Identification of Heme and Copper Ligands in Subunit I of the Cytochrome \textit{bo} Complex in \textit{Escherichia coli}\* 

Jun Minagawa, Tatsushi Mogi, Robert B. Gennis‡, and Yasuhiro Anraku§

From the Department of Biology, Faculty of Science, University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, Japan and the
\‡Departments of Biochemistry and Chemistry, University of Illinois, Urbana, Illinois 61801

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The cytochrome \textit{bo} complex is a terminal ubiquinol oxidase in the aerobic respiratory chain of \textit{Escherichia coli} (Kita, K., Konishi, K., and Anraku, Y. (1984) \textit{J. Biol. Chem.} 259, 3368-3374) and functions as a proton pump. It belongs to the heme-copper oxidase superfamily with the \textit{aa}_3-type cytochrome \textit{c} oxidases in mitochondria and aerobic bacteria. In order to identify ligands of hemes and copper, we have substituted eight conserved histidines in subunit I by alanine and, in addition, His-106, -284, and -421 by glutamine and methionine. Western immunoblotting analysis showed that all the mutations do not affect the expression level of subunit I in the cytoplasmic membrane, indicating that these histidines are not crucial for its stability. A single copy expression vector carrying a single mutation at the invariant histidines, His-106, His-284, His-333, His-334, His-419, and His-421, of subunit I was unable to support the aerobic growth of a strain in which the chromosomal terminal oxidase genes (the \textit{cyo} and \textit{cyd} operons) have been deleted. The same mutations caused a complete loss of ubiquinol oxidase activity of the partially purified enzymes. Spectroscopic analysis of mutant oxidases in the cytoplasmic membrane revealed that substitutions 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 the CO-binding high spin heme. These spectroscopic properties of mutant oxidases were further confirmed with partially purified preparations. Atomic absorption analysis showed that substitutions of His-106, -333, -334, and -419 eliminated Cu\textit{b} almost completely.

Based on these findings, we conclude that His-106 and -421 function as the axial ligands of the low spin heme as well as a pentacoordinated high spin heme (10, 11). Although both hemes have been considered as protoheme IX for long time (3), a new heme component was found recently to be also contained in the oxidase (12), together with the copper atom (Cu\textit{b}) as another prosthetic group (3). It is suggested that the low spin heme is located close to the quinol oxidation site whereas the high spin heme/Cu\textit{b} binuclear center functions as a site for the reduction of molecular oxygen to water (13). Optical absorption spectra of the oxidase show two \textit{a}\textsubscript{1} absorption bands (555 and 665.5 nm)\textsuperscript{1} which are ascribed to a contribution from the low spin heme/Cu\textit{b} system and \textit{a}\textsubscript{2} bands (555 and 665.5 nm) of the cytochrome \textit{bo} complex (14, 15). The genes (\textit{cyoABCDE}) coding for the cytochrome \textit{bo} complex have been cloned (14, 16) and sequenced (16). The products of the \textit{cyoA} and \textit{cyoB} genes are assigned to be subunits II and I, respectively (3, 14). Subunit I of the cytochrome \textit{bo} complex is related to subunit I of the cytochrome \textit{aa}_3 complexes of mitochondria and aerobic bacteria in that 37% of the amino acids are identical over a 546-amino acid overlap (16). It is likely that the sequence similarities reflect a common molecular architecture of the reaction center in the heme-copper oxidase superfamily, although the cyto-

\footnote{1 Based on our careful spectroscopic determination of cytochromes in the cytochrome \textit{bo} complex in cytoplasmic membranes and in a purified enzyme, we reevaluate that the oxidase complex has two \textit{a}\textsubscript{1} peaks (555 and 665.5 nm) at 77 K, rather than the peaks at 555 and 562 nm.}

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\* To whom correspondence should be addressed. Fax: 81-3-3812-4929.

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From the Department of Biology, Faculty of Science, University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, Japan and the
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Based on these findings, we conclude 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\textit{b}. We present a helical wheel model of the redox center in subunit I, which consists of the membrane-spanning regions II, VI, VII, and X, and discuss the implications of the model.

