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Effects of Subunit I Mutations on Redox-Linked Conformational Changes of the *Escherichia coli bo*-type Ubiquinol Oxidase Revealed by Fourier-Transform Infrared Spectroscopy[1]

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Cytochrome *bo* is the heme-copper terminal ubiquinol oxidase in the aerobic respiratory chain of *Escherichia coli*, and functions as a redox-coupled proton pump. As an extension to our mutagenesis and Fourier-transform infrared studies on ion pumps, we examined the effects of subunit I mutations on redox-linked protein structural changes in cytochrome *bo*. Upon photo-reduction in the presence of riboflavin, Y288F and H333A showed profound effects in their peptide backbone vibrations (amide-I and amide-II), probably due to the loss of Cu_B or replacement of high-spin heme *o* with heme B. In the frequency region of protonated carboxylic C=O stretching vibrations, negative 1,743 cm^{-1} and positive 1,720 cm^{-1} bands were observed in the wild-type; the former shifted to 1,741 cm^{-1} in E286D but not in other mutants including D135N. This suggests that Glu286 in the D-channel is protonated in the air-oxidized state and undergoes hydrogen bonding changes upon reduction of the redox metal centers. Two pairs of band shifts at 2,566 (+)/2,574 (−) and 2,546 (+)/2,556 (−) cm^{-1} in all mutants indicate that two cysteine residues not in the vicinity of the metal centers undergo redox-linked hydrogen bonding changes. Cyanide had no effect on the protein structural changes because of the rigid local protein structure around the binuclear center or the presence of a ligand(s) at the binuclear center, and was released from the binuclear center upon reduction. This study establishes that cytochrome *bo* undergoes unique redox-linked protein structural changes. Localization and time-resolved analysis of the structural changes during dioxygen reduction will facilitate understanding of the molecular mechanism of redox-coupled proton pumping at the atomic level.

Key words: FTIR, ubiquinol oxidase, cytochrome *bo*, redox difference spectra, protein backbone change.

Cytochrome *bo* is a four-subunit ubiquinol oxidase in the aerobic respiratory chain of *Escherichia coli* and is predominantly expressed under highly aerated growth conditions (*1, 2* for reviews). It catalyzes the two-electron oxidation of ubiquinol-8 at the periplasmic side of the cytoplasmic membrane and the four-electron reduction of dioxygen at the cytoplasmic side. Accordingly, four scalar (or chemical) protons are apparently translocated from the cytoplasm to the periplasm, generating an electrochemical proton gradient across the membrane. In addition, cytochrome *bo* can vectorially translocate four other protons per dioxygen reduction by a pump mechanism.

Subunit I binds all the redox metal centers, low-spin heme *b*, high-spin heme *o*, and Cu_B, and the heme *o*-Cu_B binuclear center serves as a reaction center for dioxygen reduction (*1, 2*). Mutagenesis and X-ray crystallographic studies on heme-copper terminal oxidases suggest that the D- and K-channels in subunit I are operative in redox-coupled proton pumping (*3, 4*). Photoaffinity cross-linking (*5, 6*) and mutagenesis studies (*7, 8*) have shown that the low-affinity quinol-oxidation site (QL) (*9*) resides in the C-terminal hydrophilic domain of subunit II. The high-affinity quinone binding site (QH) mediates intramolecular electron transfer between QL and heme *b* (*10-13*). Subunits III and IV are not involved in catalytic functions but are required for assembly of the redox metal centers in subunit I (*14-16*).

Our previous site-directed mutagenesis studies on the conserved residues in subunit I showed that not only metal ligand histidines (*17-19*) but also other charged amino acid

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Abbreviations: FTIR, Fourier-transform infrared; QH, the high-affinity quinone binding site; QL, the low-affinity quinol oxidation site.

residues in the putative proton channels (*20, 21*) are crucial for the assembly of the redox metal centers. They are likely to be involved in the vectorial translocation of pumped and/or chemical protons (*3, 4, 22-24*); thus an examination of changes in the protonation and hydrogen bonding states of the conserved charged amino residues is essential for understanding the pathways and sequences of proton translocation across the oxidase protein. In addition, the redox metal centers must play an important role in redox-linked protein structural changes to drive the dioxygen reduction cycle and proton pumping mechanism.

