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Fourier-Transform Infrared Studies on Conformation Changes in *bd*-Type Ubiquinol Oxidase from *Escherichia coli* upon Photoreduction of the Redox Metal Centers[1]

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Cytochrome *bd* is a two-subunit ubiquinol oxidase in the aerobic respiratory chain of *Escherichia coli* that does not belong to the heme-copper terminal oxidase superfamily. To explore unique protein structural changes associated with the reduction of the redox metal centers, we carried out Fourier-transform infrared and visible spectroscopic studies on cytochrome *bd*. For infrared measurements of a partially dehydrated thin sample solution, the air-oxidized enzyme was fully reduced by the intermolecular electron transfer of photo-excited riboflavin in the absence and presence of KCN, and redox difference spectra were calculated. Upon reduction, the bound cyanide was released from the heme b_{595}-heme *d* binuclear center but remained in a protein pocket as a deprotonated form. Reduction of heme b_{558}, heme b_{595}, and heme *d* resulted in large changes in amide-I and protonated carboxylic CO-stretching vibrations and also a small change in the cysteine SH-stretching vibration. The location of the redox metal centers and the effects of cyanide suggest that these protein structural changes occur at the heme-binding pockets near the protein surface. Systematic site-directed mutagenesis and time-resolved FTIR studies on cytochrome *bd* will facilitate an understanding of the unique molecular mechanisms for dioxygen reduction and delivery of chemical protons to the active center at the atomic level.

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

Cytochrome *bd* is a two-subunit ubiquinol oxidase in the aerobic respiratory chain of *Escherichia coli* under microaerobic growth conditions (Fig. 1; *1* for a recent review). Cytochrome *bd* catalyzes the two-electron oxidation of ubiquinol-8 on the periplasmic side of the cytoplasmic membrane and the four-electron reduction of dioxygen on the cytoplasmic side. Accordingly, four scalar (chemical) protons are apparently translocated from the cytoplasm to the periplasm, generating an electrochemical proton gradient across the membrane. In contrast, cytochrome *bo*, an alternative four-subunit oxidase under highly aerated growth conditions, vectorially translocates four different protons by a pump mechanism.

On the basis of the spectroscopic and ligand binding properties of cytochrome *bd*, three distinct redox metal centers have been identified as hemes b_{558}, b_{595}, and *d* (*1*). Heme b_{558} is a *hexa*-coordinated low-spin heme exhibiting absorption peaks at 562, 532, and 428 nm in the reduced state, and is a site of primary electron input from quinols (*2-4*). Heme b_{595} is a *penta*-coordinated high-spin heme that shows peaks at 595, 562, and 442 nm in the reduced state, and serves as a low-affinity ligand binding site (*2-6*). Heme *d* is a dioxygen reduction site and has an absorption maximum at 628 nm in the fully reduced state and at 647 nm in the air-oxidized state (*2-4, 7*); ferric heme *d* shows no significant absorbance around 650 nm (*8*). In cytochrome *bd* in the air-oxidized, resting state, heme *d* exists in a mainly one-electron reduced, oxygenated form [Fe(II)-O_2], due to its extremely high affinity for dioxygen (*9-11*), and partially in oxoferryl (*12*) and cyanide-sensitive ferric low-spin (*13*) forms. Spectroscopic studies indicate that hemes b_{595} and *d* form a heme-heme binuclear center (*6, 14, 15*).

The molecular mechanisms of electron transfer, dioxygen reduction, and proton transfer in respiratory terminal oxidases have attracted great interest. Extensive molecular biological and physicochemical studies have been carried out on cytochrome *bo* to probe redox-driven proton pumping by the heme-copper terminal oxidases (*1*). The determination of the crystal structures of bacterial (*16, 17*) and bovine (*18, 19*) cytochrome *c* oxidases, homologues of cytochrome *bo*, provided the structural basis for understanding the mechanisms at the atomic level. In contrast,

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Abbreviation: FTIR, Fourier-transform infrared.