The cytochrome bo complex is one of two terminal oxidases in the respiratory chain of aerobically grown \textit{Escherichia coli} (1, 2). The expression of the cytochrome bo complex is pre-
chromo bo complex uses ubiquinol as an electron donor in place of reduced cytochrome c and contains hemes different from those of subunit I (17). From the comparison of the primary structures of subunit I of the cytochrome bo complex and its counterparts of the cytochrome aa3 complexes (18), 6 His residues, His-106, His-284, His-333, His-334, His-419, and His-421, are invariable and two His residues, His-54 and His-411, are highly conserved in the terminal oxidase superfamily. Among the 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. 1). Spectroscopic studies on the cytochrome c oxidase suggest that subunit I has two axial ligands for the low spin heme, one axial ligand for the high spin heme, and three ligands for Cuo (19).

We are taking great advantage of the molecular genetics in E. coli and carrying out the structure-function studies on the cytochrome bo complex to elucidate a mechanism of the redox-coupled proton pumping (15). We have introduced a series of deletions in which each of the cyo genes is carried on a single copy expression vector (mini-F plasmid) and found that all five subunits are essential for the in vivo assembly of the functionally active enzyme. The versatile vector pCYOF2, which is particularly designed for the site-directed mutagenesis and for the expression of the cyo genes, has facilitated the detailed structure-function studies of the cytochrome bo complex (15).3

In this study, we 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 Cuo. We 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 Cuo.

**EXPERIMENTAL PROCEDURES**

**Media**—For the 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 (NH₄)₂SO₄, 12.0 mg/ml K₂HPO₄, 1 mM MgSO₄, 1% (w/v) glycerol, 10 μg/ml FeSO₄, and 0.03 μg/ml CuSO₄). For the analysis of copper content, the medium to which FeSO₄ and MgSO₄, 1% (w/v) glycerol, 10 μg/ml FeSO₄, and 0.3 μg/ml CuSO₄ was used was not supplemented and was also used in the complementation test for the aerobic growth, Davis and Mingioli minimal plate containing 1% (w/v) glucose or casein hydrolysate. The locations of the eight conserved His residues (His-54, -106, -284, -333, -334, -411, -419, and -421) are indicated in the boldface letter H. The invariable residues are indicated by the 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. Non-conserved residues are not shown for clarity.

**Constructions of pCYOF4 and pMP04**—Multicopy plasmid pCYOF4 which contains the unique Nhel site on the upstream region of the cyoB gene has been constructed from pCYOF2 (15) (Fig. 2). The construction was made via site-directed mutagenesis using an in vitro mutagenesis system (Amersham Corp.) and a primer (5' GGCTAGCGCGCGCTAGCTTTTCGAACG-3') corresponding to nucleotides 767-792 of the cyoA gene. The Nhel site was introduced without any changes in the amino acid sequence of subunit II and can be used for subcloning of the entire cyoB gene from the phagemid into a derivative of mini-F plasmid pMP01 (15). The DNA sequence between the SmaI and Nhel sites was confirmed not to contain any unexpected nucleotide changes; we found only Lys-253/Leu-254 codon changes (AAAT-CTG to AAG-CTA). The Smal-Sall fragment containing the unique Nhel site was replaced by the counterpart from wild-type plasmid pCYOF2. It is a T7 expression vector carrying the F1 replication origin and the intact cyo gene (15). The Nap (7524) V-EcoRI fragment (2.6 kb) of pMP01 which carries the F-prime-derived replication origin was replaced by the corresponding region of pCYOF4. The resultant single copy plasmid, pMP04, contains the unique Nhel site and was used throughout this study as a wild-type control of the cyo operon.

**Site-directed Mutagenesis**—Site-directed mutagenesis was done by the method of Taylor et al. (28). Reagents and enzymes for the reaction were obtained from Amersham Corp.