Fourier-transform infrared (FTIR) spectroscopy has been used to probe the molecular environment of the active center of terminal oxidases using respiratory inhibitors such as CO and cyanide (*25, 26*). Recently, the application of a spectro-electrochemical cell (*27, 28*) and the introduction of photo-activatable electron donors (*29-31*) enabled us to examine protein structural changes that occur upon reduction of the redox metal centers of respiratory terminal oxidases. Alternatively, the photodissociation of CO from reduced cytochrome *bo* at 80 K revealed local structural changes surrounding the heme-copper binuclear center (*32*).

In the present article, we applied the method of Lübben and Gerwert (*29*) to *E. coli* cytochrome *bo* and examined the effects of subunit I mutations and cyanide binding on redox-linked protein structural changes by FTIR spectroscopy. Asp135Asn, Glu286Asp, and Glu286Gln (*20*) could identify structural changes in the carboxylic side-chains at Asp135 and Glu286 in the D-channel when the protonated carboxylic CO-stretching vibrations were monitored, and Tyr288Phe (*20*) and His333Ala (*17*) could probe the possible roles of Cu_B and high-spin heme *o* in redox-linked protein structural changes in the amide-I and -II regions, and the protonated carboxylic CO- and cysteine SH-stretching vibration regions.

MATERIALS AND METHODS

Purification of Cytochrome bo—Wild-type and His333-Ala mutant enzymes were isolated from the overproducing strains GO103/pHN3795-1 (*cyo+Δcyd/cyo+*Apr) and ST4533/pHN3795-H333A (*Δcyo cyd+/cyo−*Apr), respectively, as described previously (*26, 33*). Asp135Asn, Glu286Asp, Glu286Gln, and Tyr288Phe were purified from ST4676 (*Δcyo cyd*) harboring the derivatives of a single copy plasmid pMFO9 (*cyo+ −*Apr) (*20, 34*).

FTIR and Visible Spectroscopies—Spectroscopic analyses were carried out as described previously (*31*). Photoreduction of the air-oxidized enzymes was conducted at room temperature (25°C) in the presence of riboflavin as a photoactivatable electron donor (*29*).

RESULTS AND DISCUSSION

Visible Absorption Changes upon Photoreduction—Photo-reduced *minus* air-oxidized redox difference spectra (Fig. 1) indicate that 2 min illumination in the presence of riboflavin results in the reduction of both low-spin and high-spin hemes of subunit I mutants except Glu286Gln to a level similar to the wild-type enzyme (88%). It was quite difficult to reduce Glu286Gln under the present conditions. During illumination, dissolved dioxygen in the sample must be exhausted by repeated turnover of the oxidase reaction. Redox difference spectra of the wild-type, Asp135Asn, and Glu286Asp enzymes show absorption maxima at 428, 529, and 561 nm, characteristic of the fully reduced cytochrome *bo*. It should be noted that due to the replacement of heme O at the high-spin heme binding site with heme B (*20, 21*), the Soret peak of air-oxidized Tyr288Phe shifted from 408 nm as seen in the wild-type to 413 nm (data not shown), and a trough in the difference spectrum shifted from 400 to 409 nm (Fig. 1). In contrast, other mutants showed only slight changes in the Soret region.

Infrared Spectral Changes in the 1,800-1,300 cm^{-1} Region—Photo-reduced *minus* air-oxidized FTIR difference spectra of Asp135Asn and Glu286Asp are similar to that of the wild-type which shows positive bands at 1,663, 1,653, and 1,635 cm^{-1} (Fig. 2, a-c), frequencies typical of α_{II}-helix, α_I-helix, and β-sheet structures, respectively (*35*). In contrast, marked differences were observed in the amide-I region (1,700-1,600 cm^{-1}) of Tyr288Phe and His333Ala (Fig. 2, d and e). The positive 1,653-cm^{-1} band is almost absent in His333Ala, while a sharp negative peak is present at 1,668 cm^{-1} in Tyr288Phe and His333Ala. Regular (α_I-helix) and distorted (α_{II}-helix) helices possess amide-I frequencies at 1,650-1,660 cm^{-1} and 1,660-1,670 cm^{-1}, respectively (*35*). The results on Tyr288Phe and His333Ala thus imply an alteration in the local protein structure around the heme *o*-Cu_B binuclear center, particularly in the helices. In the amide-II region, two positive peaks at 1,545 and 1,537 cm^{-1} in the wild-type, frequencies

