Site-Directed Mutants of the Cytochrome *bo* Ubiquinol Oxidase of *Escherichia coli*: Amino Acid Substitutions for Two Histidines That Are Putative Cu_B Ligands†

Melissa W. Calhoun,‡ John J. Hill,‡ Laura J. Lemieux,‡,§ W. John Ingledew,∥ James O. Alben,⊥ and Robert B. Gennis*,‡

School of Chemical Sciences, University of Illinois, Urbana, Illinois 61801, Department of Biochemistry and Microbiology, University of St. Andrews, St. Andrews, Scotland, and Department of Medical Biochemistry, The Ohio State University, Columbus, Ohio 43210

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ABSTRACT: The *bo*-type ubiquinol oxidase of *Escherichia coli* is a member of the superfamily of structurally related heme–copper respiratory oxidases. The members of this family, which also includes the aa_3-type cytochrome *c* oxidases, contain at least two heme prosthetic groups, a six-coordinate low-spin heme, and a high-spin heme. The high-spin heme is magnetically coupled to a copper, Cu_B, forming a binuclear center which is the site of oxygen reduction to water. Vectorial proton translocation across the membrane bilayer appears to be another common feature of this superfamily of oxidases. It has been proposed previously that the two adjacent histidines in putative transmembrane helix VII (H333 and H334 in the *E. coli* sequence) of the largest subunit of the heme–copper oxidases are ligands to Cu_B. Previously reported mutagenesis studies of the *E. coli bo*-type oxidase and the aa_3-type oxidase of *Rhodobacter sphaeroides* supported this model, as substitutions at these two positions produced nonfunctional enzymes but did not perturb the visible spectra of the two heme groups. In this work, six different amino acids, including potential copper-liganding residues, were substituted for H333 and H334 of the *E. coli* oxidase. All of the mutations resulted in inactive, but assembled, oxidase with both of the heme components present. However, cryogenic Fourier transform infrared (FTIR) spectroscopy of the CO adducts revealed that dramatic changes occur at the binuclear center as a result of each mutation and that Cu_B appears to be absent. To varying degrees, the protein environment monitored by CO bound to heme *o* in the mutants is perturbed by the H333 and H334 mutants. Electron paramagnetic resonance (EPR) spectroscopy of oxidation–reduction potentiometric titrations of the mutant in which H333 is replaced by Leu demonstrate that the high-spin heme is no longer magnetically coupled to Cu_B. The results confirm that both H333 and H334 are essential for the assembly and/or maintenance of Cu_B in the oxidase.

The *bo*-type ubiquinol oxidase of *Escherichia coli* is the predominant respiratory terminal oxidase when the bacteria are grown under high oxygen tension. The *cyo* operon which encodes this oxidase has been cloned and sequenced (Chepuri et al., 1990b). The deduced amino acid sequences of the subunits of the cytochrome *bo* complex have revealed a relationship between this *bo*-type ubiquinol oxidase and the aa_3-type cytochrome *c* oxidases. These enzymes contain at least two heme prosthetic groups, a six-coordinate low-spin heme, and a high-spin heme. The high-spin heme is magnetically coupled to a copper, Cu_B,[1] forming a binuclear center which is the site of oxygen reduction to water. This heme–copper binuclear center defines a superfamily of heme–copper oxidases (Hill et al., 1992; Woodruff, 1993). Proton translocation across the membrane bilayer appears to be another common feature of these enzymes (Chepuri et al., 1990a,b; Saraste et al., 1989; Puustinen et al., 1991; Hosler et al., 1993).

The combination of site-directed mutagenesis and spectroscopic characterization has recently been employed in the determination of the specific amino acid residues which ligate the heme and copper prosthetic groups (Lemieux et al., 1992; Minagawa et al., 1992; Shapleigh et al., 1992b). The two histidines which bind the low-spin heme have been identified by these methods, as has the histidine ligand to the high-spin heme component (Hosler et al., 1993; Calhoun et al., 1993).

