibility that the phenotypes of the mutant enzymes were obscured by an unexpected mutation somewhere else introduced during *in vitro* DNA manipulations.

In vivo *activities of mutant enzymes*

First, the catalytic activities of the mutant enzymes were tested on their abilities to support aerobic growth of a strain that was unable to grow aerobically. Strain ST2592 was used for this complementation analysis. It has deletion mutations in both the *cyo* and *cyd* loci. In addition, it has the *recA* mutation and is therefore unable to rescue its chromosomal *cyo* mutation by recombination with the *cyo* gene introduced on a plasmid. To transform this strain, I emplyed a mini-F plasmid. Since it contains *Eco*RI-generated f5 replicator fragment of the conjugative plasmid F (Timmis *et al.* 1975), the multi-copy suppression effect could be avoided. In this study, first, a novel unique

restriction site, *Nhe*I, was introduced into a laboratory stock mini-F plasmid, pMFO1 (Nakamura *et al.* 1990) which carries entire *cyo* operon into plasmid pMF3 (Manis and Kline 1977). Then, the restriction fragments of each mutant gene cut with *Nhe*I and *Sph*I enzymes were inserted into this plasmid. Catalytic activity of the mutant enzyme should correlate with rate of aerobic growth on a nonfermentable carbon source of the double mutant cells carrying the mutated gene on mini-F plasmid.

The anaerobic transformants of ST2592 were streaked on minimal-glycerol and minimal-glucose plates, and examined aerobic growth for two days. The results are shown in Fig III-2: Only two transformants carrying an I-H54A or I-H411A mutant gene could grow aerobically on minimal-glycerol plates like the wild-type depending on oxidative phosphorylation. This result indicates that both His-54 and His-411 in subunit I are functionally dispensable. The mini-F plasmids carrying all the other mutation failed to complement the defects in aerobic growth of the double mutant on minimal-glycerol plates, indicating that substitution of the totally conserved histidines caused complete loss of enzymatic activity. The fact that all the transformants grew aerobically on minimal-glucose plates eliminated a possibility that these mutations induced large structural perturbation of the enzyme complex that might alter membrane permeability. Hence, invariable histidines are essential for the structure-function of the terminal oxidase, such as, its ability to bind prosthetic groups.

Quinol oxidase activity of partially purified preparations

Catalytic activities of the mutant oxidases were further examined by the experiments *in vitro*. The mutant enzymes were solubilized from cytoplasmic membranes with a nonionic detergent and separated from other cytochromes, such as an alternative quinol oxidase in the aerobic respiratory chain (*i.e.*, the cytochrome *bd* complex) and the cytochrome b_{556} (*i.e.*, the succinate dehydrogenase), by ion-exchange chromatography using HPLC. As shown in Table III-2, the mutant oxidases carrying H106A, H284A, H333A, H334A, H419A, and H421A substitutions did not show ubiquinol-1 oxidase activity. Since this result is in good agreement to the finding from the *in vivo* complementation test, the invariable histidines in subunit I are likely to be involved in catalytic functions of the enzyme.

Immunological analysis of the mutant enzymes

The expression levels of the mutant oxidases encoded by the multi-copy phagemids were examined by Western immunoblotting analysis of the cytoplasmic membranes (Fig. III-3). The amounts of polypeptides cross-reacting with rabbit polyclonal antisubunit I and antisubunit II antisera were not changed significantly by His-to-Ala mutations in subunit I. These results indicate that none of the mutations altered stability or assembly of the mutant enzymes.

Spectroscopic analysis of the mutant enzymes in cytoplasmic membranes

The *E. coli* cytochrome *bo* complex shows split α–absorption band at 555 nm and 563.5 nm in its low temperature redox spectrum (Kita *et al.* 1984, Mogi *et al.* 1992), both of which have been ascribed to contribution from the low-spin heme (Puustinen *et al.* 1991). In addition, the presence of this heme group can be confirmed by the detection of a low-spin component in the EPR spectrum with a g-value of around 3.0 (Hata *et al.* 1985, Salerno *et al.* 1989). The high-spin heme can be detected in the Soret region by a peak at 416 nm and a trough at 430 nm in a CO-binding (CO-reduced *minus* reduced) spectra (Kita *et al.* 1984).

Substitutions of His-54, -333, -334, and -411 did not have any significant effects on the spectra of either the low-spin or high-spin heme (Fig.III-4A). Thus, these histidines are unlikely to be heme ligands. Of the His-to-Ala mutations, only those of His-106 and His-421 completely eliminated the trough at 563.5 nm in the second-order finite spectrum at 77K (Fig. III-5A), suggesting that these two invariable His residues are the axial ligands of the low-spin heme. This result is consistent with the results of the EPR spectra (Fig. III-6) of the low-spin component (g=3.0) of this complex. On the other hand, the amount of CO-binding high-spin heme were greatly reduced by substitution of His-106, -284, and His-419 (Figs. III-5B and III-7B). Since His-106 can be unambiguously assigned as one of the low-spin heme ligands, His-284 and -419 are candidates for a ligand of the high-spin heme.

Spectroscopic properties of partially purified preparations

The spectroscopic properties of some mutant oxidases were further examined in partially purified preparations, in which their properties were not obscured by contributions from other cytochromes (Fig. III-8). In contrast to the wild-type and H284A mutant oxidases, the H106A and H421A mutant oxidases did not show the 563.5 nm peak completely. However, the absorption around 555 nm in these low spin heme deficient oxidases was comparable to that of the wild-type oxidases, although the contribution of the high spin heme to the α absorption band in the wild-type oxidase was estimated to be less than 10 % (Puustinen *et al.* 1991). This result could be due to a loss of interactions of the high-spin heme with the low-spin heme and/or changes in the environment of the high-spin heme. Slight blue shifts (1-2 nm) of the 555 nm peak were observed in the H421A and H284A mutant oxidases, and the H421A oxidases showed a shoulder at about 550 nm. These indicate that loss of one of the hemes alters the spectral properties of the other by changing the electron distribution in the latter or the equilibrium of the conformational substates.

Copper contents in mutant oxidases

The effects of His-to-Ala mutations in subunit I on its copper content were examined (Table III-3). Cytoplasmic membranes were prepared from mutant cells grown in the absence of added copper, and used for analysis of copper contents by atomic absorption spectroscopy. The copper contents in the wild-type, His-54, -284, and -411 mutant

membranes were found to be stoichiometrically related with the amount of the cytochrome *bo* complex. For example, the wild-type membranes contained 0.75 nmol Cu atom / mg protein (Table III-3) and 0.89 nmol of cytochrome *o* / mg protein (Fig. III-4). On the other hand, the His-106, -333, -334, and -419 mutant membranes contained only negligible amounts of copper like membranes from cells harboring plasmids without the insert of the *cyo* operon. In the His-421 mutant membranes, the copper content was one fifth of that of wild-type membranes. These defects could be restored by supplementation of the medium with excess $CuSO_4$ (Table III-3). Strains carrying these mutant genes, however, were neighther unable to grow aerobically on minimal-glycerol plates supplemented with $CuSO_4$, indicating that supplementation of excess copper is not enough for functional restoration. I assume that copper ions are able to associate with mutant oxidases in which distortion of a Cu_B binding site results in decrease in affinity for copper ions.

Taken together these data and those from spectroscopic observations for heme-binding (Figs. III-4, to -7), it is probable that His-333, 334 and 419 are ligands of Cu_B and His-284 is a ligand of the high-spin heme.

Construction of a model for membrane-spanning regions of subunit I

Chepuri and Gennis (1990) proposed fifteen transmembrane helices (0 to XIV) from experimental results using the *cyoB'-lacZ* and *cyoB'-phoA* fusion genes.

Accordingly, I determined the boundarys for membrane-spanning regions so as to be in agreement with the reporter activity of the fusion gene product.

The procedure was as follows: First, hydropathic character of primary sequence of subunit I of the cytochrome *bo* complex was examined by the KKD method (Klein *et al.* 1985), which was developed specifically for membrane proteins based on Kyte-Doolittle prameters (Kyto and Doolittle 1982) and thus evaluated to be with least ambiguity and the highest accuracy in determining integral sequences (Fasman and Gilbert 1990). Original output is shown in Table III-5. Fourteen membrane-spanning regions (0 to XIV except IV) were predicted using the default parameters. Moreover, the region IV may be integral sequences due to its hydrophobic character despite the odds is relatively low. Since the topological study (Chepuri and Gennis 1990) indicated there are fifteen membrane-spanning regions in subunit I, I concluded the regions 0 to XIV (Table III-5) are integral sequences in subunit I. Second, several boundarys were modified. Because membrane hemoproteins are not the typical integral proteins—they often contain ligands for heme, the original output should be improved (Esposti 1989). The boundaries at cytoplasmic side of regions VI and VII were accordingly extended, which was based on information of the location of ligands for both hemes, and examination of the propensities of amino acid residues for the location of helix ends (Richardson and Richardson 1988). The result is shown in Table III-5, which was used to depict a model of membrane-spanning regions of subunit I (Fig. III-1).

Discussion

The *E. coli* cytochrome *bo* complex, which is encoded by the *cyoABCDE* operon (Chepuri *et al.* 1990a), consists of five subunits and has two α–absorption peaks at 555 nm and 563.5 nm in the low temperature redox spectrum (Kita *et al.* 1984, Mogi *et al* 1992). Resonance Raman spectroscopy (Uno *et al.* 1985) and EPR (Hata *et al.* 1985) studies have suggested that the high spin heme has at least one axial ligand (proximal ligand) and the low spin heme have two axial ligands. Salerno *et al.* (1990) determined the crystal field parameters of the low spin heme from EPR spectra and showed a *bis*-histidine ligation of the low spin heme. The identity of the ligand of the high spin heme remains uncertain, although His residue is most often found in other heme proteins as the proximal ligand.