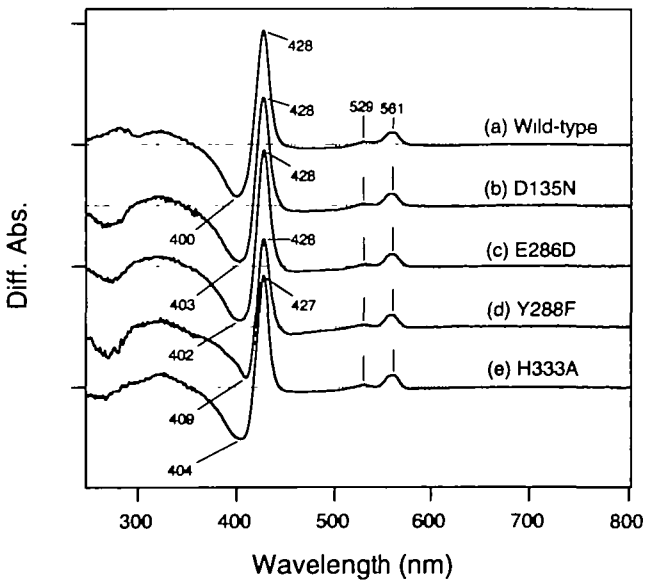

Fig. 1. **Photo-reduced *minus* air-oxidized visible absorption difference spectra of subunit I mutants of cytochrome *bo*.** Five microliters of enzyme (wild-type, 0.275 mM; D135N, 0.12 mM; E286D, 0.15 mM; Y288F, 0.09 mM; H333A, 0.24 mM) in 50 mM Tris-HCl (pH 7.4) containing 0.1% sucrose monolaurate (Mitsubishi-Kagaku Foods, Tokyo), 0.25 mM riboflavin, and 50 mM sodium EDTA was partially dehydrated *in vacuo* on a BaF_2 window, and covered with another window with a 6-mm diameter aperture (*31*). Spectra were obtained at room temperature (25°C) with a dispersive Shimadzu MPS-2000 spectrometer before and after 2 min illumination with a tungsten-halogen lamp (*31*). Spectral changes were normalized to the absorbance at 560 nm of the wild-type enzyme. One division of the *Y*-axis corresponds to 0.2 absorbance unit.

typical of α-helices, are preserved in Asp135Asn and Glu286Asp (Fig. 2, a-c). A slight shift in the positive 1,537-cm^{-1} band to 1,540 cm^{-1} in Tyr288Phe and its absence in His333Ala provide further support for structural perturbations of helices in these two mutants.

His284 and Tyr288 are both present in helix VI (*36*) where a regular α-helical structure is perturbed by Pro285 (*2, 4*). Tyr288Phe undergoes loss of the Cu_B (*20*) that is ligated to His284, His333, and His334 (*17-19, 37, 38*), a covalent bond between Tyr288 and His284 (*39, 40*), and a hydrogen bond between the OH group of Tyr288 and the OH group of hydroxyethylfarnesyl chain of the high-spin heme *o* (*41*) due to replacement with heme B (*20*). Accordingly, Tyr288Phe causes a direct alteration of the α-helical structure of helix VI and also that of helix X through His419, the high-spin heme ligand (*18, 19, 42*).

His333 and His334 are located in loop VII/VIII (*4, 39, 41*) and the imidazole ring of His333 can adopt two alternative conformations upon ligand binding to the binuclear center (*4*). His333Ala lacks Cu_B completely but has no significant effect on the bound hemes (*17, 33*). Thus His333Ala is unlikely to affect helices VII and X but might alter the structure of helix VI through His284, which has lost the bound Cu_B. In conclusion, binuclear center mutations have large effects on redox-linked protein structural changes brought about by the loss and/or substitution of metal centers.

In the protonated carboxylic C=O stretching region, the wild-type enzyme shows a redox-linked hydrogen-bonding change from 1,743 cm^{-1} to 1,720 cm^{-1} that is attributable to one Asp or Glu residue (Fig. 3, dotted line). The frequency of the oxidized form is comparable to previous reports (1,745 cm^{-1}), whereas that of the reduced form is considerably different from the 1,735 cm^{-1} reported by Lübben and coworkers (*29, 30*). Changes were also observed in Asp135Asn, Tyr288Phe, and His333Ala, however, a negative peak in air-oxidized Glu286Asp clearly shifted to 1,741 cm^{-1} with reduced intensity. It is thus concluded that this band contains the C=O stretching vibration of Glu286. The effect of Glu286 mutations on infrared changes is consistent with our recent report that such mutations affect the binuclear center structure of the air-oxidized form (*34*). In general, the protonated carboxylic C=O stretch appears in the 1,700-1,780 cm^{-1} region, and the frequency shifts lower if the hydrogen bonding is strengthened. The frequency shift from 1,743 cm^{-1} to 1,720 cm^{-1} originates from the strengthened hydrogen bonding of Glu286 upon the reduction of metalcenters.

