Demonstration by FTIR That the *bo*-Type Ubiquinol Oxidase of *Escherichia coli* Contains a Heme–Copper Binuclear Center Similar to That in Cytochrome *c* Oxidase and That Proper Assembly of the Binuclear Center Requires the *cyo*E Gene Product†

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ABSTRACT: Amino acid sequence data have revealed that the *bo*-type ubiquinol oxidase from *Escherichia coli* is closely related to the eukaryotic aa_3-type cytochrome *c* oxidases. In the cytochrome *c* oxidases, the reduction of oxygen to water occurs at a binuclear center comprised of heme a_3 and Cu_B. In this paper, Fourier transform infrared (FTIR) spectroscopy of CO bound to the enzyme is used to directly demonstrate that the *E. coli bo*-type ubiquinol oxidase also contains a heme–copper binuclear center. Photolysis of CO ligated to heme *o* at low temperatures (e.g., 30 K) results in formation of a CO–Cu complex, showing that there is a heme–Cu_B binuclear center similar to that formed by heme a_3 and Cu_B in the eukaryotic oxidase. It is further demonstrated that the *cyo*E gene product is required for the correct assembly of this binuclear center, although this polypeptide is not required as a component of the active enzyme in vitro. The *cyo*E gene product is homologous to COX10, a nuclear gene product from *Saccharomyces cerevisiae*, which is required for the assembly of yeast cytochrome *c* oxidase. Deletion of the *cyo*E gene results in an inactive quinol oxidase that is, however, assembled in the membrane. FTIR analysis of bound CO shows that Cu_B is present in this mutant but that the heme–Cu_B binuclear center is abnormal. Analysis of the heme content of the membrane suggests that the *cyo*E deletion results in the insertion of heme B (protoheme IX) in the binuclear center, rather than heme O. The insertion of the incorrect heme at this site appears to be the cause of the inability of the enzyme to function properly.

The terminal oxidases of bacterial respiratory chains display an impressive diversity. Traditionally, these enzymes are classified by their substrate (*c*-type cytochrome or quinol) and by their heme composition (Anraku, 1988; Ludwig, 1987; Poole, 1983). Many bacteria contain an aa_3-type cytochrome *c* oxidase which is closely related to the mitochondrial cytochrome *c* oxidase (Anraku, 1988; Ludwig, 1987; Saraste, 1990). There are several well-studied prokaryotic cytochrome *c* oxidases. For example, that from *Paracoccus denitrificans* has been isolated as a three-subunit enzyme (Haltia et al., 1988; Hendler et al., 1991), and the amino acid sequences of the three subunits are related to the sequences of the three mitochondrial encoded subunits (I, II, and III) of the eukaryotic oxidase (Saraste, 1990). The sequences of genes encoding subunits of the cytochrome *c* oxidases of several other bacteria suggest that subunits I, II, and III are common to this family of enzymes (Saraste, 1990). It has also become evident from sequencing data that this family of oxidases includes, in addition, oxidases that are not aa_3-type and oxidases that are quinol rather than cytochrome *c* oxidases (Gennis, 1991; Saraste, 1990). The most prominent example is the *bo*-type ubiquinol oxidase of *Escherichia coli* (Chepuri et al., 1990). The sequence of subunit I of this quinol oxidase is 40% identical to that of subunit I of bovine cytochrome *c* oxidase, even though the *E. coli* enzyme contains no heme A and does not utilize cytochrome *c* as a substrate (Chepuri et al., 1990). In this paper, it is definitively shown by Fourier transform infrared (FTIR) spectroscopy of CO bound to the enzyme that the *bo*-type ubiquinol oxidase contains a heme–copper binuclear center, similar to the catalytic site where oxygen is reduced to water in the aa_3-type cytochrome *c* oxidases (Chan & Li, 1990; Saraste, 1990).

The purified *bo*-type oxidase contains two equivalents of heme and one of Cu (Minghetti et al., 1992; Puustinen et al., 1991). The oxidase contains a low-spin heme b_{562}, which is equivalent to heme *a* in the aa_3-type oxidases, and the binuclear center contains a second heme plus Cu_B. It has recently been shown that the *bo*-type oxidase contains at least one equivalent of a chemically unique heme, designated heme O (Puustinen & Wikström, 1991; Wu et al., 1992). It is suggested in this work that the presence of heme O at the binuclear center is probably essential for proper function of the oxidase. This is shown by using FTIR spectroscopic analysis to examine the consequences of deleting the *cyo*E gene from the *cyo* operon.