Carbon monoxide (CO), which has a high affinity for oxidases, is a useful probe for the analysis of high-spin hemes in the presence of low-spin heme, since the high-spin heme component can be detected by its spectral shift upon binding carbon monoxide (Wood, 1984). The strong infrared absorption of CO can also be monitored by Fourier transform infrared (FTIR) spectroscopy. The center frequency and the band shape of the CO stretching mode are very sensitive to the coordination state and the surrounding protein "solvation shell" of the CO molecule (Alben & Fiamingo, 1984). FTIR spectroscopy of the CO adduct of the *E. coli bo*-type quinol oxidase has provided evidence that this enzyme contains a heme–copper binuclear center that is similar to those identified in other heme–copper oxidases (Alben et al., 1981, 1982;

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‡ University of Illinois.

§ Current address: Department of Pathology, Richardson Laboratory, Queen's University, Kingston, Ontario K7L 3N6, Canada.

∥ University of St. Andrews.

⊥ The Ohio State University.

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[1] Abbreviations: CO, carbon monoxide; Cu, copper; Cu—C≡O, carbonmonoxy adduct of copper; E_m, midpoint potential; EPR, electron paramagnetic resonance; Fe, heme iron; Fe—C≡O, carbonmonoxy adduct of heme; FTIR, Fourier transform infrared; $\Delta\nu_{1/2}$ and BWHH, full width of stretching frequency band at half-maximal height.

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Einarsdóttir et al., 1989; Hill et al., 1992; Shapleigh et al., 1992a; Woodruff et al., 1993). Under dark conditions, the CO molecule stably binds to the heme of the binuclear center. Upon photolysis of the heme Fe—C≡O bond, the CO can interact with Cu_B in the binuclear center. Although this Cu_B—C≡O interaction occurs only transiently at room temperature (Woodruff et al., 1991), it can be trapped by performing the photolysis at cryogenic temperatures (Alben et al., 1982).

EPR analyses of the aa_3-type oxidase from beef heart (van Gelder & Beinert, 1969) and the *bo*-type oxidase from *E. coli* (Salerno et al, 1990) have demonstrated that, under normal conditions, Cu_B is EPR-silent, apparently the result of magnetic interaction with the heme in the binuclear center. The presence of Cu_B can be inferred by the diminution of the EPR signal from the high-spin heme which occurs at high redox potentials. This interaction results in a "bell-shaped" titration curve for the heme component of the binuclear center. Removal of Cu_B from the protein would be expected to eliminate this unusual behavior.

Cu_B appears to be bound by at least two and possibly more histidine ligands (Cline et al., 1983; Powers et al., 1987; Scott et al., 1986; Ingledew & Bacon 1991; Surerus et al., 1992). It has been proposed previously that the two adjacent histidines (H333 and H334 in the *E. coli* sequence) in helix VII of subunit I of the heme–copper oxidases are ligands to Cu_B (Holm et al., 1987). The initial mutagenesis studies of the *E. coli bo*-type oxidase support this model, as leucine and alanine substitutions at these two positions produce nonfunctional enzymes but do not perturb the visible spectra of the two heme groups (Lemieux et al., 1992; Minagawa et al., 1992). In this work, several other amino acids were introduced at these two sites to test the essentiality of these two residues. All of the mutations, however, result in inactive, though assembled, oxidase, and Fourier transform infrared (FTIR) spectra of the CO adducts reveal that dramatic changes have occurred at the binuclear center, particularly at Cu_B. Potentiometric analyses of the H333L mutant demonstrate that the magnetic coupling between heme *o* and Cu_B has been eliminated. It is concluded that both H333 and H334 are essential residues and both are required for the insertion and/or maintenance of Cu_B. All amino acid substitutions at these positions result in the loss of integrity of the binuclear center where molecular oxygen is bound and reduced to water.