The strategy involved in the cassette replacement mutagenesis was as follows. The sequence of the Sall-PstI fragment for the I-H54A and I-H106A mutants or the AflII-SplI fragment (for all the other mutants) of pCYOF4 from candidate clones was confirmed to contain the desired codon change by direct plasmid sequencing (29) via the

3 The abbreviations used are: kb, kilobase pair(s); HPLC, high pressure liquid chromatography.

4 The designations for mutants make use of the standard one-letter abbreviations for amino acids. Thus, "I-H54A" signifies the mutant in which histidine 54 is replaced by alanine; elsewhere in some figures, it is simply expressed as "H54A."
were processed and analyzed on the Macintosh by using a software program using the subroutines kindly provided by Dr. K. Matsuura (WaveMetrics, Lake Oswego, OR). The amount of cytochrome o showed a complete loss of the enzymatic activities.

Preparation of Cytoplasmic Membranes and Purification of Mutant Enzymes—Mutant enzymes encoded by pCYO4 derivatives were expressed in ST470 by the F7 polymerase/2 promoter system (15). Cytoplasmic membranes were prepared according to the method of Yamato et al. (31) with slight modifications. Spheroplasts were disrupted by two passages through a French press (1000 kg/cm²). Total membrane vesicles were precipitated by centrifugation (140,000 × g, 1 h). Then, the membrane vesicles suspended in 3 mM sodium EDTA (pH 8.0) were subjected to isopnic sucrose density gradient centrifugation. The dialysis step was omitted. Mutant enzymes were solubilized by sucrose monolaurate (Mitsubishi-Kasei Food Co., Tokyo) and separated from all the other cytochromes present in the cytoplasmic membrane by HPLC on DEAE-5PW (Tosoh Co., Tokyo) as described previously (3). Digital output of the His codon changes were confirmed by direct plasmid sequencing, and the fragments were then replaced with the counterparts in the wild-type E. coli cytochrome bo complex.

Complementation Test for the Aerobic Growth.—The mini-F plasmids carrying the mutant genes were introduced into terminal cytochrome c oxidase-deficient strain ST2592 (W3110 Acyo::Cm' AcydKm' recA). Transformants were obtained on LB-ampicillin plates under anaerobic conditions using sealed jars (gas pak anaerobic system, BBL Microbiology Systems, Cockeysville, MD) and then allowed to grow aerobically on minimal glycerol and minimal glucose plates, indicating that substitutions of the totally conserved histidines caused a complete loss of the enzymatic activities. Possible involvements of these histidines as the ligands of the low spin and high spin hemes and copper atom could be tested by amino acid substitutions.

Spectroscopic Analyses—Measurements of the dithionite-reduced minus air-oxidized difference spectra of cytochromes at 77 K and the CO plus reduced minus reduced difference spectra at room temperature were performed with a UV-3000 dual wavelength spectrophotometer (Shimadzu Co., Kyoto) as described previously (3). Digital output of the His codon changes were confirmed by direct plasmid sequencing, and the fragments were then replaced with the counterparts in the wild-type E. coli cytochrome bo complex. Directional cloning of the fragments was accomplished by choosing the unique SfiI-PstI fragment (0.2 kb) for the His-54 and His-411 mutations and the AflI-SplI fragment (1.2 kb) for all the other subunit I mutations. Thus, we could eliminate the possibility that the phenotypes of the mutant enzymes would be obscured by any unexpected mutations elsewhere which could be introduced throughout the in vitro DNA manipulations.

RESULTS

Mutagenesis of the Conserved His Residues in Subunit I—In the primary sequences of subunit I of mitochondrial and bacterial cytochrome c oxidases, six His residues corresponding to His-106, -284, -333, -334, -419, and -421 in subunit I of the E. coli cytochrome bo complex are totally conserved (Table I and Fig. 1) and are implicated as the ligands of the prosthetic group. The other two histidines, His-54 and -411, are also conserved except fungal oxidases (39-41) and Bradyrhizobium japonicum oxidase (37), respectively. Possible involvements of these histidines as the ligands of the low spin and high spin hemes and copper atom could be tested by amino acid substitutions. Using oligonucleotide-directed site-specific mutagenesis, we have introduced single codon changes for replacement of these eight histidines with an Ala residue (Table II). His-106, -284, and -421 residues were also individually 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. Gln residues have a similar side chain volume and hydrophobicity to His residue, and Met residue may work as an alternative ligand for the hemes. For instance, Met residue works as the axial ligand of soluble cytochrome b₅₆₆ in E. coli (54) and of mitochondrial cytochrome c (55).