The *bo*-type oxidase is usually isolated as a four-subunit enzyme (Matsushita et al., 1984; Puustinen et al., 1991; Wu et al., 1992), and these subunits are encoded by contiguous genes in the *cyo* operon (Chepuri et al., 1990). These correspond to *cyo*A (subunit II), *cyo*B (subunit I), *cyo*C (subunit III), and *cyo*D (subunit IV). There is a fifth gene, *cyo*E, at the end of the operon that encodes a protein that is homologous to a yeast nuclear gene product, COX10, which is required for the posttranslational assembly of the mitochondrial cytochrome *c* oxidase (Nobrega et al., 1990). The

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*cyo*E gene product is also homologous to the ORF1 proteins encoded by the *cta*B genes in operons encoding aa_3-type oxidases in several prokaryotes (Saraste, 1990). It has been shown that the *cta*B gene is required for the assembly of the aa_3-type cytochrome *c* oxidase in *Paracoccus denitrificans* (Steinrücke et al., 1991).

The *cyo*E gene product may be a fifth subunit of the *bo*-type oxidase which is lost during purification of the enzyme (Minghetti et al., 1992). However, the absence of this subunit appears to have no influence on the electron transfer activity of the purified oxidase in vitro. As expected from the previous results from *Saccharomyces cerevisiae* (COX10) (Nobrega et al., 1990) and *P. denitrificans* (*cta*B) (Steinrücke et al., 1991), the deletion of *cyo*E results in improper assembly of the *bo*-type oxidase. This inactive form of the oxidase contains heme b_{562} as well as a binuclear center. FTIR of bound CO shows that Cu_B is present but that the CO senses an abnormal environment. Extraction and subsequent analysis of the hemes from the membranes containing the mutant oxidase show that no heme O is present. It is concluded that heme B must be inserted in the binuclear center as a result of the *cyo*E deletion. It appears that either the *cyo*E gene product is required for the process of inserting the correct heme O into the binding site or it is required for the biosynthesis of heme O from heme B.

MATERIALS AND METHODS

Materials. Restriction enzymes and DNA ligase were purchased from New England Biolabs and Bethesda Research Laboratory. DNA polymerase I (Klenow fragment) was purchased from Boehringer Mannheim and low melting point agarose was Sea Plaque, obtained from FMC BioProducts. The BCA protein determination kit was purchased from Pierce Chemical Co.

Construction of a Plasmid for Expression of cyoABCD. Previous work characterized a series of *cyo–pho*A in-frame fusions (Chepuri & Gennis, 1990). One of these constructs (pVPS154) has a fusion junction following amino acid residue 36 in the *cyo*E gene product. A unique *Bam*HI site located at the *cyo–pho*A fusion junction and a unique *Sph*I site at the 3′ end of the *pho*A gene was used to remove the *pho*A gene from the fusion plasmid. The fusion plasmid, pVPS154, was digested with *Bam*HI and *Sph*I, and the mixture was electrophoresed on 0.6% low melting point agarose. The 1.4-kb *pho*A gene was removed, and the larger fragment was excised from the gel. This fragment was filled in using DNA polymerase I, ligated, and transformed into strain GV102. Transformants were selected for ampicillin resistance. Restriction analysis was performed on the plasmid DNA isolated from the transformants to confirm the deletion of the *pho*A gene and the presence of the *cyo* gene. The resulting plasmid, pABCD, contains the *cyo*ABCD genes. This is a multicopy plasmid which is derived from pBR322. All molecular biology procedures were performed as described (Sambrook et al., 1989).