Hellwig *et al.* showed that redox-induced infrared changes at 1,746(−) and 1,734(+) cm^{-1} in *aa*3-type cytochrome *c* oxidase from *Paracoccus denitrificans* were associated with electron transfer from/to low-spin heme *a* and disappeared in Glu278Gln and Glu278Asp but were unaffected in Asp124Asn and Asp399Asn (*28*). During the revision of this manuscript, Lübben *et al.* reported that the 1,745/1,735 cm^{-1} signals were lost in Glu286Asp and Glu286Gln mutants of *E. coli* cytochrome *bo* but retained in Asp135Glu and Asp407Asn mutants (*30*). For an unknown reason, they were unable to express enough Asp135Asn for purification. Although the discrepancy in the frequencies reported in this study and that of Lübben *et al.* (*30*) remains to be explained, it can be concluded that the side-chain of the conserved Glu residue in the middle of helix VI is protonated in the air-oxidized state and undergoes a hydrogen bonding change upon reduction of the metal centers, because a membrane-buried carboxylic residue is

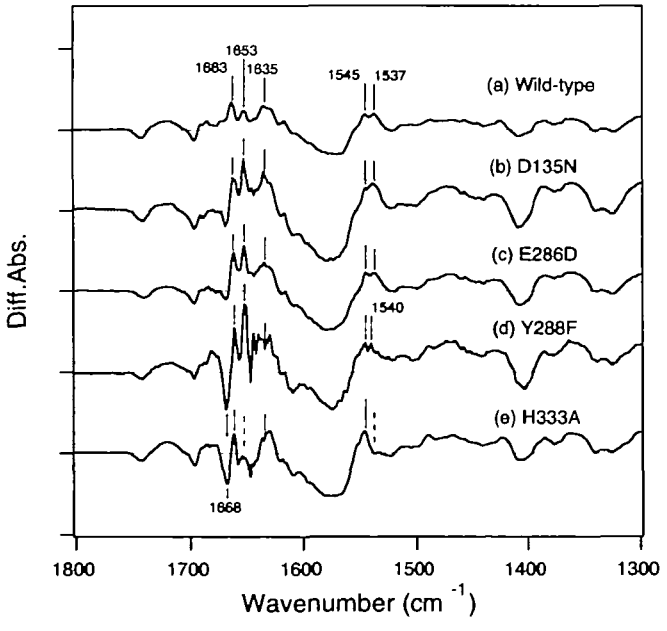

Fig. 2. **Photo-reduced *minus* air-oxidized infrared difference spectra of subunit I mutants in the amide-I and amide-II (1,800-1,300 cm^{-1}) region.** Two hundred fifty-six interferograms at 2 cm^{-1} resolution were recorded at room temperature (25°C) with a Bio-Rad FTS-40 FTIR spectrometer (*31*). One division of the Y-axis corresponds to 0.004 absorbance unit. Other details are the same as in the legend to Fig. 1.

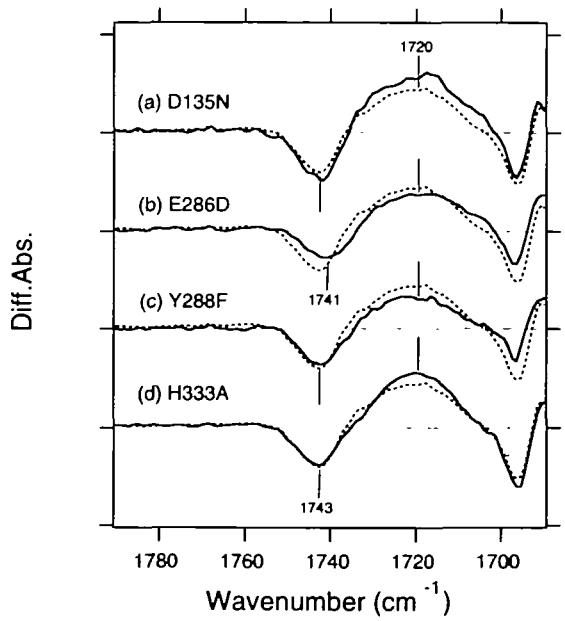

Fig. 3. **Photo-reduced *minus* air-oxidized infrared difference spectra of subunit I mutants in the protonated CO stretching frequency region.** The dotted line in each panel indicates the wild-type enzyme. One division of the *Y*-axis corresponds to 0.001 absorbance unit. Other details are the same as in the legend to Fig. 2.

expected to be protonated (*4*), and the frequencies of this band pair in fact show a downshift when the solvent is changed to D_2O (*27-30*). It should be noted that mutations at the heme-copper binuclear center (Tyr288Phe and His333Ala) and at the ends of the proton channel (Asp-135Asn, Asp135Glu, and Asp407Asn) do not affect the redox-induced protein structural changes at Glu286.