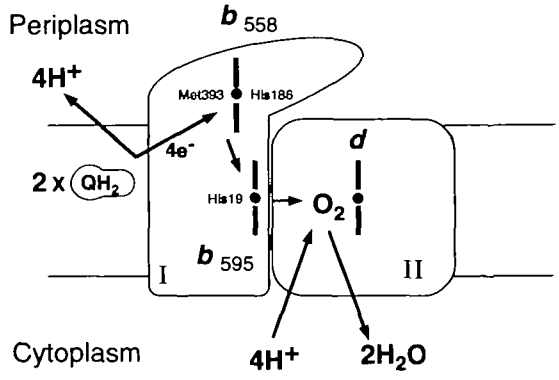

Fig. 1. **Schematic model of cytochrome *bd* from *E. coli*.**

limited structural information is available for cytochrome *bd* that belongs to a distinct terminal oxidase family.

Among various spectroscopic techniques, Fourier-transform infrared (FTIR) spectroscopy can probe the molecular environment of the active center of terminal oxidases by using π anions such as CO, CN^-, or azide (*14, 15, 20, 21*). Recently, the application of a specially designed spectro-electrochemical cell (*22, 23*) and the introduction of riboflavin as a photo-activatable electron donor (*24*) enabled the detection of protein structural changes upon the reduction of cytochrome *bo* from *E. coli* and cytochrome *c* oxidases from *Rhodobacter sphaeroides* and *Paracoccus denitrificans*. Also, the photodissociation of carbon monoxide from the reduced enzyme revealed local structural changes surrounding the active center of the *E. coli* cytochrome *bo* (*25*). These technical improvements in FTIR spectroscopy should facilitate investigations of the structure-function relationships of oxidases in great detail, as has been successfully shown for retinal proteins (*26, 27*).

In the present article, we applied FTIR and visible spectroscopies to *E. coli* cytochrome *bd* and examined protein structural changes upon photoreduction of the air-oxidized enzyme in the presence and absence of cyanide. We found that the release of cyanide from heme *d* and changes in the amide I and protonated carboxylic CO-stretching regions are associated with the full reduction of the redox metal centers of cytochrome *bd*.

MATERIALS AND METHODS

Preparation of Enzyme—Cytochrome *bd* was purified from the overproducing strain GR84N/pNG2 (*cyo+ cydA2/cyd+*; *28*), a generous gift from R.B. Gennis, as described previously (*29*), and was stored at −80°C in 50 mM sodium phosphate (pH 7.4) containing 0.1% sucrose monolaurate (SML; Mitsubishi-Kagaku Foods, Tokyo). The concentration of the enzyme was calculated from the heme B content determined by the pyridine ferrohemochromogen method (*29*). Samples for spectroscopic analysis were prepared essentially according to Lübben and Gerwert (*24*). Five microliters of reaction mixture containing 0.254 mM air-oxidized cytochrome *bd*, 0.25 mM riboflavin, 50 mM sodium EDTA, 50 mM sodium phosphate (pH 7.4), and 0.1% SML with or without 25 mM KCN was placed on a BaF_2 window and partially concentrated in a vacuum dessiccator. A high water content is not suitable for infrared spectroscopy, while too little water prohibits the efficient electron transfer reaction between riboflavin and the oxidase. The hydrated enzyme/detergent paste was then sandwiched by another BaF_2 window, and covered by a 6 mm diameter aperture. Once the oxidase was mixed with riboflavin, all procedures were carried out under dim red light conditions.