Nucleotide sequences of the restriction fragments containing the His codon changes were confirmed by direct plasmid sequencing, and the fragments were then replaced with the counterparts in the wild-type cytochrome c operon. Directional cloning of the fragments was accomplished by choosing the unique SfiI-PstI fragment (0.2 kb) for the His-54 and His-411 mutagenesis and the AflI-SplI fragment (1.2 kb) for all the other subunit I mutations. Thus, we could eliminate the possibility that the phenotypes of the mutant enzymes would be obscured by any unexpected mutations elsewhere which could be introduced throughout the in vitro DNA manipulations.

The in Vivo Activity of the Mutant Enzymes—The catalytic activities of the mutant enzymes were first tested by the in vivo complementation test on the ability to support the aerobic growth of the cyo cyd double deletion mutant, ST2592. In order to avoid multicopy suppression effect, each of the mutant genes was subcloned into a single copy expression vector, pMP04, and expressed aerobically in the double mutant. If the mutant enzymes encoded by the plasmids are functional, their catalytic activities would correlate to the rates of aerobic growth of the transformants on nonfermentable carbon sources. Thus anaerobic transformants of ST2592 with the pMP04 derivatives were streaked on minimal glycerol and minimal glucose plates and grown aerobically for 2 days. Only two mutants carrying I-H54A or I-H411A mutation could grow aerobically on minimal glycerol plates as the wild type depending on the oxidative phosphorylation (Fig. 3). This indicates that both His-54 and His-411 in subunit I are functionally dispensable. However, mini-F plasmids carrying all the other mutations failed to complement the defects in aerobic growth of the double mutant on the minimal glucose plates, indicating that substitutions of the totally conserved histidines caused a complete loss of the enzymatic activities.

Other Methods—Copper content was determined by atomic absorption analysis using a Perkin-Elmer 390 or Shimadzu AA-640 atomic absorption spectrophotometer. The output signals were calibrated by running standards of copper (ranging from 0.02 to 1.0 ppm). Ubiquinol-1 oxidase activities were assayed according to Kita et al. (3). Protein concentration was determined by the BCA method (32) with bovine serum albumin as a standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was done essentially by the method of Laemmli (33). Western immunoblotting was performed by the method of Towbin et al. (34) with the following modifications. Filters were blocked in 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 for 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 MgCl₂. Anti-subunit I and anti-subunit II antisera were kindly provided by Dr. K. Kita (University of Tokyo).

Chemicals—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 U. S. Biochemical Corp. Isopropyl D-galactopyranoside was from Nova Chemicals. [32P]dCTP (111 TBq/mmol) was from ICN Radiochemicals. Sep-Pak C18 cartridge was from Millipore Co. Triethylammonium bicarbonate was purchased from Wako Chemicals, Kyoto. Other chemicals were commercial products of analytical grade.


**Heme and Copper Ligands of E. coli Cytochrome bo Complex**

**Table I**

Sequence alignment of His residues in subunit I of the E. coli cytochrome bo complex with corresponding residues in subunit I of the cytochrome aa₃ complexes

Amino acid sequences aligned are: E. coli (16); thermophilic bacillus PS3 (T. PS3, (35)); Bacillus subtilis (B. subtilis (36)); Bradyrhizobium japonicum (37), Pseudomonas denitrificans (38), Saccharomyces cerevisiae (yeast) (39), Neurospora crassa (40), Aspergillus nidulans (41), Chlamydomonas reinhardtii (42), maize (43), soybean (44), Trypanosoma brucei (45), Paramecium aurelia (46), Drosophila melanogaster (fruit fly) (47), Paracentrotus lividus (48), Xenopus laevis (frog) (49), bovine (50), and human (51). The numbering refers to the E. coli sequence. Deletions are marked with —. The His residues altered in this study are marked by asterisks.