Subunit I has been identified as the *cyoB* gene product and found to be the binding sites for the low- and high-spin hemes (Nakamura *et al.* 1990, Mogi *et al.* 1992). The presence of both hemes was detected in membranes containing overexpressed *cyoBCDE*. This would initially suggest that the low-spin heme of the complex may be ligated by residues that are present in one of the subunits encoded by *cyoBCDE*. Kita *et al.* (1984), however, have purified a two subunit complex of tye chtochrome *bo* complex that contains both hemes and copper. This preparation contains only subunits I and II and has been found to be active. This suggests that *cyoCDE* are not involved in ligating either of the hemes. Since both hemes

are present in the *cyoBCDE* containing membranes, it would appear that *cyoB* contains both hemes of the cytochrome *bo* complex. The Cu_B component of this enzyme has not been experimentally localized to subunit I. However, since this prosthetic group is part of the binuclear center, it is likely that it is also localized to subunit I. These low spin and high spin hemes correspond to cytochrome $b_{563.5}$ and *o* components, respectively and equivalent to cytochrome *a* and a_3 of the cytochrome *c* oxidases, respectively. It is suggested that the low-spin heme is located close to quinol oxidation site whereas the high spin heme/Cu_B binuclear center functions as a site for the reduction of molecular oxygen to water (Salerno *et al.* 1990).

Of twenty His residues in subunit I of the cytochrome *bo* complex, six His residues, His-106, -284, -333, -334, -419, and -421, were found to be invariable in the aa_3-type cytochrome *c* oxidases of mitochondria and aerobic bacteria (Table III-1) and are located in the transmembrane regions II, VI, VII, and X (Fig. III-1). Two other His residues, His-54 and -411 are highly conserved and located in the putative hydrophilic loop 0-I and IX-X, respectively. Spectroscopic studies, such as MCD, EXAFS, and ENDOR, of the cytochrome *c* oxidases indicate that heme *a* is ligated by two His residues (Babcock *et al.* 1981, Eglinton *et al.* 1984), heme a_3 by one His residue (Blokzijl-Homan and Gelder 1971, Stevens and Chan 1981), and Cu_B by three His residues (Cline *et al.* 1983, Li *et al.* 1987, Scott *et al.* 1988). The fourth ligand of Cu_B is thought to form a bridge to heme a_3. The identity of the fourth ligand is still controversial (Capaldi 1990). Since the *E.coli*

cytochrome *bo* complex belongs to the heme-copper oxidase superfamily (Gennis 1991), at least three His residues function as the heme ligands and the other three His residues are involved in binding of Cu_B, which is electronically coupled to the high spin heme.

In order to test the idea, I have carried out the oligonucleotide-directed site-specific mutagenesis of these His residues and examined the spectroscopic properties and the copper content in the mutant oxidases. Since manipulations of a large DNA segment (i.e., 5 kb of the *cyo* operon) may result in unexpected mutations elsewhere in the structure genes. I accordingly designed mutagenesis experiments so that phenotypes of the mutant oxidases would not be obscured by any unexpected mutations, and sequenced the mutagenized fragments thoroughly to confirm the desired codon change (see Fig. II-2). Then I introduced the fragments into the wild-type *cyo* operon for expression of the mutant genes. Moreover, expression of the *cyo* operon was tightly controlled by the T7 promoter in vector pCYOF4 during DNA manipulations, and the expression levels of the mutant oxidases were mimicked at the level of the chromosomal copy by using a single copy vector that I have developed for expression of membrane proteins.

Western immunoblotting analysis of the mutant oxidases (Fig. III-3) suggested that Ala substitutions of conserved histidines do not affect the assembly of the enzyme complex into the cytoplasmic membrane or the stability against proteolytic degradation. An *in vivo* complementation test (Fig. III-2) and *in vitro* quinol

oxidase assay (Table III-2) both demonstrated that the six invariable histidines are essential for the catalytic functions of the cytochrome *bo* complex. In contrast, the possibility that His-54 and His-411 are ligands of the prosthetic groups of this complex is excluded. This is quite possible since based on the current topological model of subunit I (Fig. III-1), the location of the H54A and H411A mutations would be in the cytoplasmic and periplasmic space, respectively.

The defects of enzymatic acitivity by the mutations at conserved His residues were further examined by optical spectroscopy and copper analysis. Of the mutant oxidases, only the H106A and H421A oxidases showed loss of the 563.5 nm peak in the low temperature redox spectra of cytoplasmic membranes (Fig. III-5) and of partially purified preparations (Fig. III-8). These results indicate that His-106 and His-421 are the axial ligands of the low-spin heme. EPR characterization of the H106A and H421A oxidases in cytoplasmic membranes confirmed loss of the low-spin signal. By copper analysis of mutant membranes, I found that substitutions of His-106, -333, -334, and -419 resulted in complete absence of copper in the mutant enzymes. Since His-106 and -421 have been shown unambiguously to be the ligands of the low-spin heme, His-333, -334, and -419 are suggested to be ligands of Cu_B.

Substitutions of His-419 resulted in a large extent of reduction of CO-binding activity, and those of His-333 and -334 resulted in the slight reduction. These results are consistent with those obtained by Salerno *et al.* (1990). They reported that the copper associated with the high-

spin heme exert a strong effect on the strength of CO-binding to that heme. Supplementation of the growth medium with $CuSO_4$ restored the defect in binding of copper atoms, suggesting that these His substitutions altered the affinity of the Cu_B binding site for copper atom due to loss of one ligand and/or perturbation of a tertiary structure of the redox center. From the assignments of His-333, -334, and -419 as ligands of Cu_B, I conclude that the His-284 residue is a proximal ligand of the high-spin heme. Defects of CO-binding in the H106A mutant enzyme could be due to perturbation of the high-spin heme / Cu_B binuclear center. In the His-284 mutant enzyme, a binding pocket for the high-spin heme is still present in the redox center and a ligand of the high-spin heme may be provided from the nearby Cu_B binding site (i.e., His-333 and Tyr-288 could be an alternative ligand (see Fig. III-8). Alternatively, portions of the hemes in the low-spin heme binding site may be able to bind CO in this mutant. These results confirmed possible interactions of His-284, -333, -334, and -419 in the binuclear center. I also obtained similar results on substitutions of His-106, -284, and -421 by Gln and Met residues. Lemieux *et al.* (1992) replaced seven conserved His residues in subunit I by other residues such as Leu and Gly. All of these mutants had similar phenotypes to those of Ala mutations in this study, indicating that constraints at positions 106, 284, 333, 334, 419, and 421 in functional enzymes are limited to His residues.

Thus, I conclude from this study that: 1) His-106 and -421 are the axial ligands of the low-spin heme, 2) His-284 probably functions as the proximal ligand of the high-

spin heme, and 3) His-333, -334, and -419 are possible ligands of Cu_B. I also suggest that these six invariable histidines also serve as ligands in the cytochrome *c* oxidases. Furthermore, identifications of the ligands for the prosthetic groups in subunit I lead me to propose a helical wheel projection model (Schiffer and Edmundson 1967) of the redox reaction center in the cytochrome *bo* complex (Fig. III-9). The reaction center is provided by at least four putative transmembrane helices II, VI, VII, and X in subunit I, which all carry invariable histidines. The low-spin heme binding site is provided by helices II and X, and the binuclear center is formed by helices VI, VII, and X. A four-α-helix bundle is one of the common packing motifs found in protein structures with a wide range of functions (Weber and Salemme 1980). In the bacterial photosynthetic reaction center, two helices of two distinct subunits form this arrangement (Yeates *et al.* 1987). However, the redox center of the cytochrome *bo* complex is not an anti-parallel helix bundle which is an energetically favorable arrangement (Gilson and Honig 1989). Thus, Another putative transmembrane helix may be present in between these helices. Such a helix with a helix dipole whose direction is opposite to those of helices II, VI, and X could increase the stability of the helical bundle through electrostatic interactions. Since His-106 and -421 are so crucial in binding of the low-spin heme and Cu_B, they may also serve as key residues for driving a bundle of putative transmembrane helices to form the redox reaction center in subunit I through association of the transmembrane region II and X via heme ligation (see Fig. III-8). In the model, electron flow from ubiquinol-8 to the high-spin heme / Cu_B binuclear center is mediated via the low-spin

heme and the committed reduction of molecular oxygen to water takes place in the binuclear center. Proton pumping must be coupled to these redox reactions. Besides conserved histidines only two (potentially) charged residues (Glu-286 and Tyr-288 in helix VI) are present in the redox center. These charged residues may be involved in proton translocation by the heme-copper oxidases, as in a light-driven proton pump, bacteriorhodopsin (Mogi *et al.* 1988). Invariable aromatic residues such as Tyr-288 in helix VI may be involved in these electron transfer reactions. In the photosynthetic reaction center of *Rhodobacter sphaeroides*, Tyr-210 in subunit M has been identified as a key residue in primary electron transfer (Finkele *et al.* 1990, Nagarajan *et al.* 1990).

Tables

Table III-1 Sequence alignment of His residues in subunit I of the *E. coli* cytochrome *bo* complex with corresponding residues in subunit I of the aa_3-type cytochrome *c* oxidases. The amino acid sequences aligned are: *Escherichia coli* (Chepuri *et al.* 1990a); *Halobacterium halobium* (Denda *et al.* 1992); *Bacillus subtilis* (Saraste *et al.* 1991b); thermophilic bacillus PS3 (Ishizuka *et al.* 1990); *Bradyrhizobium japonicum* (Bott *et al.* 1990), *Paracoccus denitrificans* (Holm *et al.* 1987), *Saccharomyces cerevisiae* (yeast)(Bonitz *et al.* 1980), *Neurospora crassa* (Burger *et al.* 1982), *Aspergillus nidulans* (Waring *et al.* 1984), *Chlamidomonas reinhardi* (Boer *et al.* 1985), maize (Isaac *et al.* 1985), Soy bean (Grabau 1986), *Trypanosoma brucei* (Hensgens *et al.* 1984), *Paramecium aurelia* (Pritchard *et al.* 1986), *Drosophila melanogaster* (fruit fly) (De Bruijn 1983), *Paracentrotus lividus* (Cantatore *et al.* 1989), *Xenopus laevis* (flog) (Roe *et al.* 1985), bovine (Anderson *et al.* 1982), and human (Anderson *et al.* 1981) sequences. The numbering refers to the *E. coli* sequence. Deletions are shown as -. The His residues altered in this study are marked by asterisks.