MATERIAL AND METHODS

Materials. Restriction endonucleases and DNA-modifying enzymes were obtained from New England Biolabs, Bethesda Research Laboratories, or United States Biochemical Corp. Oligonucleotides used in the generation of mutants and for DNA sequencing were obtained from the Biotechnology Center at the University of Illinois at Urbana–Champaign. Sequenase enzyme and kits were purchased from U.S. Biochemical Corp. Carbon monoxide (CO) was purchased from Matheson and was 99.5% pure. All other materials were scientific grade.

Construction of Mutants. The constructions of the H333L and H334L mutants have previously been described (Lemieux et al., 1992). The other mutants described in this work were constructed and confirmed according to previously published methods (Lemieux et al., 1992). All mutant proteins are expressed from the native *cyo* promoter on plasmid derivatives of the plasmid pL1 (Lemieux et al., 1992), which have been previously described (Lemiuex et al., 1992; M. W. Calhoun, unpublished results; J. W. Thomas, unpublished results).

Expression and Spectroscopic Characterization of Mutants. The growth, expression, complementation analysis, and characterization of the mutants by visible spectroscopy were performed as described (Lemieux et al., 1992). Characterization of the mutants was performed with membranes isolated from the host strain GL101 (*cyo sdh recA*) containing the overexpressed plasmid-borne *cyo* operon (Lemieux et al., 1992). In addition to the cytochrome *bo* complex, these bacterial strains contain chromosomal levels of the alternate *E. coli* oxidase, the cytochrome *bd* complex.

The collection of Fourier transform infrared spectra of carbonmonoxy adducts of the *E. coli* oxidase was performed as previously described (Hill et al., 1992). Dehydrated, CO-bound samples were pressed to a desired thickness between two CaF_2 windows (Janos Technology, Inc.). Infrared spectra were collected with a Mattson Sirius 100 FTIR interferometer at 0.5 cm^{-1} resolution. Full double-sided interferograms were signal-averaged 512 times and triangularly apodized with the real part of the Fourier transform, yielding a single-beam spectrum. A liquid nitrogen cooled indium antimonide detector was used to observe the spectra in the 1750–3000 cm^{-1} range, which permitted the observation of both the iron-bound and the photodissociated CO. Low temperatures of 10–20 K were measured and maintained by a Lake Shore Cryotronics closed-cycle helium refrigerator. The spectra are presented as difference spectra, with the spectrum prior to photolysis ("dark") subtracted from the spectrum following photolysis ("light"). Photodissociation was achieved by continuous irradiation from a focused 500-W tungsten lamp, with collection of the light spectrum initiated after 10 min of illumination. Heat and UV radiation were attenuated by passage of the photolyzing beam through water and glass. Subtraction of the least-squares fits of a cubic polynomial to the base-line regions of the spectra was used for base-line correction if noted in the figure captions. There was no further averaging, smoothing, or other correction of the spectra.

The membranes used for analysis by EPR were prepared in the same way as for visible spectroscopy, with the following modifications. After the membranes were pelleted at 160000*g* using the 60Ti rotor, they were resuspended using a tissue homogenizer in 50 mM TES and 2 mM EDTA (pH 7.0). The membranes were then centrifuged at 160000*g* for 2 h. The washed membrane pellet was then resuspended in a minimal volume of 50 mM TES and 2 mM EDTA (pH 7.0) to approximately 40 mg of membrane protein/mL. The concentrated membrane samples were either used immediately or stored frozen at −80 °C.