Bacterial Strains and Growth Conditions. Two strains were used in this work, GV102/pABCD and GV102/pRG110. GV102 carries a deletion of the chromosomal *cyo* operon (Oden et al., 1990). This strain can grow aerobically by utilizing the alternate *bd*-type oxidase. By harvesting the cells during the early exponential phase of growth, the amount of the *bd*-type oxidase in the membrane is low, since the *bd*-oxidase is induced to high levels in response to low oxygen concentration (Cotter et al., 1990; Fu et al., 1991; Iuchi et al., 1990). GV102/pABCD overexpresses the mutant *bo*-type oxidase (*cyo*ABCD), and GV102/pRG110 (Au & Gennis, 1987; Oden et al., 1990) overexpresses the fully functional *bo*-type oxidase. Growth conditions have been previously described.

Genetic Complementation. The ability of pABCD (*cyo*ABCD) to produce a functional oxidase was tested by genetic complementation using strain RG129 (Au & Gennis, 1987), which is *cyo cyd* and cannot grow aerobically on nonfermentable substrates. The control strain, RG129/pRG110, can grow aerobically due to the plasmid-encoded wild-type *bo*-type oxidase. Details of the complementation procedure have been previously described (Lemieux et al., 1992).

Preparation of Membranes and UV/Visible Spectroscopy. The preparation of membranes for UV/visible spectroscopic analysis was as previously described (Lemieux et al., 1992), as was the procedure used to obtain low-temperature (77 K) reduced-minus-oxidized spectra and room temperature CO/reduced-minus-reduced difference spectra (Lemieux et al., 1992). The spectra were recorded using an Aminco DW-2 spectrophotometer.

FTIR Spectroscopy. Membranes were prepared as previously described (Lemieux et al., 1992). The membranes were suspended in 50 mM phosphate buffer, pH 7.0. The CO adduct was prepared in an anaerobically sealed, Beckman, 60Ti centrifugation bottle; 67 mM sodium dithionite (final concentration), which had been made anaerobic with CO gas, was added to the sample. The membranes were pelleted by centrifugation at 55 000 rpm in a Beckman 60Ti rotor (1 h, 4 °C). Pelleted membranes were overlayed with CO-saturated glycerol for at least 8 h for concentration and desiccation. The resulting pellet was pressed between a pair of CaF_2 windows with an optical path of 40 μm and mounted in a cryostat.

Infrared spectra were obtained with a Mattson Sirius 100 Fourier transform infrared interferometer at 0.5-cm^{-1} resolution. A liquid nitrogen cooled indium antimonide detector was used to observe the spectra in the 1750–3000-cm^{-1} range, which permitted observation of both the iron-bound and photodissociated CO. Interferograms were recorded in both directions of mirror movement through a 16-bit A/D converter and signal averaged, 512 times in each direction, into 32-bit words, which were used for all further computations. Full double-sided interferograms were triangularly apodized, with the real part of their Fourier transform resulting in the single-beam spectra. Low-temperature spectra were ratioed against spectra from the same sample after photodissociation with a 500-W tungsten lamp focused through a slide projector and optically filtered through glass and water to remove ultraviolet and infrared radiation. Subtraction of the least-squares fits of a cubic polynomial to the base-line regions of the spectra were used for base-line correction. There was no further averaging, smoothing, or other correction of the spectra.

Low temperatures were maintained and measured with a Lake Shore Cryotronics closed-cycle helium refrigerator (Model LTS-21-D70C) and a Palm Beach Cryophysics Model 4025 cryogenic thermometer/controller.

Heme Analysis. Analysis of hemes was essentially performed according to Puustinen and Wikström (1991) as follows. A membrane sample containing approximately 80 nmol of oxidase was extracted with 5 volumes of acetone/HCL/water (9:1:1) and then vortexed vigorously and pelleted by centrifugation at 4 °C. The supernatant was transferred to a new tube, and the hemes were extracted with ether. The heme-containing upper phase was washed twice with water, the ether was evaporated under a steam of nitrogen, and the residue was resuspended in 1 mL of methylene chloride/95% ethanol (1:1). Hemes were applied to a 1.5-mL column of