	13	54	97	98	106	195	262	277	284	333	343	378	411	419	421	472	492	550	557	579	584	609	650
		◆			✻				✻	✻	✻		◆	✻	✻								
E. coli	H	H	H	H	H	Q	H	N	H	H	H	H	H	H	H	Y	H	Y	H	H	H	H	H
H. halobium	T	H	S	L	H	T	T	H	H	H	H	T	H	H	H	L	T	K	P	G	D	S	-	-
B. subtitlis	-	H	Q	A	H	Q	N	H	H	H	H	T	H	H	H	H	W	Y	H	S	L	G	L	-
T. PS3	-	H	G	L	H	Q	N	H	H	H	H	T	H	H	H	H	L	N	S	V	-	-	-	-
B. japonicum	-	H	H	T	H	H	T	H	H	H	H	R	Q	H	H	H	G	H	R	Q	-	-	-	-
P. denitrificans	-	H	H	L	H	H	Q	H	H	H	H	K	H	H	H	H	Y	H	K	E	-	-	-	-
S. cerevisiae	-	A	Q	L	H	H	S	H	H	H	H	A	H	H	H	H	G	H	V	S	-	-	-	-
N. crassa	-	A	Q	L	H	H	S	H	H	H	H	T	H	H	H	H	G	H	L	S	F	Y	-	-
A. nidulans	V	A	Q	L	H	H	S	H	H	H	H	T	H	H	H	H	G	H	L	S	-	-	-	-
C. reinhardi	-	H	Q	L	H	H	A	H	H	H	H	F	H	H	H	H	G	H	V	R	-	-	-	-
Wheat	-	H	Q	I	H	H	T	H	H	H	H	K	H	H	H	H	G	H	T	K	-	-	-	-
Soybean	-	H	Q	L	H	H	T	H	H	H	H	K	H	H	H	H	G	H	A	K	-	-	-	-
T. brucei	-	H	Q	F	H	H	S	H	H	H	H	C	H	H	H	H	F	D	C	C	F	F	-	-
P. aurelia	-	H	I	K	H	V	S	H	H	H	H	D	H	H	H	F	G	V	N	F	V	V	-	-
D. melanogaster	-	H	Q	I	H	H	S	H	H	H	H	S	H	H	H	H	T	H	L	T	-	-	-	-
Sea urchin	-	H	Q	I	H	H	T	H	H	H	H	E	H	H	H	H	L	H	P	T	-	-	-	-
Frog	-	H	Q	I	H	H	T	H	H	H	H	D	H	H	H	H	L	H	V	I	-	-	-	-
Bovine	-	H	Q	I	H	H	T	H	H	H	H	S	H	H	H	H	M	H	Y	N	-	-	-	-
Human	-	H	H	I	H	H	T	H	H	H	H	S	H	H	H	H	T	H	Y	K	-	-	-	-

✻ : Invariant His residues
◆ : Highly conserved His residues
- : Deletions

Table III-2. Ubiquinol oxidase activities of subunit I mutants.

Mutant	Ubiquinol-1 oxidase activity	
	μmol Q_1H_2/min /mg protein[a]	%[b]
WT	57.2	100
I-H54A	39.1	48.0
I-H106A	0.2	1.8
I-H284A	0.5	0.9
I-H333A	1.6	2.1
I-H334A	0.9	1.2
I-H411A	57.1	56.0
I-H419A	0.2	2.9
I-H421A	0.2	0.8

[a]Average of at least three determinations.

[b]Specific activity were normalized on the basis of the amount of subunit I in each membrane preparation, which was determined by densitometric analysis of the subunit I band in Western blots using a Shimadzu double-wavelength flying spot scanner CS-9000

WT: wild type.

Table III-3 Copper contents of subunit I mutants.

Mutant	+ Cu medium		– Cu medium	
	nmol/mg protein[a]	*%*[b]	*nmol/mg protein*[a]	*%*[b]
WT	1.34±0.02	100	0.75±0.00	100
control	0.08±0.00	0	0.10±0.01	0
I-H54A	1.00±0.02	76	0.57±0.05	80
I-H106A	1.17±0.02	74	0.10±0.01	1
I-H284A	1.40±0.14	122	0.59±0.03	81
I-H333A	1.18±0.03	96	0.11±0.00	4
I-H334A	1.11±0.07	74	0.11±0.01	2
I-H411A	1.05±0.06	62	0.55±0.00	82
I-H419A	1.25±0.07	85	0.11±0.00	2
I-H421A	1.11±0.05	76	0.20±0.02	22

[a]Average ± S. D for at least three determinations.

[b]Specific contents of copper were normalized on the basis of the amount of subunit I in each membrane preparation, which was determined by densitometric analysis of the subunit I band in Western blots using a Shimadzu double-wavelength flying spot scanner CS-9000.

Table III-4 Summary of biochemical and growth properties of the mutant oxidases.

Strain	Cyt.$b_{563.5}$	Cyt.*o*	Cu_B	Quinol oxidase	Complementation test
WT	++	+++	++	+	+
control	-	-	-	-	-
I-H54A	++	+++	++	+	+
I-H106A	-	+	-	-	-
I-H106Q	-	+	N.T.	N.T.	-
I-H106M	-	+	N.T.	N.T.	-
I-H284A	++	++	++	-	-
I-H284Q	++	+	N.T.	N.T.	-
I-H333A	++	++	-	-	-
I-H334A	++	+++	-	-	-
I-H411A	++	+++	++	-	+
I-H419A	++	++	-	-	-
I-H421A	-	++	+	-	-
I-H421Q	-	+	N.T.	N.T.	-
I-H421M	-	+	N.T.	N.T.	-

N.T.: not tested.

Table III-5 Predicted boundarys of the membrane-spanning regions in subunit I.

Regions	Original Boundarys from KKD argolithm	Odds P(Ext):P(Int)	Boundarys Proposed in This Study (Length)
0	14(-1,+2)—— 3 6(-4,+4)	-9.92	14—— 36(23)
I	55(-1,+2)—— 76(-3,+3)	-6.72	55—— 76(22)
II	104(-3,+3)——128(-5,+5)	-8.76	101——123(23)
III	144(-1,+2)——164(-2,+2)	-4.94	144——164(21)
IV	196(-1,+2)——212(-2,+2)	2.28	196——212(17)
V	233(-1,+2)——255(-2,+2)	-9.02	233——255(23)
VI	286(-1,+2)——305(-2,+2)	-4.46	278——303(26)
VII	314(-1,+2)——333(-2,+2)	-7.11	316——340(25)
VIII	347(-1,+2)——364(-2,+2)	-0.37	347——364(18)
IX	385(-1,+2)——404(-2,+2)	-4.19	385——404(20)
X	413(-1,+2)——432(-2,+2)	-3.35	413——432(20)
XI	458(-1,+2)——479(-2,+2)	-4.67	458——479(23)
XII	494(-1,+2)——518(-2,+2)	-9.71	494——518(25)
XIII	590(-1,+2)——609(-2,+2)	-5.57	589——607(19)
XIV	609(-1,+2)——628(-2,+2)	-4.09	610——630(21)

Figures

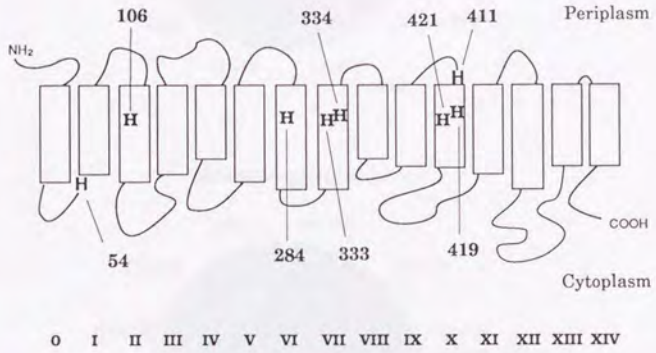

Figure III-1 Secondary structure model for subunit I of the cytochrome *bo* complex showing locations of the conserved residues. The secondary structure model based on the computer-aided prediction of membrane-spanning regions using the algorithm of Klein et al. *(Klein,* et al. *1985)* has been modified by the results of gene fusion experiments *(Chepuri and Gennis 1990).* Membrane-spanning regions are indicated by rectangles with the numbers of amino acids residues at the beginning and the end of each transmembrane domain connected by hydrophilic loops. The locations of the eight conserved His residues (His-54, -106, -284, -333, -334, -411, -419, and -421) are indicated by bold letters 'H'. The invariant residues cited in Table III-1 are indicated by standard one-letter abbreviations: These residues are mostly located in the membrane-spanning regions I, II, VI, VII, VIII, X, and XI, and the loop II-III. For simplicity, non-conserved residues are not shown.

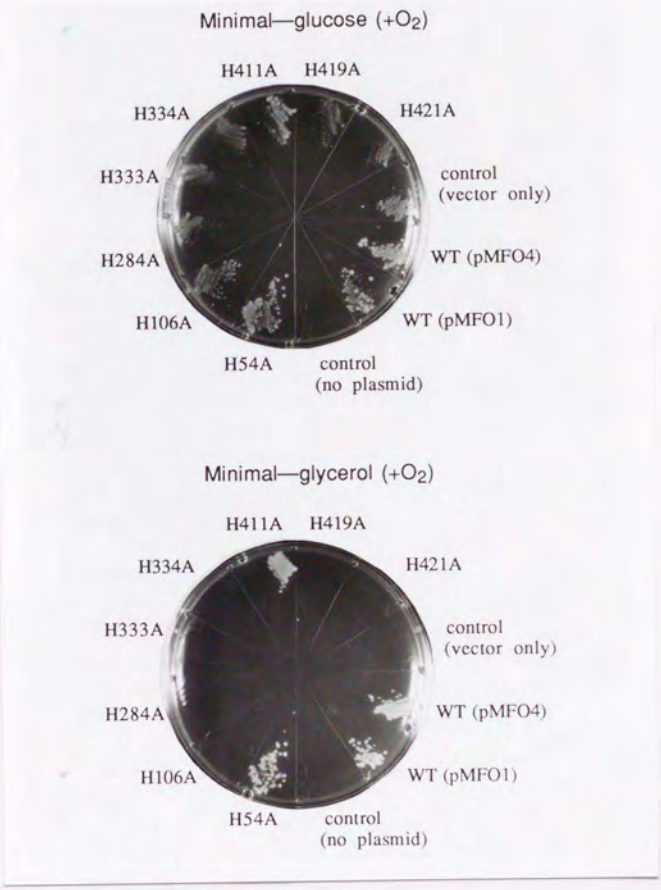

Figure III-2 Complementation test for aerobic growth of the Δcyo Δcyd double mutant ST2592 with the mini-F plasmid pMFO4 containing a single His-to-Ala mutation. A vector (pHNF-2) without the insert of the cyo operon was used as a control. Minimal medium plates containing 1 % glucose (upper panel) and 1 % glycerol (lower panel) were used for aerobic growth at 37°C for two days.