Redox titrations were performed as previously described (Salerno et al., 1990), with the following modifications. Membrane samples were diluted at least 4-fold in 100 mM MOPS (pH 7.0). The redox mediator dibromophenol/indophenol was omitted, and redox mediators were used at concentrations of 4–20 μM. The samples were frozen immediately in a mixture of 5:1 isopentane/methylcyclohexane in a liquid nitrogen bath and transferred to liquid nitrogen. Spectra were obtained using an X-band instrument (either a Bruker ER220D or a Bruker ESP300 spectrometer at approximately 9.46 GHz) equipped with an Oxford Instruments Cryostat with flowing liquid helium. Conditions were as follows: 100-kHz modulation frequency, 10-G modulation amplitude, 4000-G sweep width, 2100-G center field, and 5-mW power. The time constant was 163.84 ms, resulting in a sweep time of 335.544 s. For each titration, a constant temperature was maintained (±0.5 K), and temperatures for separate titrations ranged from 12 to 20 K. Each titration

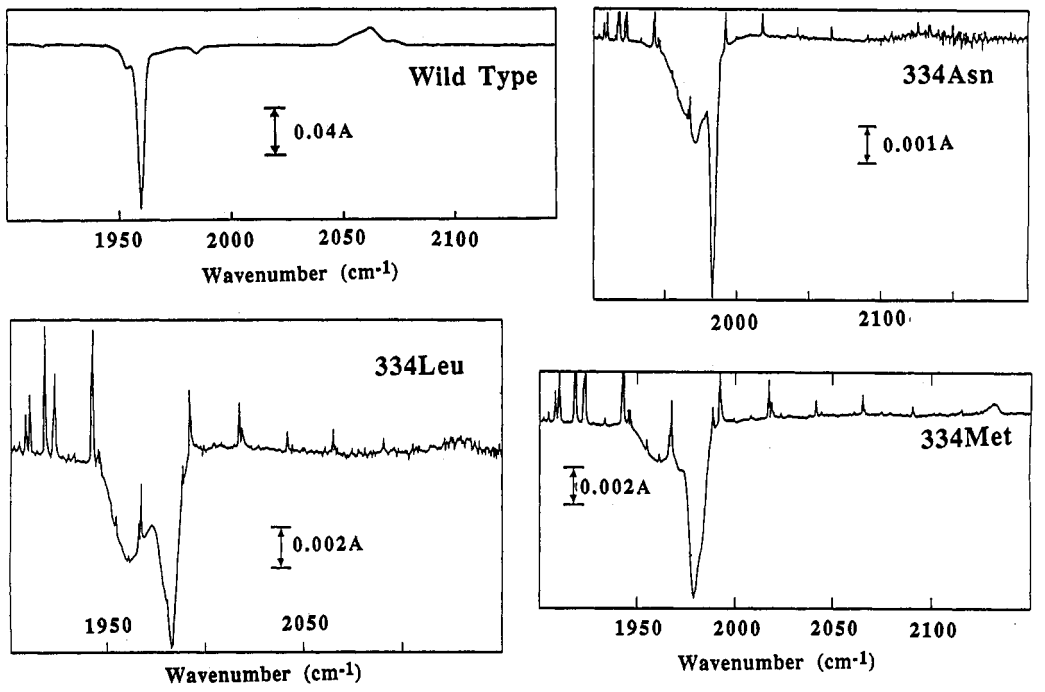

FIGURE 1: FTIR spectra of CO adducts of the H334 mutants. Spectra are presented as light *minus* dark spectra, as defined in the text. The spectra were recorded at the temperatures with the noted sample path lengths. If the spectrum was subjected to base-line correction (see Materials and Methods), the note "bc" is present. The Fe—C≡O band (trough) at 1984 cm^{-1} in the mutant samples arises from the alternate *E. coli* oxidase, the cytochrome *bd* complex. The very sharp peaks in some of the spectra arise from water vapor. The frequencies of the Fe—C≡O bands attributed to the *bo*-type oxidase are listed. Spectra: wild-type 15 K, 19.5 μm, 1959 cm^{-1}, bc; 334Asn 13 K, 25 μm, 1970 cm^{-1}; 334Leu 11 K, 96 μm, 1960 and 1979 cm^{-1}; 334Met 10 K, 27 μm, 1961 and 1979 cm^{-1}.

was plotted with the maximum value being 100% and the minimum being 0%.