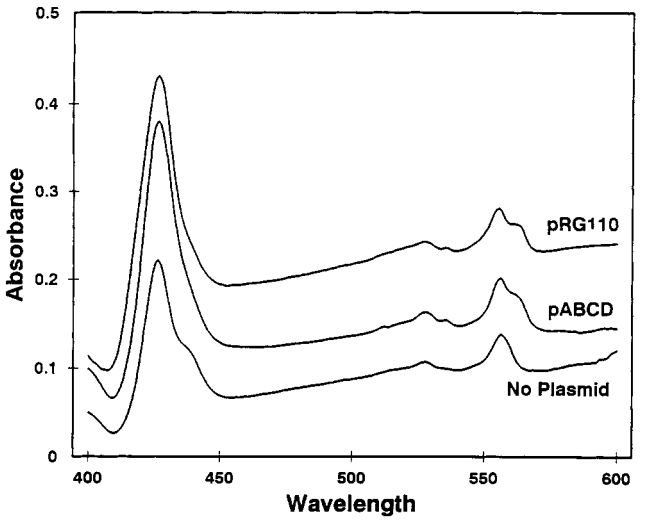

FIGURE 1: Low-temperature (77 K) dithionite reduced-minus-air oxidized difference spectra of membrane suspensions from GV102/pRG110, which contains the wild-type *bo*-type oxidase, GV102/pABCD, which contains the mutant *bo*-type oxidase resulting from the *cyo*E deletion, and GV102 with no plasmid. The membrane concentration was 8 mg/mL, and the spectra were obtained as previously described.

DEAE-Sepharose (Fast Flow, Pharmacia), previously washed with 10 mL of 1% ammonium acetate in aqueous 80% acetone, and equilibrated with 10 mL of methylene chloride/95% ethanol (1:1). The column was washed with 2 mL of 95% ethanol, and the hemes were eluted with 95% ethanol/acetic acid/water (70:17:13). The hemes were typically collected in four or five 200-μL fractions. Hemes were separated on a reverse-phase HPLC column (Microsorv-MV C-18 100 Å; 4.6 mm × 250 mm) at a flow rate of 1 mL/min (2350 psi [162.07 bar]) in 95% ethanol/acetic acid/water (70:17:7) with a Milton-Roy CM4000 system. Heme-containing fractions were detected by following the absorption at 406 nm by using a Milton-Roy SpectroMonitor 3100.

RESULTS

Plasmid pRG110 contains the entire *cyo*ABCDE operon, and the expression of this plasmid-encoded operon confers to RG129 (*cyo cyd*) the ability to grow aerobically on nonfermentable substrates (Au & Gennis, 1987). In contrast, truncation of the *cyo* operon within the *cyo*E gene (pABCD) results in expression of an inactive form of the oxidase. This is shown by the inability of RG129/pABCD to grow aerobically. Despite the fact that the *cyo*E protein is not a required subunit for the activity of the purified oxidase, this polypeptide is required to assemble the oxidase in an active form.

The cytoplasmic membranes prepared from GV102/pRG110 (*cyo*ABCDE) and from GV102/pABCD (*cyo*ABCD) are bright red due to overproduction of the *bo*-type oxidase. Western immunoblotting (not shown) confirms the presence of subunits I (*cyo*B) and II (*cyo*A) in the membranes of the strains expressing *cyo*ABCD. Antibodies directed against subunits III (*cyo*C) and IV (*cyo*D) are not available to confirm the presence of these subunits in the membranes of GV102/pBRABCD.

Figure 1 shows the low-temperature (77 K) reduced-minus-oxidized spectra of membrane suspensions. The main feature to note is the α-band near 560 nm. The purified *bo*-type oxidase has a low-spin heme b_{562} component which has a split α-band with a distinct peak at 563.5 nm (Minghetti et al., 1992; Pusstinen et al., 1991). The presence of this peak in the spectrum is diagnostic of the presence of heme b_{562} of the *bo*-type oxidase, since no other heme-containing protein in the *E. coli* membrane absorbs at this wavelength (Lemieux et al., 1992). The spectra in Figure 1 clearly show that the inactive oxidase resulting from the deletion of *cyo*E (GV102/pABCD) contains heme b_{562} at levels comparable to those of the wild-type control. Note that the amount of the *bd*-type oxidase in these membranes is relatively low due to the fact that the cells were grown with high oxygen levels in the growth medium. Hence, the overproduced *bo*-type oxidase dominates spectroscopically (Lemieux et al., 1992).