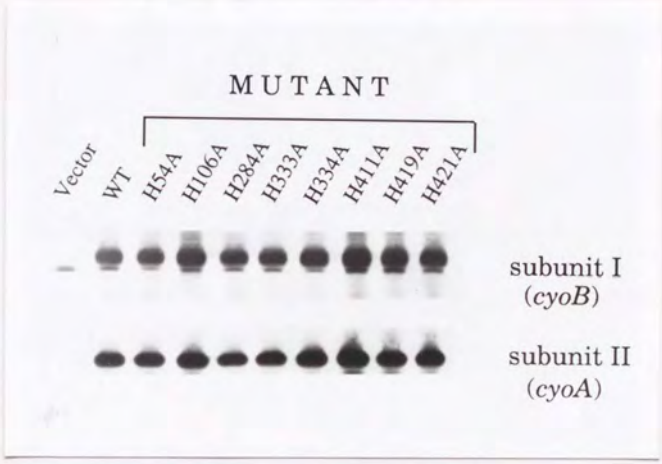

Figure III-3 Immunoblotting analysis of cytoplasmic membranes from strains expressing the mutant *cyo* operons with anti-subunit I and anti-subunit II antisera. Cytoplasmic membranes were prepared from strain ST4700 harboring pCYOF4 derivatives which contain a single His-to-Ala mutation. Samples of 5 μg of membrane proteins per lane were loaded on SDS-12.5 % polyacrylamide gel.

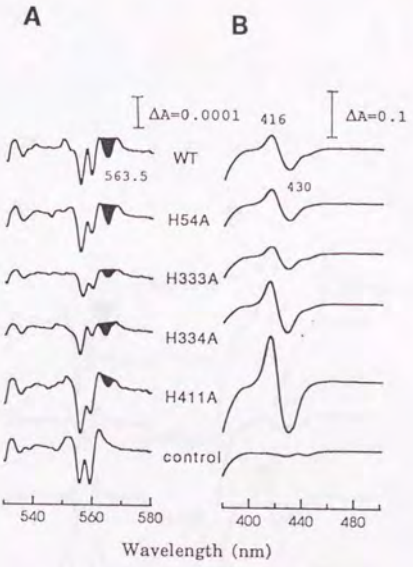

Figure III-4 Second order finite spectra of dithionite-reduced *minus* air-oxidized difference spectra (A) and CO-reduced *minus* reduced difference spectra (B) of cytoplasmic membranes from the I-H54A, I-H333A, I-H334A and I-H411A mutants. A, Spectra were recorded with a Shimadzu UV-3000 spectrophotometer at 77K, with a spectral band width of 1 nm and light path of 1 mm. The scanning rate was 50 nm/min and the protein concentrations were 3 mg protein per ml of 30 mM Tris-HCl (pH7.4). B, Conditions were as for A, except that measurements were done at room temperature with light path of 1 cm and protein concentrations were 0.5 mg protein per ml. Treatment with CO gas was carried out as described *(Kita,* et al. *1984)*. Strains carrying plasmid pCYOF8 and pCYOF1 were used as the wild-type control (WT) and a negative control (control), respectively.

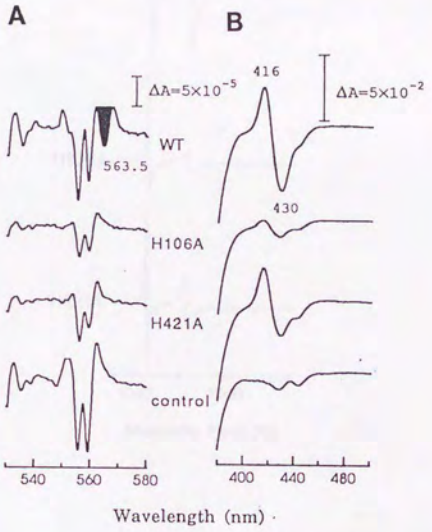

Figure III-5 Second order finite spectra of dithionite-reduced *minus* air-oxidized difference spectra (A) and CO-reduced *minus* reduced difference spectra (B) of cytoplasmic membranes from the I-H106A and I-H421A mutants. Conditions and procedures were as described in the legend to Fig. III-4.

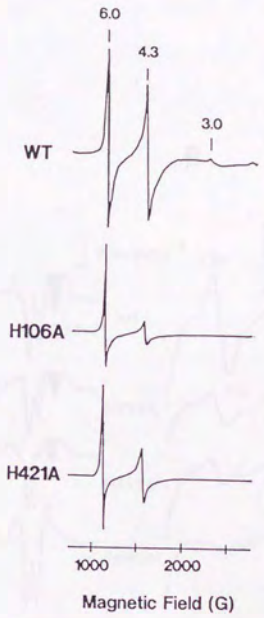

Figure III-6 EPR spectra at 15K on oxidized membranes from the I-H106A and I-H419A mutants. Cytoplasmic membranes from strain ST4700 (Δ*cyo*, *cyd*$^+$) carrying either H106A , H421A mutant gene, or wild type gene (pCYOF2) were poised at 280 mV by TMPD and ammonium persulfate. Protein concentrations of H106A, H421A, , and wild type membranes were 28.1, 21.6, and 22.9 mg/ml, respectively.

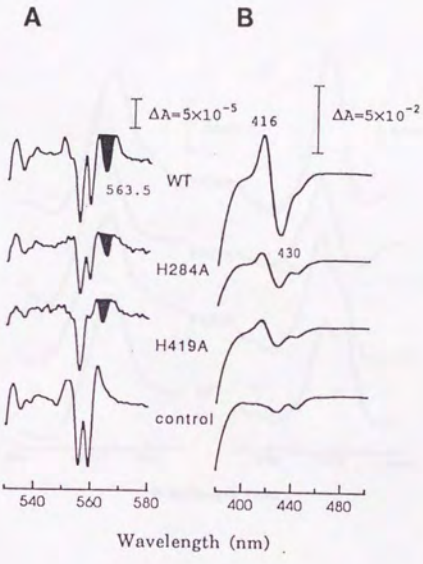

Figure III-7 Second order finite spectra of dithionite-reduced *minus* air-oxidized difference spectra (A) and CO-reduced *minus* reduced difference spectra (B) of cytoplasmic membranes from the I-H284A and I-H419A mutants. Conditions and procedures were as described in the legend to Fig. III-4.

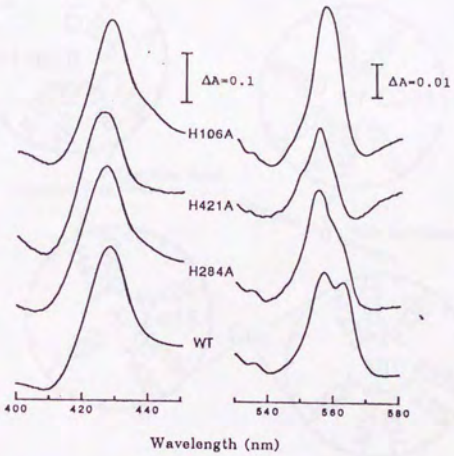

Figure III-8 Dithionite-reduced *minus* air-oxidized difference spectra of partially purified preparations from the I-H106A, I-H284A, and I-H421A mutants Spectra were normalized by the height of the Soret peak. Conditions and procedures were as described in the legend to Fig. III-4

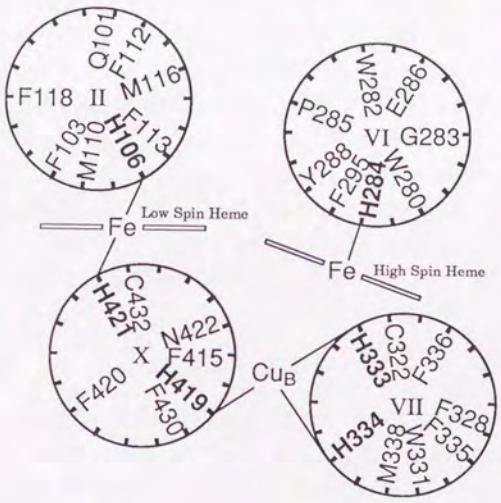

Figure III-9 Helical wheel projection model of the redox center in subunit I. The model shows possible spatial interactions between the low and high spin hemes and Cu_B, and arrangements of putative transmembrane helices II, VI, VII, and X. The rotational orientations of the helices were selected on the basis of the hydrophobic moment of the helices *(Eisenberg,* et al. *1984)* and the results of site-specific mutagenesis in this study. Both hemes are oriented with their planes perpendicular to the membrane plane *(Salerno and Ingledew 1991)*. See text for details.

Chapter IV. Replacements of Conserved Aromatic Amino Acid Residues

Introduction

Mutagenesis study of subunit I of the cytochrome *bo* complex in *E. coli* has been used to insight the structure of this enzyme. Analysis of His-to-Ala mutant enzymes has led to the identification of both axial-ligands of the low-spin heme as well as a potentail ligand of the high-spin heme and three putative ligands of Cu_B. To insure the identification of ligands of the prosthetic groups and to further examine the structure of subunit I, in particular, molecular structure of the redox reaction center in subunit I, conserved aromatic amino acid residues were altered in this chapter. Since the crystal structure of this enzyme has not yet been determined, side-directed mutagenesis is one of the best way to study tertially structure of membrane protein at a molecular level.