<table>
<thead>
<tr>
<th>Species</th>
<th>Codons changed</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>HHHHHHQQHHHHHHH</td>
</tr>
<tr>
<td>T. PS3</td>
<td>HGLHGLQNHHTHHLHLYA</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>HQAHHQNHHTHHLHLYA</td>
</tr>
<tr>
<td>B. japonicum</td>
<td>HHTHHTHHTHHRQHHGGHRQ</td>
</tr>
<tr>
<td>P. denitrificans</td>
<td>HHLHHQHHHHKHHHHYKE</td>
</tr>
<tr>
<td>Yeast</td>
<td>AQLHHSHHHHAHHHHGHV</td>
</tr>
<tr>
<td>N. crassa</td>
<td>AQLHHSHHHHTHHHHGH</td>
</tr>
<tr>
<td>A. nidulans</td>
<td>VAOQLHHSHHHHTHHHL</td>
</tr>
<tr>
<td>C. reinhardtii</td>
<td>HQOLHHAHHHHFHHGHHVR</td>
</tr>
<tr>
<td>Maize</td>
<td>HQIHHHTTHHHKHHGH</td>
</tr>
<tr>
<td>Soybean</td>
<td>HQLHLHTHHHHKHHG</td>
</tr>
<tr>
<td>T. brucei</td>
<td>HQFFHHSHHHHHHFFDCC</td>
</tr>
<tr>
<td>P. aureus</td>
<td>HIKHVSHHHHHHHFVG</td>
</tr>
<tr>
<td>D. melanogaster</td>
<td>HQIHTSSHHHHTHHL</td>
</tr>
<tr>
<td>P. lividus</td>
<td>HQIHHHTTHHHHHHHHHLHPT</td>
</tr>
<tr>
<td>X. laevis</td>
<td>HQIHHHTHHHHHHDHHHLHVI</td>
</tr>
<tr>
<td>Bovine</td>
<td>HQIHHHTHHHHHHHMMYN</td>
</tr>
<tr>
<td>Human</td>
<td>HHIHHTTHHHHSHHHHTYK</td>
</tr>
</tbody>
</table>

**Table II**

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

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Mutagenic oligonucleotide</th>
<th>Codon change</th>
</tr>
</thead>
<tbody>
<tr>
<td>I-H54A</td>
<td>3'-AGCGACCTCGGATTTGCGGAG-5'</td>
<td>CAT → GCT</td>
</tr>
<tr>
<td>I-H106A</td>
<td>3'-AAATTGGCGCCGGCCGACTAA-5'</td>
<td>CAC → GCC</td>
</tr>
<tr>
<td>I-H109Q</td>
<td>3'-ATGGGCCCTCCCGCAGTAGT-5'</td>
<td>CAC → CAG</td>
</tr>
<tr>
<td>I-H106M</td>
<td>3'-GGAATTGCGCTACCATATT-5'</td>
<td>CAC → ATG</td>
</tr>
<tr>
<td>I-H284A</td>
<td>3'-CGGACGCCCGGAGGGCTTCA-5'</td>
<td>CAC → GCT</td>
</tr>
<tr>
<td>I-H284Q</td>
<td>3'-GACGGCCTTGCCTTCAAAA-5'</td>
<td>CAC → CAG</td>
</tr>
<tr>
<td>I-H284M</td>
<td>3'-CGGACCCCCTGACCGCTTCAAA-5'</td>
<td>CAC → ATG</td>
</tr>
<tr>
<td>I-H333A</td>
<td>3'-GAAACCGAACGGGTGAAAGAA-5'</td>
<td>CAC → GCC</td>
</tr>
<tr>
<td>I-H334A</td>
<td>3'-ACCGACCTGGCGGAAAGATG-5'</td>
<td>CAC → GCC</td>
</tr>
<tr>
<td>I-H411A</td>
<td>3'-AGCGCAAGCCTGGTTTGCGGAC-5'</td>
<td>CAT → GCT</td>
</tr>
<tr>
<td>I-H419A</td>
<td>3'-GACTACCGCGGCAAGATTTG-5'</td>
<td>CAT → ATG</td>
</tr>
<tr>
<td>I-H421A</td>
<td>3'-GCCGGTGAGCTTGCTTACAGT-5'</td>
<td>CAT → CAG</td>
</tr>
<tr>
<td>I-H421M</td>
<td>3'-GCCGCTACTGCCAAGCTTACAGT-5'</td>
<td>CAT → ATG</td>
</tr>
</tbody>
</table>

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

In addition, since all the mutants did grow aerobically on minimal glucose plates via glycolysis, we could eliminate a possibility that these mutations induced a large structural perturbation of the enzyme complex which may alter membrane permeability. Thus, these six histidines are essential for the structure-function of the terminal oxidase, such as binding of the prosthetic groups.