Thus FTIR spectroscopy is demonstrated to be a powerful tool with which to probe the protonation and hydrogen bonding states of carboxylic residues in the proton channel, since X-ray crystallographic studies on cytochrome *c* oxidases fail to detect such a redox-induced microenvironmental change (*4, 39-41*). Upon reduction of the metal centers, two chemical protons are delivered to the heme-copper binuclear center through the K-channel to compensate for the two additional negative charges (*3, 43*). However, the side-chain of Glu286 is located on the side opposite to His284, which ligates Cu_B at the end of the K-channel, and protrudes into the D-channel. Redox-linked hydrogen bonding changes at Glu286 cannot be involved in the translocation of the initial two chemical protons, but may be related to the proton release to the periplasm through the D-channel during subsequent steps of dioxygen reduction (*3, 44*).

Infrared Spectral Changes in the SH Stretching Region—We were able to extend infrared studies on redox-linked protein structural changes of terminal oxidases (*27-30*) to 2,620 cm^{-1} (*31*). Upon hydrogen bonding change, the SH stretching vibration of cysteine residues show a small frequency shift in the 2,580-2,525 cm^{-1} region (*45-47*). We found two positive and negative peaks at 2,566 and 2,546 cm^{-1}, and 2,574 and 2,556 cm^{-1}, respectively, in the wild-type and mutant enzymes other than Tyr288Phe (Fig. 4). This indicates that one SH group corresponding to the 2,574 cm^{-1} band forms a new hydrogen bond upon reduction (*46, 47*), and that the other corresponding to the 2,556 cm^{-1} band undergoes stronger hydrogen bonding. The large decrease in the signal intensity for Tyr288Phe may be related to the replacement of heme O at the high-spin heme binding site (*20*), a lack of a covalent bond between the side chains of Tyr288 and His284 (*39, 40*), or a direct proton donor to the binuclear center (*3, 40*).

Subunit I of cytochrome *bo* binds all the redox metal centers (*17-19, 37, 38, 42*) and contains four cysteines at the cytoplasmic end of helices V (Cys234), X (Cys432), and XII (Cys511) and the middle of helix VII (Cys322) (*36*). Although all these residues are functionally dispensable (*32*), redox-linked changes in the cysteine SH stretching vibrations may represent protein structural changes associated with the opening of the proton channel(s) or the delivery of two chemical protons (*3, 43*). These results indicate that at least two cysteines undergo hydrogen bonding changes between the oxidized and reduced states, and that they are not located in the vicinity of the binuclear center. The assignment of such cysteine residues by site-directed mutagenesis will lead to a better understanding of redox-linked protein structural changes in cytochrome *bo*.

Infrared Spectral Changes in the CN Stretching Region—To localize redox-linked protein structural changes, the effect of cyanide binding to the binuclear metal center was examined. The air-oxidized wild-type enzyme exhibits an absorption maximum at 414 nm in the presence of KCN, and 92% of the cyanide-inhibited enzyme is photo-reduced upon 2 min illumination resulting in absorption maxima identical to those of the control enzyme. In the 2,180-2,025 cm^{-1} region of the photo-reduced *minus* air-oxidized FTIR difference spectrum, we found a negative band at 2,146 cm^{-1} assignable to the CN stretching vibration of the Fe^{3+}-C=N-Cu_B^{2+} bridging structure (*26*) and a positive band at 2,080 cm^{-1} with a broad shoulder on the higher frequency side (Fig. 5). It has been reported that free HCN and CN^- (deprotonated form) have CN stretching vibrations at