Aromatic ring may mediate electron-transfer

Living organisms move electrons over considerable distances, with great speed and specificity. An appropriate arrangement of transition-metal centers can mediate electron-transfer because these metals readily accept and donate electrons, but in biological systems such electron-transfer occurs rapidly over large molecular distances (10-20 Å). So far, many examples have been found where such a long-range electron-transfer is considered to be mediated by the aromatic amino acid residue(s).

play important role in primary electron transfer from the special pair (P) to bacteriopheophytin (H_L)(Yeates *et al.* 1988), and recently two groups obtained the supporting evidence by site-directed mutagenesis (Finkele *et al.* 1990, Nagarajan *et al.* 1990). Site-directed mutagenesis on the photosystem II of cyanobacteria indicated that the electron donor to P680+ was a Tyr-161 of the D1 polypeptide (Barry and Babcock 1987, Debus *et al.* 1988, Vermaas *et al.* 1988). In addition, Tyr-83 of plastocyanin involves the binding of plastocyanin to cytochrome *f* and is thought to form part of the main route of electron-transfer (He *et al.* 1991). Poulos and Kraut (Poulos and Kraut 1980) first suggested a critical role of the Phe-82 of tuna cytochrome *c* in the electron-transfer between cytochrome *c* and cytochrome *c* peroxidase complex with the aid of a compluter-graphics. This hypothesis has been probed by site-directed mutagenesis of yeast's enzyme. (Liang *et al.* 1986, Pielak *et al.* 1987). The Phe-82 is also suggested to involve cytochrome *c*-cytochrome b_5 electron-transfer from the computational calculation (Wendoloski *et al.* 1987).

Aromatic ring bulkiness may serve as binding pocket for prosthetic group

In a membrane protein which contains prosthetic group, aromatic amino acid residues are thought to play a structural role, probably providing bulkiness and hydrophobicity for a tightly packed hydrophobic environment about the buried portion of the heme, or providing π-π type interaction which stabilize a chromophore. The retinal environment of bacteriorhodopsin has been suggested to involve four

aromatic amino acid residues, Trp-86, -182 and -189, and Tyr-185 (Mogi *et al.* 1989). They seem to stabilize the chromophore with their bulky aromatic side chains or by providing a binding pocket within the bundle of helices (Henderson *et al.* 1990). In yeast iso-1-cytochrome *c*, Tyr-48 and Trp-59 form hydrogen bonds to the deeply buried heme propionate-7 on pyrrole ring IV, and Tyr-67 forms a hydrogen bond to sulfur of Met-80 which is an axial ligand of heme *c*. Moreover, all six aromatic amino acid residues in cytochrome b_{562} from the cytoplasm of *E. coli* lie in the vicinity of the heme group (Mathews 1985)

The aim of this study

In the previous chapter, a molecular model for the redox reaction center in subunit I of the cytochrome *bo* complex was presented. Although it is consistent with the results so far obtained, there are some unresolved problems. For example, Is His-284 really a proximal ligand of the high-spin heme ? Why the reaction center is made up of energetically unfavoured "non" antiparallel helix bundle ? In this study, I try to identify the aromatic amino acid residues which play significant role in the enzymatic activity, such as electron-transfer and formation of heme-binding pocket. Using the findings from the alterations of conserved aromatic amino acids, the four-α-helix bundle model is evaluated critically. Finally, based on the results throughout the thesis work, a improved model, five-a-helix bundle model, will be proposed.

Results

Mutagenesis of conserved aromatic amino acid residues in subunit I

Aromatic amino acid residue often play a significant role on long-range electron-transfer and stabilization of prosthetic group in a binding pocket. To identify the residues commited such a role in the redox reaction on the cytochrome *bo* complex, I altered the conserved aromatic amino acid residues in subunit I. This polypeptide contains 58 Phe, 20 Tyr, and 26 Trp residues in total. Table I-3 shows the conservancy in heme-copper oxidase superfamilly and that positions 147, 170, 288, 331, 391, and 420 are restricted to Trp, Trp, Tyr, Trp, Phe, and Phe residue, respectively, and positions 263, 282, 336, 348, 383, 415, and 439 are occupied by either of aromatic amino acid in any sequences. Other than those residues indicated above, there are many aromatic amino acid residues which are conserved at more than 60 % of the oxidases in the super familly. Of these residues, I selected the following twelve residues—Tyr-61 and -288, Phe-112, -113, -208, -328, -336, -348, and -415, and Trp-280, -282, and -331. These residues are highly conserved and located in the membrane-spanning regions of subunit I on a secondary structure model (Fig. IV-1).

Twelve mutated genes were constructed—I-Y61F, I-F112L, I-F113L, I-F208L, I-W280L, I-W282F, I-Y288L, I-W331L, I-F336L, I-F348L, and I-F415W by

oligonucleotide directed site-specific mutagenesis using Eckstein method (Taylor *et al.* 1985). Once these mutated genes were subcloned, sequencing was used to establish the presence of the mutation in the final clone.

Enzymatic Activities in vivo

Mutants were initially characterized by determining whether or not they could reestablish aerobic growth in a strain, ST2592, that was unable to grow aerobically. All twelve mutated genes were subcloned into the mini-F plasmid pMFO4 using *Nhe*I and *Sph*I restriction sites, following which ST2592 was transformed with them. Mini-F plasmids pMFO9 and pMFO4-Δ(*Hin*dIII-*Hin*dIII) and were constructed and employed as a positive and negative control, respectively. The results are shown in Fig. IV-2. Mutants W280L, Y288L, F328L, W331L, and F348L were unable to grow on non-fermentable carbon source, indicating that Trp-280, Tyr-288, Phe-328, Trp-331, and Phe-348 play important role on the terminal oxidase activity.

Immunological identification.

In order to examine the expression level of the enzyme, Western blotting analysis of subunit I (Fig. IV-3) and II of these mutant enzymes was performed using rabbit polyclonal antisera. Although Western blots indicated that both subunits I and II were present on the cytoplasmic membranes of all the mutants, only a replacement of Phe-112 by Leu residue caused a reduction, compared to the wild-type, in the amount of subunit I antigen found in the membrane. This indicates that all

these mutations but F112L did not affect gross structure of this enzyme complex. Leu at position 112 may affect the amount of polypeptide correctly inserted in the cytoplasmic membrane or afffect the feasibility of those proteolytic lability. Phe-112 is accordingly a key residue for the folding and/or stabilizing the polypeptide.

Copper content.

The effect to copper binding was examined by atomic absorption spectrum using cytoplasmic membranes prepared from mutant cells, which had grown in the absence of added copper (Table IV-1). Copper content in the cytoplasmic membranes of the mutant W280L, Y288L, W331L, and F348L were greatly reduced compared to those of wild-type enzyme. Alteration of these aromatic amino acid residues might cause the constraints of the ligand(s) of the high-spin heme/Cu_B binuclear center.

From the study of His-to-Ala mutant, the redox reaction center of subunit I of the cytochrome *bo* complex was localized to a four-α-helix bundle which is comprised of transmembrane helices II, VI, VII, and X. On this model, Trp-280 and Tyr-288 locate in the helix VI, and Trp-331 locates in the helix VII. A defferent residue at their position might constrain the ligand (s) of the binuclear center. Therefore, the results that replacements of these three aromatic amino acid affected the enzymatic activity as well as copper-binding ability is in good agreement with the four-α-helix bundle model. In addition, Phe-348 in helix VIII was found to be important for enzymatic activities and copper binding, suggesting that helix VIII is located adjacent to helices VI and VII.

Optical spectra.

Dithionite-reduced minus air-oxidized difference spectra at 77K, their second-order finite spectra (Fig. IV-4), and CO-binding spectra (Fig. IV-5) of the mutants were obtained. All mutations appear to have nearly wild-type spectra, indicating that all the aromatic amino acid residues are dispensable for the heme-binding ability to bind both hemes, and furthermore, they don't involve heme-binding directly. In the mutant W280L and W282F, CO-binding abilities were reduced. They may potential candidates for members contributing to formation of the high spin heme-binding pocket. Mutations at Phe-112 and -113 effected to red shift of α-band, suggesting that these mutations effected conformational change of α-helix structure of the helix II and the effect was transfered to the low-spin heme through axial ligand bonding by His-106 causing change of electron density of the low-spin heme. On the other hand, the mutant F336L was found to have an additional peak at 552.1 nm. Because the bands of 555 nm and 563.5 nm were still present, the 552.1 nm band appear to be caused by alteration of environment at position 336. Although, additional experiments are required to draw any explanation, one possible explanation of this is that the mutation effected shift of the spin equilibrium of the high-spin heme toward the low-spin state, which brought an additional α-absorption band at 552.1 nm.

It is concluded from a combination of the experimental results that the aromatic amino acid residues, Phe-112 and -113 are suggested to be in the same helix contributing to the axial ligand of the low-spin heme, Trp-280, Tyr-288, Trp-331, and Phe-348 are suggested to

involve the binding pocket of the binuclear center, and Phe-336 may locate proximal to the sixth ligand position of the high-spin heme. Phe-328 also play an important role on the enzyme activity which is not relevant to prosthetic group binding. More experiments are required to determine which role Phe-328 plays in the enzyme complex.

Modeling

Membrane-exposed residues in the photosynthetic reaction center are more hydrophobic than buried interior residues in the transmembrane helices (Eisenberg *et al.* 1984), and are relatively poorly conserved (Komiya *et al.* 1988). Since these nature may be useful for packing of transmembrane α-helices in subunit I of the cytochrome *bo* complex, I examined periodicity of hydrophobicity (Fig. IV-6A) and mutability (Fig. IV-6B) in the integral sequences. As shown in Fig. IV-6A, none of the fifteen helices except IV show any convincing periodicity. This may have implications for its three-dimensional structure suggesting that they are not peripheral helices. Since the cytochrome *bo* complex is a multi-subunit complex in that each subunit has multiple transmembrane helices, subunit I can be accordingly surrounded by the transmembrane helices of other subunits and be shielded from lipids.

On the other hand, convincing periodicity of mutability were shown in several helices (Fig. IV-6B). I propose that the conserved side of these helices faces the interior of the helical bundle, where the prosthetic groups probably locate. Based on these characters of each helices and information from the mutagenesis work, a most

reliable two-dimentional model for subunit I of the cytochrome *bo* complex is constructed (Fig. IV-7 and 8).