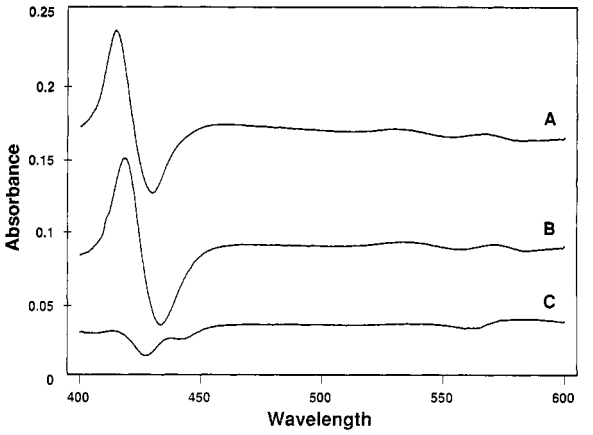

FIGURE 2: CO/reduced-*minus*-reduced difference spectra recorded at 25 °C of membranes suspended at 2 mg/mL. Spectrum A: GV102/pRG110 (wild-type *bo*-type oxidase). Spectrum B: GV102/pABDC (*cyo*E deletion). Spectrum C: GV102 host strain (no *bo*-type oxidase). Details are described elsewhere.

Figure 2 shows the spectroscopic perturbations due to the binding of CO to the dithionite-reduced membranes. The wild-type control (spectrum A) shows the expected peak and trough at 415 nm and 430 nm, respectively, similar to the features exhibited by the purified *bo*-type oxidase (Puustinen et al., 1991). The membranes from the host strain (spectrum C) shows that the signal due to CO binding to the *bd*-type oxidase is relatively small by comparison. The CO difference spectrum of the strain expressing *cyo*ABCD (spectrum B) shows substantial CO binding, but the spectrum is shifted by about 5 nm to longer wavelengths compared to that of the wild-type control (spectrum A). This result indicates that there is a CO-binding heme component associated with the inactive *bo*-type oxidase but that it is slightly perturbed in comparison to that of the wild-type control.

Low-temperature, "light-minus-dark" FTIR spectra of the CO bound to the oxidase yield additional information. This technique utilizes the stretching frequency of CO as a probe of its environment (Alben & Fiamingo, 1984; Fiamingo et al., 1982). For each membrane sample, two spectra are recorded. First, the FTIR spectrum of CO-saturated membranes is recorded in the dark. The CO is bound to the heme component of the binuclear center under these circumstances. Following this, a second spectrum is recorded while the membranes are exposed to visible light, which results in photolysis of CO from the heme. At low temperatures (e.g., 30 K), the CO binds stably to the nearby Cu in the binuclear center after photolysis (Fiamingo et al., 1982). Hence, the only difference between the two spectra (light vs dark) is that in the dark there is a heme Fe–CO present and in the light a Cu–CO is present. Subtraction of these two spectra reveals that the Fe–CO and Cu–CO complexes have distinct CO stretching frequencies. The technique has been used to characterize the heme–Cu binuclear center of the bovine aa_3-type cytochrome *c* oxidase (Einarsdóttir et al., 1988; Fiamingo et al., 1982,

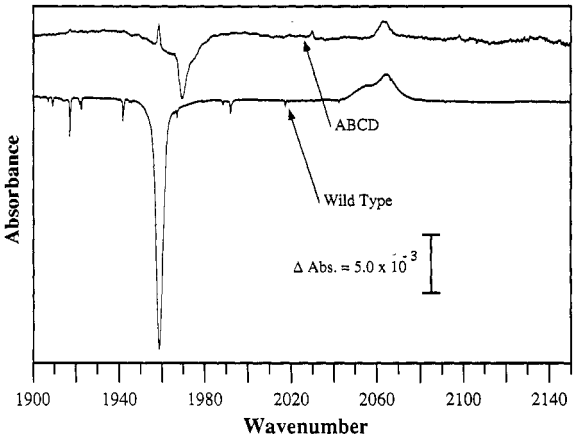

FIGURE 3: FTIR absorbance spectra (light-minus-dark) of CO complexes of glycerol-extracted membranes from GV102/pRG110 (wild-type *bo*-type oxidase) and GV102/pABCD (inactive oxidase resulting from the *cyo*E deletion). The spectra were recorded at 32 K (wild type) and 15 K (mutant). The difference in temperature does not influence the spectra. The wild-type Fe–CO absorption band is centered at 1959 cm^{-1}. The major Cu–CO band is centered at 2063 cm^{-1} with a shoulder at 2054 cm^{-1}. The mutant oxidase has an Fe–CO absorption band centered at 1975 cm^{-1}, and the Cu–CO band is centered at 2062 cm^{-1}.