Visible and FTIR Spectroscopies—Visible absorption spectra were measured with a dispersive Shimadzu MPS-2000 spectrometer. FTIR spectra were recorded with a Bio-Rad FTS-40 spectrometer (*30, 31*). Oxford cryostats (DN-1704) were used in both the visible and FTIR spectrometers, and the sample was attached to the sample holder for the cryostat. The experiments were conducted according to the following scheme; (a) measurement of the visible spectrum of the air-oxidized form, (b) measurement of the FTIR spectrum of the air-oxidized form, (c) illumination with a white light from a tungsten-halogen lamp for 2 min for full (photo)reduction, (d) measurement of the FTIR spectrum of the fully reduced form, and (e) measurement of the visible spectrum of the fully reduced form. The reduced *minus* air-oxidized difference spectra were calculated by (e) − (a) and (d) − (b) in the visible and infrared regions, respectively. For the FTIR spectroscopy, the 256 interferograms at 2 cm^{-1} resolution were recorded and converted to absorption spectrum according to a reference interferogram recorded in the absence of the sample. The spectral difference before and after irradiation was compared with the base line as the difference between the two spectra without intervening irradiation, and, if necessary, the base line was subtracted from the data. All experiments were conducted at room temperature (25°C).

RESULTS AND DISCUSSION

Visible Absorption Changes upon Photoreduction of Cytochrome bd—Figure 2 shows the visible absorption spectra of cytochrome *bd* before and after photoreduction. Riboflavin has two peaks at 373 and 445 nm with molar extinction coefficients of 10,600 and 12,500, respectively (*32*). For the numerical estimation below, the spectral contribution of riboflavin in Fig. 2 was subtracted, although it is considerably smaller than the Soret band of cytochrome *bd* (*15*).

In the absence of KCN, the air-oxidized form exhibits the Soret peak at 411 nm and the α peaks of heme *d* at 643 and 680 nm (Fig. 2a, dotted line). The latter two peaks are ascribable to the oxygenated (Fe^{2+}-O_2) and oxoferryl (Fe^{4+}=O) forms of heme *d*, respectively. Upon illumination, absorption maxima characteristic of the fully reduced form appear at 427, 529, 560, and 628 nm (solid line). The difference spectrum shows negative peaks at 400, 648, and 680 nm and positive peaks at 429, 529, 560, and 628 nm (Fig. 2c, solid line). These data indicate that the photoexcitation of riboflavin causes an intermolecular electron transfer from riboflavin to cytochrome *bd*, as reported for the heme-copper terminal oxidases *bo*-type ubiquinol oxidase from *E. coli* and *aa3*-type cytochrome *c* oxidase from *Rb. spaheroides* (*24*). At the steady state, dissolved dioxygen in the sample must be exhausted by repeated turnover of the oxidase reaction. On the basis of the molar extinction coefficients of the air-oxidized and fully reduced forms, 223,000 at 414 nm and 303,000 at 429 nm, respec-

tively (*15*), the proportion of the reduced form was estimated to be 95%.

In the presence of KCN, the Soret band of the air-oxidized, CN^- inhibited form shows a broader peak at 407 nm and heme *d* exhibits no significant features around 650 nm (Fig. 2b, dotted line). Upon illumination, the reduced form (solid line) shows absorption maxima identical to those in the absence of KCN. The difference spectrum is also similar to that in the absence of KCN (Fig. 2c, dotted line). In the Soret region, heme $b_{558}{}^{2+}$ shifts only slightly to 428 nm and heme $b_{595}{}^{2+}$ loses some intensity at 438 nm. The loss of negative features around 650 nm indicates that heme *d* is in a CN^- bound form. On the basis of the molar extinction coefficients of the air-oxidized, CN^- bound and fully reduced forms, 185,000 at 416 nm and 303,000 at 429 nm, respectively (*15*), 92% of the enzyme is estimated to be in the reduced form.

Infrared Absorption Changes upon Photoreduction in the CN Stretching Region—Conformational changes in the protein moiety upon reduction of the redox metal centers in terminal oxidases can be studied by FTIR spectroscopy (*22-24*). The reduced *minus* oxidized FTIR difference spectra of cytochrome *bd* were measured in the absence and presence of KCN to probe structural changes localized at the active center. Then, on the basis of visible absorption changes (Fig. 2), the FTIR spectra were normalized so that the signals coming from the same molar amount (9.80×10^{-7} M) of enzyme could be quantitatively compared.