Discussion

In the previous chapter, a four-α-helix bundle model which depicts the redox reaction center of the cytochrome *bo* complex was presented. In the model, His-284 was ascribed to a proximal ligand of the high-spin heme based on the results that 1) His-284 is one of the six totally conserved His residues in subunit I. All of the totally conserved His residues had shown to be dispensable for the enzymatic activity *in vivo* and *in vitro* , and 2) the H284A enzyme contained Cu_B in a normal extent, while H333A, H334A, and H419A enzyme lost copper completely. Since it is very difficult to obtain direct evidence showing that His-284 is the proximal ligand, in this study, I attempted to identify the important aromatic amino acid residues in the vicinity of the heart of this enzyme. Twelve conserved aromatic amino acid residues, Phe-112, -113, -208, 328, -336, -348, and -415, Tyr-61 and 288, and Trp-280, -282, and -331 (Fig. IV-1) were individually replaced by Leu, Phe, or Trp and tested whether they had influences on the absorption spectra of the hemes and on copper binding ability. Aromatic amino acids were selected for the reason of that they are potential mediators of electron transfer and potential contributors of binding pocket for prosthetic groups. Accordingly, it is possible that these residues may involve ligand binding by stabilizing the ligand molecules with their bulky aromatic side-chains or by providing a binding pocket within the bundle of helices.

The twelve mutants were correctly constructed and expressed. The following discussion of the influence of the mutations on the enzymatic activity and the environment of prosthetic group is based on the assumption that the effects of the mutations originate from the molecular properties of the replaced amino acid and not from a secondary structural disorder. This assumption is supported by the results from Western immunoblotting with polyclonal antibodies which show that there are no gross alterations in the structure of antigen. First, Trp-280, Tyr-288, and Phe-328, -348 and -415 were found not to be dispensable for *in vivo* enzymatic activity. It implies that these residues locate in the vicinity of the heart of the enzyme and may play central role for the enzymatic activity, such as electron transfer and proton pumping.

Second, a mutation Trp-280 to Leu, and Trp-282 to Phe were identified to cause a half reduction of the amount of CO bound to the high-spin heme. The side-chain of Leu residue is smaller than those of Trp residue and lacks the stabilizing influence of the rigid aromatic ring. Phe residue is somewhat smaller than Trp residue and carry the stabilizing influence of the aromatic ring. It is accordingly tempting to suggest that Leu and Phe in the place of Trp-280 and -282, respectively, destabilized the high-spin heme in the heme pocket, and thus a half population of the apo-polypeptide of subunit I could not bind the heme stably. These findings are consistent with the four-α-helix bundle model proposed in the previous chapter, which localized Trp-280 and -282 to the transmembrane helix VI together with an axial ligand of the high-spin heme, His-284.

Third, Leu at Phe-112 and -113 effected red-shift of the α-band of the enzyme which recently has been determined to be due to the low-spin heme of this enzyme (Puustinen *et al.* 1991). Since these two Phe residues are dispensable for the enzymatic activity, these shifts were not caused by the disorder of heme-heme interaction, but might be caused by only the conformational changes of the α-helix on which they located, transmembrane helix II. These are consistent with the four-α-helix bundle model proposed in the previous chapter, which localized Phe-112 and -113 to the helix II together with an axial ligand for the low-spin heme, His-106.

Fourth, the substitutions of Leu residue for Trp-280 and -331, Tyr-288, and Phe-348 caused complete lack of Cu_B. The side-chain of leu residue is approximately similar in size to those of Tyr and Phe residue, suggesting that stabilizing effect by aromatic ring at these position is necessary to bind Cu_B. That is, they appear to involve a binding pocket for the Cu_B pocket. Of five essential aromatic amino acid residues for the enzymatic activity, all but Phe-328 were identified to also play important role on binding Cu_B. Which role does Phe-328 play on to drive the enzymatic activity ? Unfortunately, not any data which suggests the role of Phe-328 have been obtained. One possible explanation is as follows: Phe-328 locates the cytoplasmic half of the helix VII. The essential residues, Tyr-288 and Trp-331 occupy similar positions in the transmembrane helices, whereas nonessential residues, Trp-282 and Phe-336 locate the periplasmic half of the helices. Some significant local structure may be constituted in the cytoplasmic half of the transmembrane regions,

which is prerequisite, for example, for the redox communication between both hemes. Phe-348 locates in helix VIII, which was not depicted in a previous four-α-helix model. Hence, it is better to append the helix VIII to the model of the redox reaction center in subunit I of the cytochrome *bo* complex. Since the resultant five helix bundle become far from antiparallel, appending of helix VIII decreases the stability of the helical bundle through dipole interactions. Hence, other transmembrane helices may be present in between these helices. It is worth noting that the cytochrome *bo* complex comprised thirty two transmembrane helices in a whole complex (Chepuri and Gennis 1990), and thus, the relative unstability of a couple of helices can be compensated for by a number of surrounding helices which form an energetically stable bundle (Fig. IV-7).

A more reliable model for the redox reaction center in subunit I of the cytochrome *bo* complex is proposed from a combination of the experimental findings throughout this thesis work (Fig. IV-8). The redox center is comprised of by five transmembrane helices II, VI, VII, VIII, and X in subunit I. Because both hemes are oriented with their planes perpendicular to the membrane plane (Salerno and Ingledew 1991), they are suspended between adjacent sections of transmembrane α-helices. The low-spin heme is sandwiched by helices II and X, and the binuclear center is formed by helices VI, VII, VIII, and X. Since His-106 and -421 are so crucial in binding of the low-spin heme and Cu_B, and alteration of Phe-112 on helix II effected the expression level of the enzyme, helix-helix interaction between helices II and X through binding of the low-spin

heme may play a crucial role on folding of the whole enzyme.

Helix VI carries the largest number of conserved residues in subunit I (Fig. IV-8). This is likely to be the most important helix in this complex. In particular, sequences between positions 283 and 288, —GHPEVY—, are totally conserved. In this sequence, there is Gly and Pro residues at positions 283 and 285, respectively, which can disrupt the regular hydrogen-bonding pattern of α-helix. The sequence of Gly-His-Pro make the helix VI to form rather flexible conformation, which may enable the high-spin heme to bind exogenous ligand at the distal site and may enable to communicate with the low-spin heme through conformational changes (Bisson 1990).

In the model, electron flow from ubiquinol-8 to the high-spin heme / Cu_B binuclear center is mediated via the low-spin heme and the committed reduction of molecular oxygen to water takes place in the binuclear center. Proton pumping must be coupled to these redox reactions. Recently, Ingledew and Bacon determined the distance between the two hemes in this enzyme to approximately 14 Å (Ingledew and Bacon 1991). This distance falls within the estimated range of heme-heme distances (12-16 Å) calculated for *aa*$_3$-type cytochrome c oxidase (Ohnishi *et al.* 1982), and thus, a long-range electron-transfer which is observed in many other redox proteins occurs in the heme-copper oxidase superfamily probably in a similar manner. In this study, several important aromatic amino acid residues were identified, which are potential candidate for electron mediator. The most probable residue is Tyr-288 from a consideration of spatial location. Unfortunately, to

examine this possibility is, however, rather difficult. Effect of the alteration of Tyr-288 could be obscured by the the lack of Cu_B in the mutant enzyme.

Table

Table IV-1. Effects of Replacements of the Conserved Aromatic Amino Acid Residues

Strain	Transmembrane Helix	Cu_B %[a]	Aerobic Growth	Low-Spin Heme	CO-Binding %[a]
WT		100	+	+	100
Vector		0	–	–	–
Y61F	I	89	+	+	71
F112L	II	85	+	+(red shift)	60
F113L	II	148	+	+(red shift)	82
F208L	IV	80	+	+	101
W280L	VI	<1	–	+	54
W282F	VI	34	+	+	42
Y288L	VI	<1	–	+	64
F328L	VII	68	–	+	89
W331L	VII	<1	–	+	67
F336L	VII	57	+	+	96
F348L	VIII	<1	–	+	98
F415W	X	103	+	+	86

[a]Normalized by the amounts of subunit I polypeptide as described in the legend to Table III-2.

Figures

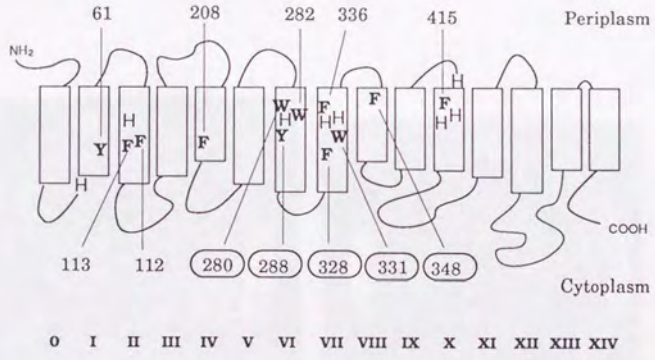

Fig. IV-1 Location of the aromatic amino acid residues altered in this study were shown in the secondary structure model of subunit I. The secondary structure model based on the computer-aided prediction of transmembrane helices using the algorithm of Klein et al. *(Klein,* et al. *1985)* has been modified by the results of gene fusion experiments *(Chepuri and Gennis 1990)*. Transmembrane regions are indicated by *rectangles* connected by hydrophilic loops. Locations of the conserved aromatic amino acid residues altered in this study are shown. Residues found to be essential for the enzymatic activities are indicated with *oval.*

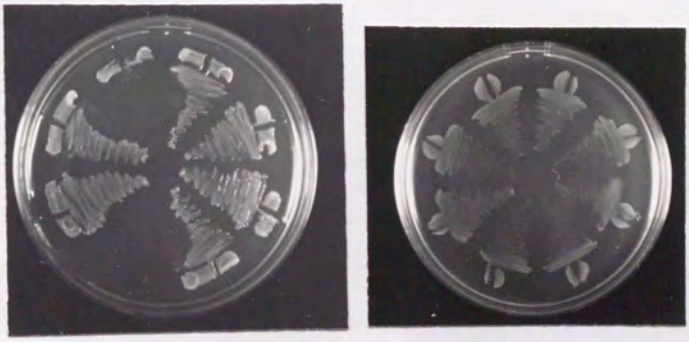

Fig. IV-2 (A) Complementation test for aerobic growth of the Δcyo Δcyd double mutant ST2592 with a mini-F plasmid carrying Y61F(a), F112L(b), F113L(c), F208L(d), W280L(e), and W282F(f) mutant gene. The mini-F plasmid pHNF-2(g) and pHNFO-4 (h)were used as a negative and positive control, respectively. Minimal-medium plates containing 1 % glycerol were used. Plates were incubated under aerobic (left panel) and anaerobic (right panel) conditions at 37°C for two days.