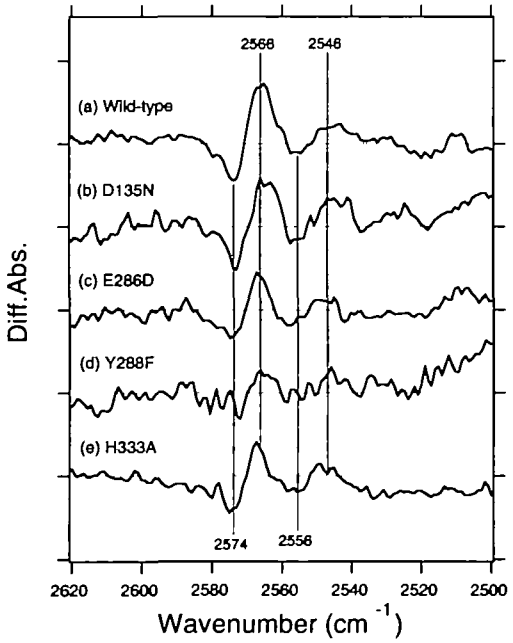

Fig. 4. **Photo-reduced *minus* air-oxidized infrared difference spectra of subunit I mutants in the cysteine SH stretching frequency (2,620-2,500 cm^{-1}) region.** One division of the *Y*-axis corresponds to 0.0001 absorbance unit. Other details are the same as in the legend to Fig. 2.

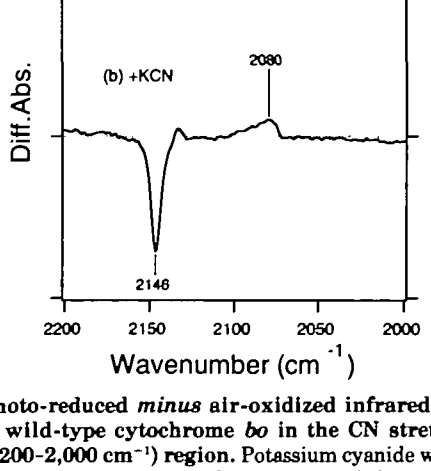

Fig. 5. **Photo-reduced *minus* air-oxidized infrared difference spectra of wild-type cytochrome *bo* in the CN stretching frequency (2,200-2,000 cm^{-1}) region.** Potassium cyanide was added to the enzyme solution at 1.4 mM. One division of the *Y*-axis corresponds to 0.0005 absorbance unit. Other details are the same as in the legend to Fig. 2.

2,093 and 2,079 cm^{-1}, respectively (*48*). Thus, the bound cyanide was released from the heme-copper binuclear center upon reduction of the binuclear metal center. This is consistent with the fact that the reduced form has the same visible absorption spectrum regardless of the presence or absence of cyanide. The concentration of cyanide in the sample (*i.e.*, 1.4 mM before partial dehydration) is much larger than the K_i value (0.01 mM) for ubiquinol-1 oxidation (*49*), but not enough to hold cyanide on the reduced high-spin heme *o* as on the oxidized form, because the K_d value for the reduced form is 7 (*50*) or 12 mM (M. Tsubaki, personal communication). The K_d value for the reduced form is different from those of *aa*3-type cytochrome *c* oxidase and *ba*3-type ubiquinol oxidase [K_d, 0.1-0.55 mM (*51*)] where the heme *a*3-Cu_B center serves as the ligand binding site.

This study reveals that cyanide bound to the binuclear center does not affect redox-linked changes in the amide-I and -II regions, the protonated carboxylic C=O and cysteine SH stretching vibrations before and after photoreduction (data not shown). The binuclear center appears to contain a ligand in the air-oxidized state, perhaps a water molecule and a hydroxyl ion (*38*) or a peroxide (*40*) as found in the crystal structures of cytochrome *c* oxidases. Alternatively, the protein structure around the binuclear center is so rigid that cyanide binding does not affect the redox-linked protein structural changes.

The molecular mechanism of vectorial proton translocation has been studied extensively for light-driven proton pump bacteriorhodopsin by site-directed mutagenesis (*52*) and FTIR spectroscopy (*53-55*). We extended our mutagenesis and FTIR studies on ion pumps, and found that hydrogen bonding changes at Glu286 and protein backbone changes around the metal centers are associated with reduction of the metal centers of *E. coli* cytochrome *bo*. We recently found that similar redox-linked protein structural changes take place in cytochrome *bd* (*31*), an alternative ubiquinol oxidase, under microaerobic growth conditions, although it cannot pump protons (*1*). Therefore, systematic site-directed mutagenesis to localize such changes and time-resolved FTIR studies during dioxygen reduction are essential for understanding the molecular mechanism of the vectorial translocation of chemical and pumped protons in heme-copper terminal oxidases.

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