Changes in the CN stretching vibration of cyanide were studied in the 2,180-2,025 cm^{-1} region. As shown in Fig. 3a, there is no contribution from the protein. In the presence of KCN (Fig. 3b), photoreduction causes a negative band at 2,160 cm^{-1} assignable to the CN stretching vibration of the heme $d(Fe^{3+})$-C≡N-heme $b_{595}(Fe^{3+})$ bridging structure (*15*) to change to a positive peak at 2,084 cm^{-1}. The CN stretching frequencies of free HCN and CN^- are reported to be 2,093 and 2,079 cm^{-1}, respectively (*33*); thus the full reduction of cytochrome *bd* can release bound cyanide from the heme-heme binuclear center but the released cyanide in a deprotonated form remains within the

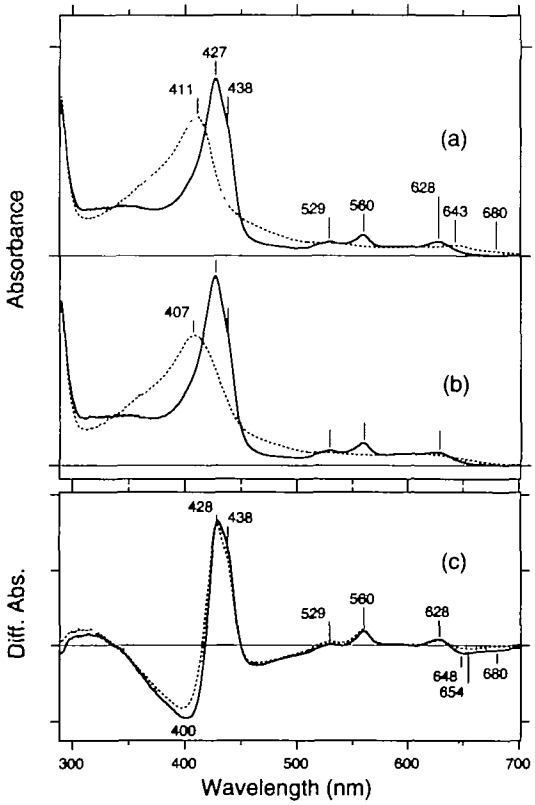

Fig. 2. **Visible absorption spectra of cytochrome *bd* in the absence (a) and presence (b) of KCN.** Dotted and solid lines represent the spectra of the air-oxidized, O_2-bound and photo-reduced forms, respectively. One division of the Y-axis corresponds to 0.35 absorbance unit. (c) The difference spectra formed by subtracting the before illumination spectrum in the absence [solid line; from (a)] and presence [dotted line; from (b)] of KCN. One division of the Y-axis corresponds to 0.1 absorbance unit.

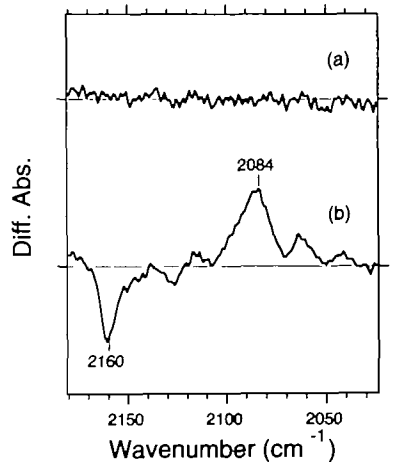

Fig. 3. **Infrared spectral changes in the CN stretching frequency region (2,180-2,025 cm^{-1}) produced by the photoreduction of air-oxidized cytochrome *bd* in the absence (a) and presence (b) of KCN.** These spectral changes in the infrared region correspond to the visible changes in Fig. 2. One division of the Y-axis corresponds to 0.0002 absorbance unit.