Fig. IV-2 (B) Complementation test for aerobic growth of the Δcyo Δcyd double mutant ST2592 with a mini-F plasmid carrying Y288L(a), F328L(b), W331L(c), F336L(d), F348L(e), and F415W(f) mutant gene. The mini-F plasmid pMFO4Δ(*Hin*dIII-*Hin*dIII) (g) and pMFO9 (h) were used as a negative and positive control. Minimal-medium plates containing 1 % glycerol were used. Plates were incubated under aerobic (left panel) and anaerobic (right panel) conditions at 37°C for two days.

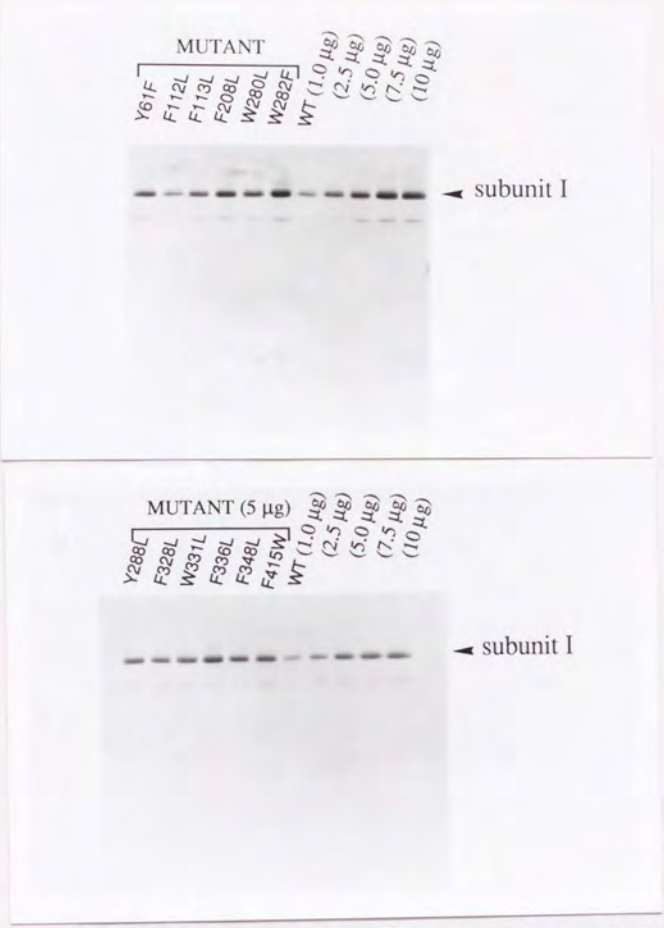

Fig. IV-3 Immunoblotting analysis of cytoplasmic membranes from strains expressing the mutant cyo operons with anti-subunit I antisera. Cytoplasmic membranes were prepared from strain ST4700 harboring pCYOF9 derivatives which contain a single mutation at conserved aromatic amino acid residues. Cytoplasmic membrane samples were loaded on SDS-12.5 % polyacrylamide gel.

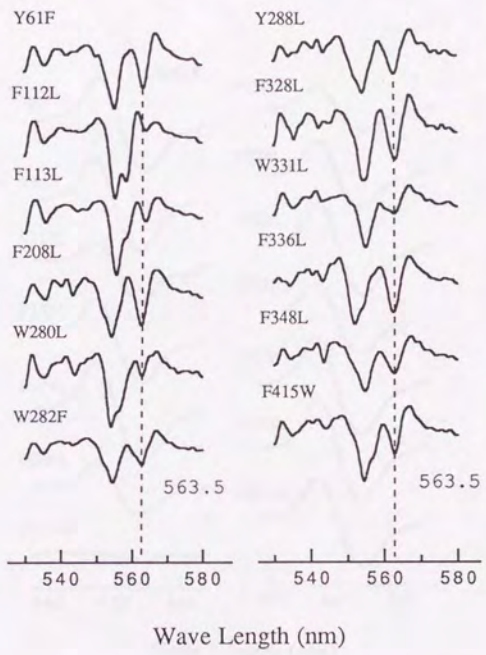

Fig. IV-4 Second order finite spectra of dithionite-reduced minus air-oxidized difference spectra at 77K of cytoplasmic membranes from the mutants. Spectra were recorded with a Shimadzu UV-3000 spectrophotometer at 77K, with a spectral band width of 1 nm and light path of 1 mm. The scanning rate was 50 nm/min and the protein concentrations were 3 mg protein per ml of 30 mM Tris-HCl (pH7.4).

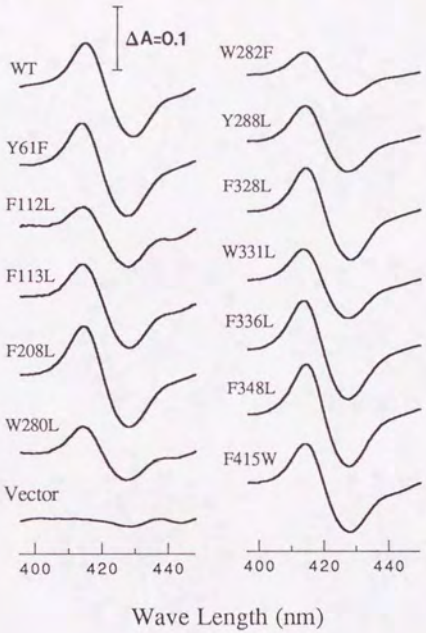

Fig. IV-5 CO-binding spectra at room temperature of cytoplasmic membranes from the mutants. Conditions were as for Fig. IV-4, except that measurements were done at room temperature with light path of 1 cm and protein concentrations were 1 mg protein per ml. Treatment with CO gas was carried out as described *(Kita,* et al. *1984).* Strains carrying plasmid pCYOF8 and pCYOF1 were used as the wild-type control (WT) and a negative control (control), respectively.

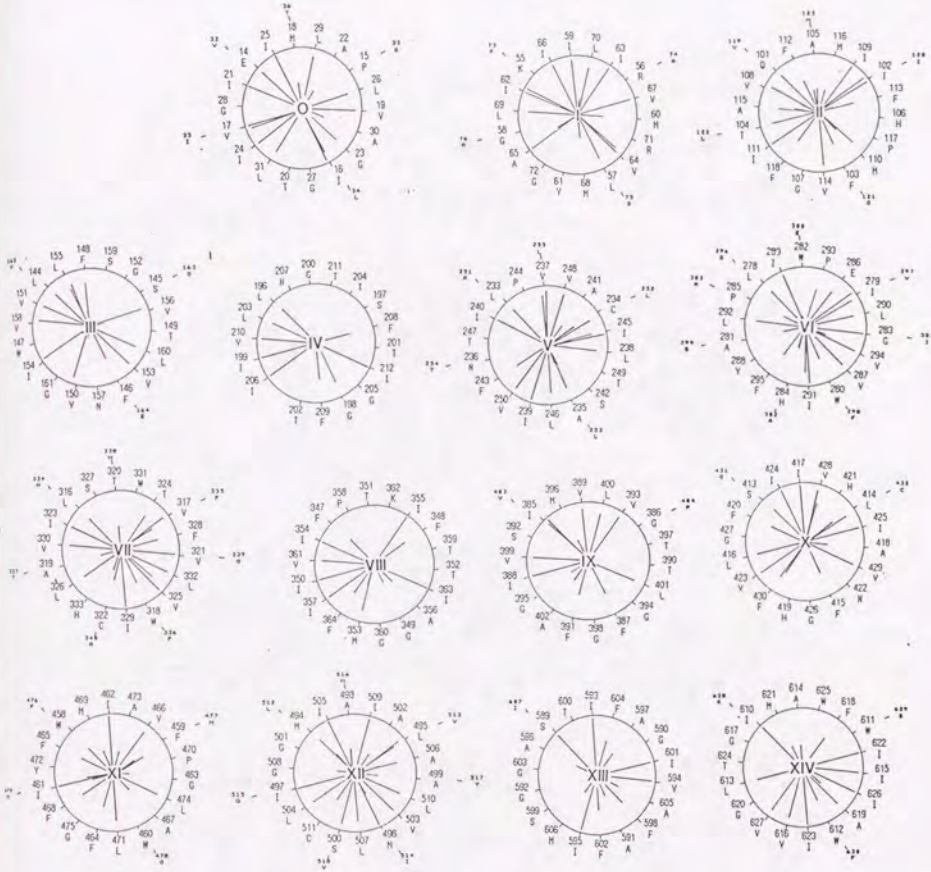

Fig. IV-6 (A) Spatial distribution of the hydrophobic side chains in transmembrane α-helices of subunit I. Hydrophobic residues are represented as lines extending out from the center of the helical wheel of the α-helix toward the location of residue. Hydrophilic residues are represented by lines extenting out from the center of the helical wheel toward the anti-direction of the locations of residues Hydropathy index from Kyto and Doolittle *(Kyto and Doolittle 1982)* was used in the analysis. Each helical wheel are depicted as if viewed from the N-terminal side of the helix.