RESULTS

Twelve different point mutations (His to Asn, Arg, Cys, Gln, Leu, and Met at each of two positions) were characterized in this study. Cys and Met residues are known to be copper ligands in other proteins and might be able to substitute for the histidine residues (Adman, 1991a,b). Asn and Gln are the residues most often substituted for histidine in evolutionarily related proteins (Dayhoff, 1978) and might maintain function if the histidine residues were not acting as metal ligands. Genetic complementation of each of the mutant *cyo* alleles was performed using a strain unable to grow aerobically on nonfermentable substrates unless the plasmid-borne *cyo* allele expresses a functional oxidase. None of the mutants at H333 or H334 is able to support aerobic growth, demonstrating that all of the amino acid substitutions at these positions examined to date create nonfunctional versions of the oxidase.

To further characterize the mutants, their heme components were analyzed by visible spectroscopy. The 550–570-nm region of the visible spectra of the leucine mutations at H333 and H334 has been previously reported (Lemieux et al., 1992). The low-spin heme b_{562} center has a split α-band with peaks at 555 and 563.5 nm, which is most clearly observed at low temperature (77 K) (Puustinen et al., 1991; Minghetti et al., 1992). This feature is clearly present in the H333 and H334 mutants, demonstrating that these mutations do not adversely affect assembly of the low-spin heme center, although in some cases the amount of *bo*-type oxidase expressed appears to be decreased by the mutations (data not shown).

The spectroscopic shift of the Soret band upon the binding of CO to reduced cytochrome *bo* was used to monitor the integrity of the high-spin heme *o* component. Since CO binding causes a blue shift in the absorbance peak, the difference spectrum contains a diagnostic feature with a peak at 415 nm and a trough at 430 nm (Kita et al., 1984; Cheesman et al., 1993). Most of the mutants at H333 and H334 contain this diagnostic CO spectrum at approximately the same level as the native enzyme, suggesting that most of the mutants contain approximately the same levels of CO-binding heme as the wild-type enzyme (data not shown). The membranes containing the H333C, H333R, and H334R mutants bind lower levels of CO per milligram of total membrane protein. This may be the result of the expression of a lower quantity of the mutant *bo*-type oxidase rather than a decreased level of CO binding per oxidase.

FTIR Spectroscopy. Figures 1 and 2 show the low-temperature light minus dark FTIR spectra of a representative set of the H333 and H334 mutants. Peaks in the spectra represent the CO stretching modes which are formed upon photolysis, while troughs are due to CO bound to the heme under dark conditions. The Fe—C≡O band at 1984 cm^{-1} arises from the alternate *E. coli* oxidase, the cytochrome *bd* complex, which is not a heme–copper oxidase (Hill et al., 1993). None of the mutants has obvious CO stretching modes in the range of the Cu—C≡O modes (2030–2100 cm^{-1}).

In addition to the lack of Cu—C≡O modes, pronounced perturbations have occurred in the Fe—C≡O stretching modes. In all cases, either the center of the mode has shifted to a higher frequency and the band width has increased or multiple forms of the Fe—C≡O species are evident.

The integrated area of the Fe—C≡O bands can be used to predict the band area of CO which, if transferred to Cu, would be occupied by a Cu—C≡O band (Alben & Fiamingo, 1984). This quantitation is important, since the signal-to-noise ratio may limit the detection of bands which are substantially broadened by heterogeneity. In the extreme case, allowing for the decrease in integrated molar absorptivity as a function of increasing frequency (Alben & Fiamingo, 1984),