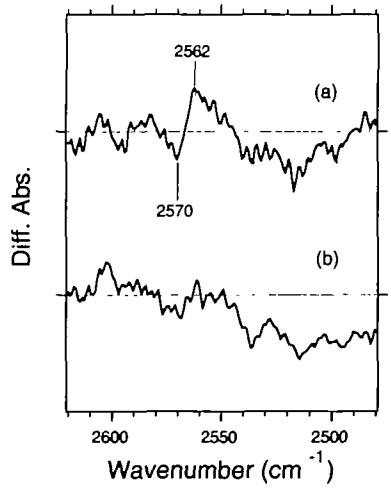

Fig. 4. **Infrared spectral changes in the SH stretching frequency region (2,620-2,480 cm^{-1}) produced by the photoreduction of air-oxidized cytochrome *bd* in the absence (a) and presence (b) of KCN.** One division of the Y-axis corresponds to 0.0001 absorbance unit.

protein where the apparent pH is considerably higher than the pK_a value of cyanide (9.22).

Infrared Absorption Changes upon Photoreduction in the SH Stretching Region—Changes in hydrogen bonding cause the SH stretching vibration of cysteine residues to undergo a small frequency shift in the 2,580-2,525 cm^{-1} region (*34*) as demonstrated for hemoglobin (*35-37*), photosynthetic reaction center (*38*), rhodopsin (*30, 39*), and gene-engineered bacteriorhodopsin (*40*). In the absence of KCN, photo reduction produces the appearance of a negative peak at 2,570 cm^{-1} and a positive peak at 2,562 cm^{-1}, indicating that one cysteine residue changes its hydrogen bonding (Fig. 4a). Since this change can be suppressed by KCN (Fig. 4b), it may occur in the vicinity of the CN^--binding site (*i.e.*, heme b_{595}-heme *d* binuclear center). Subunits I and II of cytochrome *bd* each contain three cysteine residues (*41*). Cys479 of subunit I and Cys214, Cys296, and Cys358 of subunit II are present in the C-terminal non-conserved domains (*41*); thus their sidechains are less likely to be close to the metal centers and would not be influenced by the redox and ligation states of the metal centers. Cys396 of subunit I is in close proximity to Met393, one of the heme b_{558} ligands (*42*), however, the *hexa*-coordinated low-spin heme b_{558} should not change its redox and ligation states upon CN binding. Unless heme b_{558} is proximal to the heme-heme binuclear center, Cys396 would not its change hydrogen bonding. Cys128 and His19, a putative heme b_{595} ligand (*29, 43*), are both present in the N-terminal half of subunit I. If they are topologically closer, Cys128 may sense redox-induced changes in the protein structure. The use of site-directed mutants will facilitate the identification of such a localized protein structural change in cytochrome *bd*.

Infrared Spectral Changes in the 1,780-1,510 cm^{-1} Region upon Photoreduction—Remarkable effects of KCN on the infrared spectrum were observed in the protonated carboxylic C=O stretching region (Fig. 5). In the absence of KCN, three positive and three negative bands appear at 1,752, 1,731, and 1,720 cm^{-1} and at 1,760, 1,738, and 1,726 cm^{-1}, respectively. Although the corresponding bands have to be identified exactly, it appears that three bands (1,760, 1,738, and 1,726 cm^{-1}) shift to the lower frequencies (1,752, 1,731, and 1,720 cm^{-1}, respectively), indicating that hydrogen bonding is strengthened upon reduction. While the spectral features are similar in the presence of KCN (Fig. 4d), the positive 1,752-cm^{-1} band loses much of its intensity, suggesting that CN^- binding induces the deprotonation of a carboxylic residue buried in a non-polar protein environment that provides a high pK_a (*44*). It is likely that this carboxylic acid newly would form a hydrogen bond upon the reduction of cytochrome *bd*, whereas the presence of cyanide releases the proton.