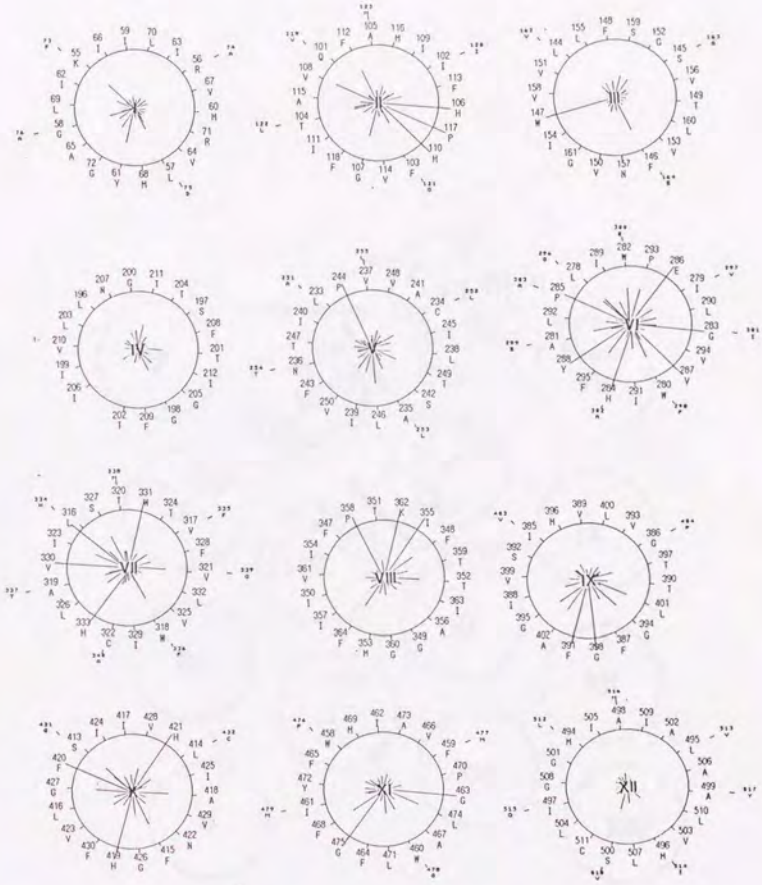

Fig. IV-6 (B) Spatial distribution of the mutability in transmembrane α-helices of subunit I. The mutability is taken as 28 / Vj where 28, the number of sequences used in the analysis; Vj, the number of different amino acids at each position in the sequence alignment. The magnitudes of mutability are indicated as length of lines extending out from the center of helical wheel toward the position of residue. Each helical wheel are depicted as if viewed from the N-terminal side of the helix.

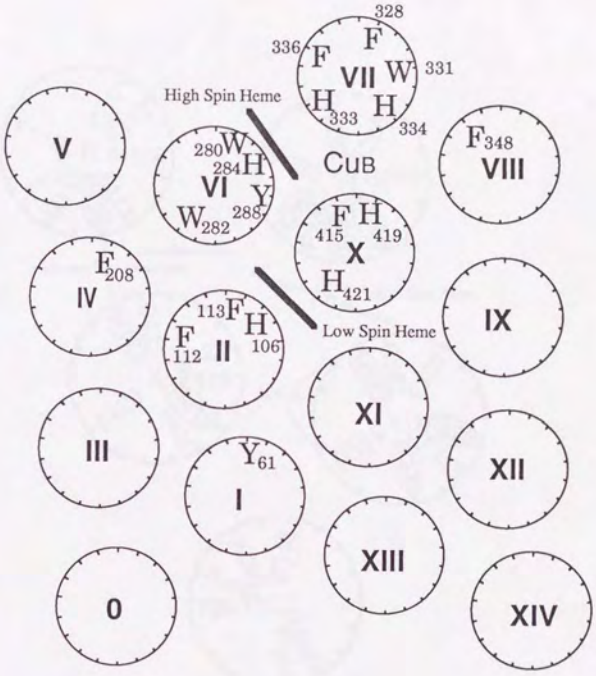

Fig. IV-7 Helical wheel depiction of all fifteen helical regions arranged as if viewed from the periplasmic side of the membrane.

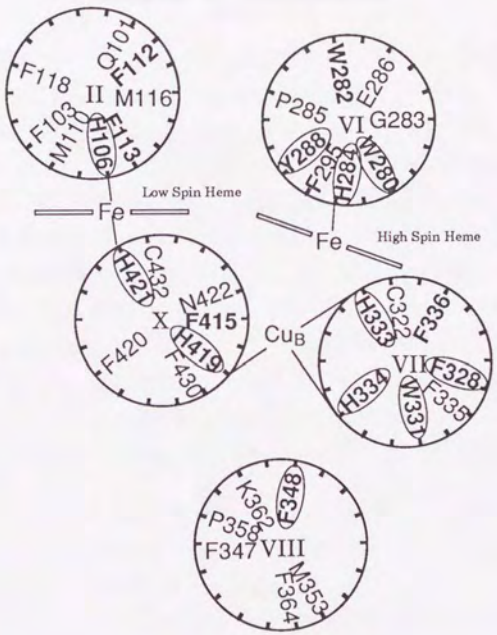

Fig. IV-8 A model of the redox reaction center in subunit I of the cytochrome *bo* complex: Five-helix bundle model.

Chapter V. Conclusion

The cytochrome *bo* complex is a terminal ubiquinol oxidase in the aerobic respiratory chain of *E. coli* and functions as a proton pump. It belongs to the heme-copper oxidase superfamily with the aa_3-type cytochrome *c* oxidases in mitochondria and aerobic bacteria. In this thesis, I replaced eight conserved His residues in subunit I of the cytochrome *bo* complex by Ala, Gln, and Met residues (Chapter III) and replaced twelve conserved aromatic amino acid residues by Leu, Phe, or Tyr residues (Chapter IV). All the mutant enzymes were characterized using genetical, immunological, biochemical, and spectroscopical methods. The results were summarized as follows:

1) H106A and H421A enzymes did not contain the low-spin heme, which indicates that these His residues are the axial ligands of the low-spin heme.

2) H333A, H334A, and H419A enzymes did not contain Cu_B, which indicates that these His residues are the ligands of Cu_B.

3) H284A enzyme contained copper atom to a normal extent, but contained largely reduced amount of CO-binding ability, which indicates that this His residue is an proximal ligand of the high-spin heme.

4) F112L and F113L enzymes contained the low-spin heme whose α-bands were shifted to longer wavelength, which indicates that these Phe residues locates in the vicinity of a ligand of the low-spin heme.

5) W280L and Y288L enzymes contained a half amount of CO-binding heme, which indicates that these aromatic amino acid residues are in the neighbor of the high-spin heme.

6) W280L, Y288L, W331L, and F348L enzymes did not contain Cu_B, which indicates that these aromatic amino acid residues involve a binding pocket of the binuclear center.

7) His-54, His-411, Tyr-61, Phe-208, Phe-336, and Tyr-415 residues could be altered without eliminating enzymatic activity, heme-binding, and copper-binding, which indicates that these are dispensable residues in this enzyme.

Based on these findings, I finally presented a helical wheel model (working model) of the redox reaction center in subunit I that consists of the transmembrane helices II, VI, VII, VIII and X. And implications of the model were discussed.

During long time many researchers have studied the molecular structure of the cytochrome *c* oxidase. So far, a number of structure models of the redox reaction center of the cytochrome *c* oxidase have been proposed (Welinder and Mikkelsen 1983, Holm *et al.* 1987, Lundeen and Chance 1987, Saraste 1990, Saraste *et al.* 1991b, Wrigglesworgh 1991), which, however, are not based on the experimental findings. This study on the cytochrome *bo* complex in *E. coli* first elucidate the molecular structure

of the redox reaction center in terminal oxidase, which is applicable to the eucaryotic counterpart. Recently, several site-directed mutagenesis studies which were oriented to the determination of a ligand of prosthetic groups, e.g., (Fang *et al.* 1988, Fridén and Hederstedt 1990, Nakai *et al.* 1990, Van de Kamp *et al.* 1990, Chang *et al.* 1991, Pakrasi *et al.* 1991, Yun *et al.* 1991). Some of them seems to have obtained rather ambiguous results. These might originate from a secondary effect of the exchaged amino acid, which is ussually very difficult to rule out. To examine the character of only the exchanged amino acid, we should always check whether there are gross alterations in the global structure. In this study, I confirmed that all the mutant enzymes were detected immunologically on the cytoplasmic membranes, and thus obtained rather clear results. However, the possibilities could not be excluded completely that the effects observed in this study was originated from a long-range structural alterations.

Finally, it is noteworthy that the structural study of the terminal oxidase proceed to the next step. The findings from this study are essential for studying the catalytic mechanisms of 1) long-range electron-transfer and 2) proton-pumping in the enzyme. The best studied enzyme which catalizes long-range electron-transfer is cytochrome *c* (Williams 1991), whereas which catalizes proton-pumping is bacteriorhodopsin (Khorana 1988). However, these knowledge are not simply applicable to the terminal oxidase, where long-range electron-transfer and pronton-pumping are coupled. The coupling mechanism has not elucidated at al. This is why the enzyme attracts many researchers all over the world. From now on,

catalytic mechanisms of 1) long-range electron-transfer, 2) proton-pumping, and 3) coupling mechanism of 1) and 2) in the heme-copper oxidase will attract more and more interests.

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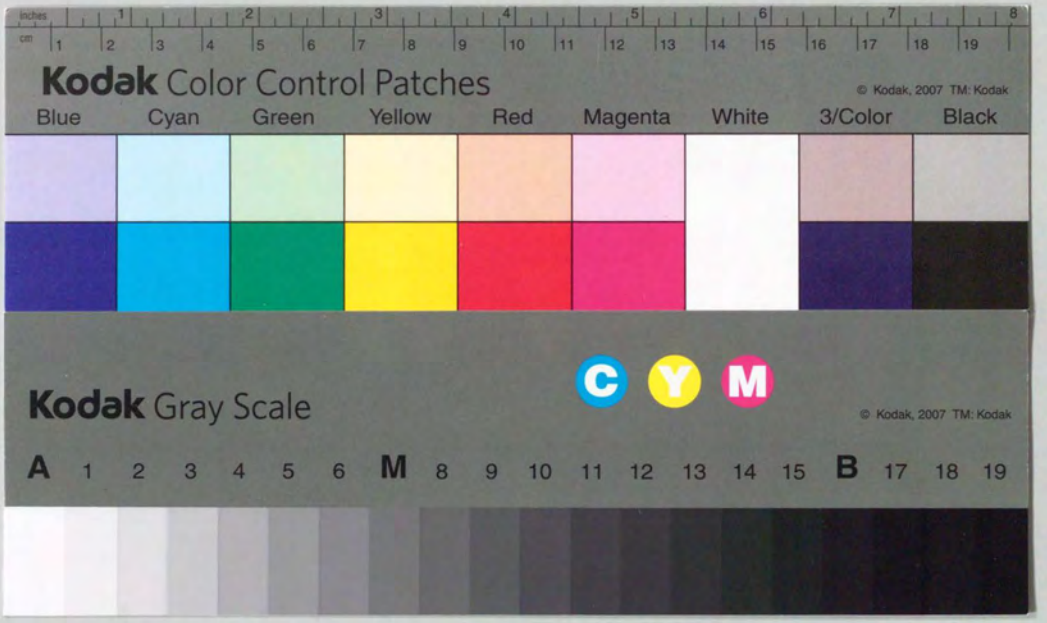
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