**Quinol Oxidase Activity of Partially Purified Preparations**

The catalytic activities of mutant oxidases were further examined in vitro. The oxidases 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), by ion exchange chromatography using HPLC. As shown in Table III, the mutant oxidases carrying H106A, H333A, H334A, H411A, and H421A were almost completely purified from their respective cytochrome complexes.

![Fig. 3. Complementation test for the aerobic growth of the Δcyo Δcysd double mutant ST2592 with the mini-F plasmid pMF04 containing a single His to Ala mutation.](image)

A vector (pHNF2) without the insert of the cya operon was used as a control. Minimal medium plates containing 1% glucose (upper panel) and 1% glycerol (lower panel) were used for the aerobic growth at 37°C for 2 days.
H284A, H333A, H334A, H419A, and H421A substitutions lost completely the ubiquinol-1 oxidase activity. Since these mutant oxidases were also defective in the in vivo complementation test, the invariant histidines in subunit I are likely to be involved in the catalytic functions of the oxidase.

Immunological Analysis of the Mutant Enzymes—Expression levels of the mutant oxidases encoded by the multicopy plasmids were examined by Western immunoblotting analysis of the cytoplasmic membranes (Fig. 4). The amounts of polypeptides cross-reacted with rabbit polyclonal anti-subunit I and anti-subunit II antisera did not change significantly by His to Ala mutations in subunit I. These results indicate that all the mutations did not alter stability and assembly of the mutant enzymes.

Spectroscopic Analysis of the Mutant Enzymes in Cytoplasmic Membranes—The low spin heme, cytochrome b563.5, was quantitated by a peak at 563.5 nm in the dithionite-reduced minus air-oxidized difference spectra at 77 K, which is specific to the cytochrome bo complex in the aerobic respiratory chain. The high spin heme, cytochrome o, was determined by CO-reduced minus reduced difference spectra at room temperature. Cytochrome o has typical features with a peak at 416 nm and a trough at 430 nm.

Substitutions of His-54, -333, -334, and -411 did not show significant effects on the extents of both low spin and high spin hemes (Fig. 5). Thus, these histidines are unlikely to be heme ligands. Among all the His to Ala mutations, only substitutions of His-106 and His-421 completely eliminated a trough at 563.5 nm in the second-order finite spectra at 77 K (Fig. 6A), suggesting that these two invariant His residues are the axial ligands of the low spin heme.

On the other hand, the amounts of the CO-binding high spin heme were greatly reduced by substitutions of His-106, -284, and -419 (Figs. 6B and 7B). Since His-106 can be unambiguously assigned as one of the low spin heme ligands, His-284 and -419 are left as possible ligands of the high spin heme. We have also substituted His-106, -284, and -421 by Gln and Met residues and found that their spectroscopic properties are similar to the Ala mutations (data not shown).

Spectroscopic Properties of Partially Purified Preparations—Spectroscopic properties of some mutant oxidases were further examined in partially purified preparations; thus these properties would not be obscured by contributions from other cytochromes (Fig. 8). In contrast to the wild-type and H284A mutant oxidases, the H106A and H421A mutant oxidases lost completely the 563.5 nm peak as in cytoplasmic membranes. However, the absorption around 555 nm in these low spin...
These results indicate that a loss of one of the hemes alters the spectral properties of the other heme by changing the electron proximal ligand. 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 peak at around 550 nm. Conditions and procedures were as described in the legend to Fig. 5.