The spectral difference between the absence and presence of KCN is more prominent in the amide-I region where carbonyl C=O stretching vibrations of the peptide backbone appear. In the absence of KCN, a pair of strong peaks appears at 1,660 cm^{-1} (+) and 1,652 cm^{-1} (−). These are in the typical frequency region of an α-helix, and the shift to a higher frequency from 1,652 to 1,660 cm^{-1} indicates that the hydrogen bonding of the α-helix is weakened upon the reduction of the redox metal centers. Spectral changes other than those attributable to the α-helix are also observed at 1,693 (−), 1,682 (+), and 1,672 (−) cm^{-1}. In

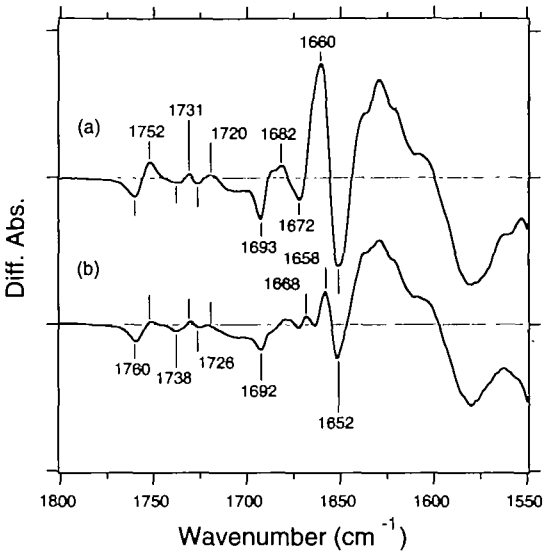

Fig. 5. **Infrared spectral changes in the 1,800-1,550 cm^{-1} region produced by the photoreduction of air-oxidized cytochromes *bd* in the absence (a) and presence (b) of KCN.** These spectral changes in the infrared region correspond to the visible changes in Fig. 2. One division of the Y-axis corresponds to 0.004 absorbance unit.

the presence of cyanide, the intensity of the pair in the α-helix region is largely reduced, indicating that the released cyanide still affects the protein structure of the reduced form (Fig. 5b). The structural change of the peptide backbone in the presence of cyanide is possibly coupled to that of carboxylic acids.

The localization of the axial ligands of hemes b_{558} and b_{595} (*7, 29, 42, 43*) and the proximity of heme *d* to heme b_{595} (*14, 15*) suggest that redox reactions in cytochrome *bd* take place at the protein surface. Accordingly, such structural changes associated with the rearrangement of the hydrogen bonding network occurring in carboxylates may be related to the delivery of chemical protons to the heme-heme binuclear center producing charge neutralization at the active center and/or local structural changes surrounding the active center.

In this study, we examined redox-induced structural changes in *bd*-type ubiquinol oxidase by FTIR spectroscopy and show for the first time that unique protein structural changes occur upon the reduction of the redox metal centers. Tendencies for stronger hydrogen bonding and the release of the bound cyanide in the reduced form have also been found in the *E. coli bo*-type ubiquinol oxidase (Yamazaki, Y., Kandori, H., and Mogi, T., unpublished results), a member of the proton pumping heme-copper terminal oxidases (*1*). It should be noted that except for the cysteine SH stretching vibration, changes in the amide-I and carboxylic C=O stretching vibrations are much smaller in *bo*-type ubiquinol oxidase (Yamazaki, Y., Kandori, H., and Mogi, T., unpublished results) even though two chemical protons must be delivered to the heme-copper binuclear center through the K-channel (*45*). Large structural changes in *bd*-type ubiquinol oxidase may be restricted to the active center domain at the protein surface. Systematic site-directed mutagenesis and time-resolved FTIR studies on *bd*-type ubiquinol oxidase will facilitate understanding of

the molecular mechanisms of dioxygen reduction and the delivery of chemical protons to the active center at an atomic level.

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