1990) as well as several prokaryotic cytochrome *c* oxidases (Einarsdóttir et al., 1989; Shapleigh et al., 1992b).

Figure 3 shows the FTIR difference spectra of the *E. coli* membrane samples. The membranes containing the wild-type *bo*-type oxidase have an Fe–CO band at 1959 cm^{-1} with a bandwidth at half-peak height of 3.8 cm^{-1}. This is a sharp trough in the difference spectrum. Following photolysis, the CO now absorbs at higher frequency, indicative of a Cu–CO complex (Blackburn et al., 1990; Casella et al., 1988; Fiamingo et al., 1982). The absorbance band is at 2063 cm^{-1} with a shoulder at 2054 cm^{-1}. This spectrum is identical to that of the purified oxidase (not shown). For comparison, the bovine mitochondrial aa_3-type cytochrome *c* oxidase has an α-form with an Fe–CO absorption at 1964 cm^{-1} and Cu–CO absorption at 2055 cm^{-1} and 2065 cm^{-1} (Fiamingo et al., 1982). The absorption of CO following photolysis is in a frequency range expected for Cu–CO complexes and would be at a substantially higher frequency if the CO were not ligated to the metal (Alben & Fiamingo, 1984). These data demonstrate conclusively that there is a Cu within the distal pocket of the CO-binding heme component of the *bo*-type oxidase.

The FTIR spectrum of membranes of the strain expressing *cyo*ABCD is also shown in Figure 3. The Fe–CO band is substantially shifted to 1975 cm^{-1} and is slightly broader (5.5-cm^{-1} bandwidth). The Cu–CO absorbance is at 2062 cm^{-1}. The absorbance at 2062 cm^{-1} clearly shows that Cu is present in the inactive mutant oxidase. Hence, the deletion of *cyo*E does not prevent the insertion of Cu into the binuclear center. The spectrum also shows that both the Cu–CO and the Fe–CO are perturbed in relation to the wild-type control. The shift to higher frequency and increased breadth of the Fe–CO absorption band suggest some alteration of the heme–CO complex, as is also concluded from the change in the CO difference spectrum monitored by the changes in the ultraviolet (Soret) band of the heme (Figure 2).

It is important to note that the FTIR of CO bound to the *bd*-type oxidase is negligible in the spectra shown in Figure 3 due to the relatively low concentration of this oxidase. The Fe–CO of CO bound to this oxidase absorbs at 1984 cm^{-1}, and there is no Cu–CO that can form in the *bd*-type alternate oxidase (unpublished data).

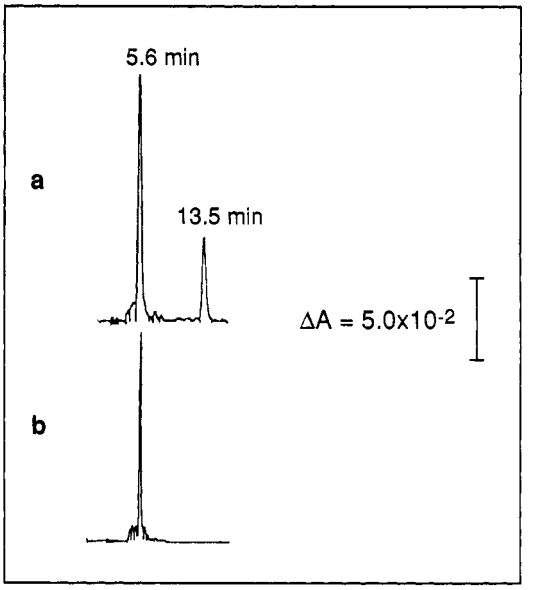

FIGURE 4: Reverse-phase HPLC analysis of the hemes extracted from membranes containing (a) the wild-type *bo*-type oxidase (GV102/pRG110) and (b) the inactive mutant *bo*-type oxidase (GV102/pABCD). The elution times for protoheme IX (heme B) and heme O are 5.6 min and 13.5 min, respectively. The data show no heme O present when *cyo*E is deleted. Procedures are given in the text.