DISCUSSION

The E. coli cytochrome bo complex, which is encoded by the cyoABCDE operon (16), consists of five subunits and has two α-absorption peaks at 555 and 563.5 nm in the low temperature redox spectrum (3, 15). Subunit I has been identified as the cyoB gene product and found to be the binding sites for the low and high spin hemes (14, 15). Copper content was assumed to be present in the redox center in subunit I.

Resonance Raman spectroscopy (10) and EPR (11) studies have suggested that the high spin heme has at least one axial ligand (proximal ligand) and the low spin heme has two axial ligands. Salerno et al. (13) determined the crystal field parameters of the low spin heme from EPR spectra and showed a bishistidine 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.

Among 20 His residues in subunit I of the cytochrome bo complex, 6 His residues, His-106, -284, -333, -334, -419, and -411, are ligands of CuB.

### Table IV

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Copper contents of subunit I mutants</th>
<th>%</th>
<th>-Cu medium</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol/mg protein</td>
<td></td>
<td>nmol/mg protein</td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>1.34 ± 0.02</td>
<td>100</td>
<td>0.75 ± 0.00</td>
<td>100</td>
</tr>
<tr>
<td>Control</td>
<td>0.08 ± 0.00</td>
<td>0</td>
<td>0.10 ± 0.01</td>
<td>0</td>
</tr>
<tr>
<td>I-H54A</td>
<td>1.00 ± 0.02</td>
<td>76</td>
<td>0.57 ± 0.05</td>
<td>80</td>
</tr>
<tr>
<td>I-H106A</td>
<td>1.17 ± 0.02</td>
<td>122</td>
<td>0.10 ± 0.01</td>
<td>1</td>
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<tr>
<td>I-H284A</td>
<td>1.40 ± 0.14</td>
<td>122</td>
<td>0.59 ± 0.03</td>
<td>81</td>
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<tr>
<td>I-H333A</td>
<td>1.18 ± 0.03</td>
<td>96</td>
<td>0.11 ± 0.00</td>
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<td>I-H334A</td>
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<td>74</td>
<td>0.11 ± 0.01</td>
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<tr>
<td>I-H411A</td>
<td>1.05 ± 0.06</td>
<td>62</td>
<td>0.55 ± 0.00</td>
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<tr>
<td>I-H419A</td>
<td>1.25 ± 0.07</td>
<td>85</td>
<td>0.11 ± 0.00</td>
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<tr>
<td>I-H421A</td>
<td>1.11 ± 0.05</td>
<td>76</td>
<td>0.20 ± 0.02</td>
<td>22</td>
</tr>
</tbody>
</table>

* Average values from at least three determinations with ±S.D.

* Specific contents of copper were normalized by the amounts of subunit I in each membrane preparation, which were determined by densitometric analysis of the subunit I band in Western blots using a Shimadzu double-wavelength flying spot scanner CS-9000.
-421, were found to be invariant in the cytochrome aa₃ complexes of mitochondria and aerobic bacteria (Table I) and are located in the transmembrane regions II, VI, VII, and X (Fig. 1). Two other His residues, His-54 and -411, are highly conserved and located in the putative hydrophobic loop 0-I and IX-X, respectively.

Spectroscopic studies, such as magnetic circular dichroism, extended x-ray absorption fine structure, and electron nuclear double resonance, of the cytochrome aa₃ complexes indicate that heme a is liganded by two His residues (56, 57), heme a₃ by one His residue (58, 59), and CuB by three His residues (60-62). The fourth ligand of CuB is thought to form a bridge to heme a₃. The identity of the fourth ligand is still controversial (19). Since the E. coli cytochrome bo complex belongs to the heme-copper oxidase superfamily (17), at least three His residues function as the heme ligands, and the other three His residues are involved in the binding of CuB, which is electronically coupled to the high spin heme. In order to test the idea, we have carried out the oligonucleotide-directed site-specific mutagenesis of these histidines 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, we have designed our mutagenesis experiments so that phenotypes of the mutant oxidases would not be obscured by any unexpected mutations. Thus, a portion of the mutagenized fragment has been thoroughly sequenced to confirm a desired codon change (see Fig. 2) and reintroduced into the wild-type cyo operon for expression of the mutant genes. In addition, the expression of the cyo operon was tightly controlled by the T7 promoter in vector pCYOF4 during DNA manipulations, or the expression level of the mutant oxidases was mimicked to the level of the chromosomal copy by using a single copy vector which we have developed for expression of the membrane proteins.