The spectra in Figures 1, 2, and 3 demonstrate that all three metal centers associated with the native *bo*-type oxidase are also present in the mutant resulting from the deletion of the *cyo*E gene. However, the binuclear center is improperly assembled (Figures 2 and 3). Following the suggestion of Dr. T. Mogi (University of Tokyo, personal communication), the heme content of the membranes were analyzed by HPLC (Puustinen & Wikström, 1991). The results are shown in Figure 4. In part a, the hemes extracted from the membranes of the wild-type control are shown to contain heme B and heme O. Previous work has shown that the purified *bo*-type oxidase contains at least one equivalent of heme O, which has a farnesylhydroxyethyl side chain similar to that in heme A (Puustinen & Wikström, 1991; Wu et al., 1992). The membranes contain more heme B than heme O because of the presence of other heme B-containing enzymes (Gennis, 1987), e.g., complex II (Kita et al., 1989) and the *bd*-type oxidase. By contrast, membranes from the strain containing a comparable amount of the inactive *bo*-type oxidase (GV102/pABCD) contain no detectable heme O. The simplest conclusion is that the *cyo*E gene product is essential for either the synthesis of heme O or its insertion into the *bo*-type oxidase. Since the binuclear center is spectroscopically perturbed due to the deletion of *cyo*E, it follows that the most likely explanation is that heme B rather than heme O has been inserted at the binuclear center. The fact that the oxidase is enzymatically inactive and that there are spectroscopic alterations observed upon CO binding are most readily explained as consequences of this improper heme insertion.

DISCUSSION

The amino acid sequence comparisons make it clear that the *bo*-type ubiquinol oxidase from *E. coli* is a member of a superfamily of respiratory oxidases that includes the aa_3-type cytochrome *c* oxidases (Chepuri et al., 1990; Saraste, 1990). Subunit I, in particular, shows a striking degree of sequence conservation. It is this subunit that contains the ligands to three metal centers: a six-coordinate low-spin heme and a

heme–Cu binuclear center where oxygen is reduced to water (Capaldi, 1990; Chan & Li, 1990; Saraste, 1990). The *E. coli bo*-type oxidase offers some experimental advantages for addressing questions relating to the structure and functional mechanism of this entire group of oxidases, including the eukaryotic cytochrome *c* oxidase. The *bo*-type oxidase can be overproduced and either studied in situ in the membrane or purified in large quantity, and it is amenable to genetic manipulations. For example, by site-directed mutagenesis, the two histidines that are the axial ligands to the low-spin heme b_{562} component of the oxidase have been identified (Lemieux et al., 1992; Minagawa et al., 1991). Heme b_{562} is the analogue of heme A in the aa_3-type oxidases (Minghetti et al., 1992; Puustinen et al., 1991). The equivalent two histidines in subunit I of the aa_3-type cytochrome *c* oxidase from *Rhodobacter sphaeroides* have been identified recently as the heme A ligands (Shapleigh et al., 1992a), thus confirming the relationship of the enzymes within thissuperfamily.