Western immunoblotting analysis of the mutant oxidases (Fig. 4) 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. The in vivo complementation test (Fig. 3) and the in vitro quinol oxidase assay (Table III) both demonstrated that the six invariant histidines are essential for the catalytic functions of the cytochrome bo complex. The defects were further examined by optical spectroscopy and copper analysis. Among mutant oxidases, only the H106A and H421A oxidases lost the 563.5 nm peak in the low temperature redox spectra of cytoplasmic membranes (Fig. 6) and of partially purified preparations (Fig. 8). The result indicates that His-106 and His-421 are the axial ligands of the low spin heme. Preliminary EPR characterization of the H106A and H421A oxidases in cytoplasmic membranes confirmed the loss of the low spin signal. From copper analysis of mutant membranes, we found that substitutions of His-106, -333, -334, and -419 caused a complete loss of copper in the mutant oxidases. Since His-106 and -421 are unambiguously assigned to the ligands of the low spin heme, His-333, -334, and -419 are suggested to be ligands of CuB. Supplementation of CuSO₄ to the growth medium restored the defect in binding of copper atoms, suggesting that these His substitutions altered an affinity for copper atom in the CuB-binding site due to a loss of one ligand and/or perturbations of a tertiary structure of the redox center. Substitutions of His-106, -284, and -419 reduced the amount of the CO-binding high spin heme, indicating their involvement in the high spin heme/CuB binuclear center. Assignments of His-333, -334, and -419 as ligands of CuB lead us to conclude that the His-284 residue is a proximal ligand of the high spin heme. A defect of the high spin heme binding in the His-106 and -419 mutant enzymes could be due to a perturbation of the high spin heme/CuB 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 CuB-binding site (i.e. His-333 could be an alternative ligand (see Fig. 9). Alternatively, a portion of 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. We have also substituted His-106, -284, and -421 by Gln and Met residues and obtained similar results. Lemieux et al. (63) substituted seven conserved His residues in subunit I by another residue, such as Leu and Gly. All of these mutations have similar effects to the Ala mutations in this study, indicating that constraints at positions 106, 284, 333, 334, 419, and 421 in functional enzymes are limited to the His residue.

Thus, we conclude from the site-directed mutagenesis studies that 1) His-106 and -421 are the axial ligands of the low spin heme and copper analysis. Among mutant oxidases, only the H106A and H421A oxidases lost the 563.5 nm peak in the low spin heme.
spin heme; 2) His-284 functions as the proximal ligand of the high spin heme; and 3) His-333, -334, and -419 are ligands of CuB. We suspect that these six invariant histidines also serve as the ligands in the cytochrome c oxidases.

Furthermore, identifications of the prosthetic groups in subunit I lead us to propose a helical wheel projection model (64) of the redox center in the cytochrome bo complex (Fig. 9). The redox center is provided by at least four putative transmembrane helices, II, VI, VII, and X, in subunit I which all carry the invariant 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. Four-a-helix bundle is one of the common packing motifs found in protein structures with a wide range of functions (67). In the bacterial photosynthetic reaction center, two helices of two distinct subunits form this arrangement (68). However, the redox center of the cytochrome bo complex is not the anti-parallel helix bundle which is an energetically favorable arrangement (69). Thus, it is possible that 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-100 and -421 are so crucial in binding of the low spin heme and CuB, they may also serve as the key residues which drive a bundling of the putative transmembrane helices to form a redox center in subunit I through association of the transmembrane region II and X via the heme ligation (see Fig. 9).

In the model, electron flow from ubiquinol-8 to the high spin heme/CuB 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; however, 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 oxidoforms, as in a light-driven proton pump, bacteriorhodopsin (70, 71). Invariant 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 the primary electron transfer (72, 73). It is possible that these residues may be involved in ligand binding by stabilizing the ligand molecules with their bulky aromatic side chains or by providing a binding pocket within the bundle of helices (74).

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