One of the purposes of this paper is to demonstrate the value of low-temperature FTIR spectroscopy of enzyme-bound CO as a tool for probing the integrity of the binuclear center in oxidase mutants. The light-minus-dark difference technique allows one to probe the environment of both the heme and the Cu_B components of the binuclear center (Alben & Fiamingo, 1984; Fiamingo et al., 1982, 1990). Certainly, the most significant result presented in this work is the fact that the *bo*-type quinol oxidase has an FTIR difference spectrum similar to those of the cytochrome *c* oxidases. Previous electron spin resonance studies have concluded that the quinol oxidase probably has a heme–copper binuclear center similar to that of the eukaryotic oxidase (Salerno et al., 1990), but the FTIR data presented here provide a direct demonstration that there is a Cu in the distal pocket of the CO-binding heme component of the quinol oxidase. The extraordinarily narrow bandwidth of the Fe–CO absorption band (3.8 cm^{-1}) is also observed with other oxidases in this family (Einarsdóttir et al., 1989; Fiamingo et al., 1982; Shapleigh et al., 1992b) and suggests that CO is sensing a homogeneous environment. The FTIR spectra of Cu bound to the eukaryotic aa_3-type oxidase (Fiamingo et al., 1982), as well as that of *Rb. sphaeroides* (Shapleigh et al., 1992b), show two conformers (α and β) with distinct FTIR characteristics. However, there is only one Fe–CO band observed with the *E. coli* oxidase. The significance of this is not clear. Multiple FTIR frequencies are seen in CO bound to myoglobin and can be ascribed to differences in the conformation of a single amino acid side chain in the heme distal pocket (Oldfield et al., 1991).

The value of this technique is demonstrated by examining the result of deleting the *cyo*E gene from the *cyo* operon. The *cyo*E gene is homologous to COX10 from *S. cerevisiae* (Nobrega et al., 1990) and to the *cta*B genes that are found in several prokaryotic operons that encode subunits of aa_3-type cytochrome *c* oxidases (Saraste, 1990; Steinrücke et al., 1991). Both COX10 and the *cta*B gene product has been shown to be required for the posttranslational assembly of the cytochrome *c* oxidases in yeast and in *P. denitrificans*, (Nobrega et al., 1990; Steinrücke et al., 1991), respectively. The exact nature of the step that requires either COX10 or the *cta*B gene product is not known. It is demonstrated in this paper that the *cyo*E gene product is also required for some posttranslational modification needed for the assembly of the active *bo*-type ubiquinol oxidase. The FTIR data (Figure 3) show that the insertion of Cu_B does not require *cyo*E. However, the unique heme O that is associated with the functional *bo*-type oxidase is not present in the strain that lacks *cyo*E (Figure 4). It is most likely that either *cyo*E is required for the insertion of heme O into the oxidase or it is required for the biosynthesis of heme O. Presumably, the insertion of an incorrect heme in the binuclear center causes the spectroscopic perturbations of both the Fe–CO and Cu–CO which are observed with the inactive, mutant oxidase. Other explanations are possible, but this is the most simple interpretation.

Heme O contains the same farnesylhydroxyethyl side chain that is also present in heme A (Wu et al., 1992). However, heme O lacks the formyl modification found in heme A (Puustinen & Wikström, 1991; Wu et al., 1992), and as a result, the absorption properties of heme O are more similar to those of heme B (protoheme IX) than to those of heme A. The *cyo*E gene product could be directly responsible for the addition of the farnesylhydroxyethyl side chain. Since the same side chain is present in heme A, one could also postulate the same role for COX10 and the ORF1 proteins (*cta*B gene products). One or more additional factors must be required for the addition of the formly group to form heme A which would not be required to form heme O.

Additional experiments will be required to resolve whether *cyo*E is necessary for the biosynthesis of heme O or simply for its transfer to the proper binding site in the *bo*-type oxidase. It is interesting to note that it appears that the *cyo*E gene product can be isolated as a fifth subunit of the oxidase (Minghetti et al., 1992), so, whatever its role, this polypeptide is probably associated with the enzyme in vivo. It is even possible that the conversion of heme B to heme O could occur following assembly in the oxidase.

Finally, the fact that the deletion of *cyo*E results in the coincidental loss of heme O and perturbation of the CO-binding heme in the oxidase strongly suggests that the presence of heme O at this site is required for catalytic activity. In contrast, recent data suggest that either heme B or heme O can occupy the low-spin heme site (heme b_{562}) with no effect on enzyme function (Puustinen et al., 1992). Data from other laboratories suggest that heme substitutions in the respiratory oxidases of other prokaryotes may be controlled by growth conditions, though additional studies are needed to demonstrate that the polypeptides remain unchanged (Matsushita et al., 1992; Sone & Fujiwara, 1991). The reason why heme B cannot replace heme O in the binuclear center of the *E. coli bo*-type oxidase remains to be determined.

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