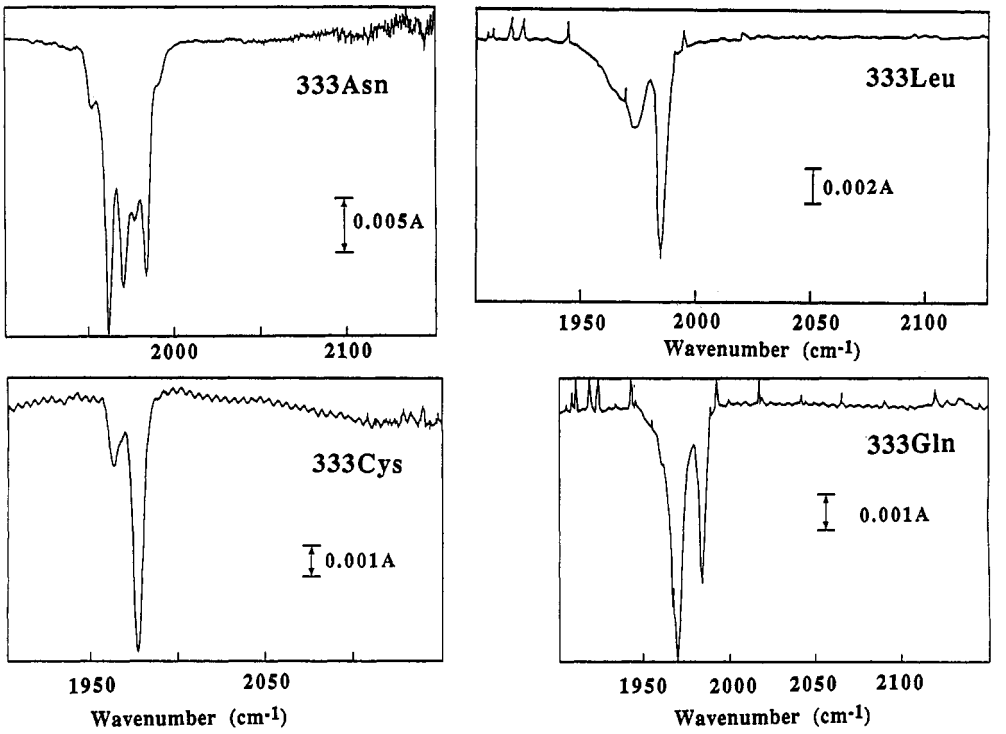

FIGURE 2: FTIR spectra of CO adducts of the H333 mutants. Spectra are presented as light *minus* dark spectra, as defined in the text. The spectra were recorded at the temperatures with the noted sample path lengths. If the spectrum was subjected to base-line correction (see Materials and Methods), the note "bc" is present. The Fe—C≡O band (trough) at 1984 cm^{-1} in the mutant samples arises from the alternate *E. coli* oxidase, the cytochrome *bd* complex. The very sharp peaks in some of the spectra arise from water vapor. The frequencies of the Fe—C≡O bands attributed to the *bo*-type oxidase are listed. Spectra: 333Asn 18 K, 51 μm, 1952, 1962, 1970, and 1977 cm^{-1}; 333Leu 11 K, 27 μm, 1971 cm^{-1}; 333Cys 15 K, 16 μm, 1962 and 1978 cm^{-1}, bc; 333Gln 13 K, 25 μm, 1970 cm^{-1}.

the Fe—C≡O band of the H334M mutant (132 cm^{-1}·mA)[2] could hypothetically yield a disordered Cu—C≡O band at 2063 cm^{-1} (52 cm^{-1}·mA) with a $\Delta\nu_{1/2}$ (BWHH[3]) of 60 cm^{-1} and an absorbance (0.86 mA) which is significantly above the noise level. This is roughly 6 times the $\Delta\nu_{1/2}$ of the wild-type Cu—C≡O mode. From these calculations, it appears unlikely that a Cu—C≡O complex is present in the photolyzed state of these enzyme CO adducts.

At 120 K, CO photolyzed from cytochrome *c* oxidase is quite stable as the Cu—C≡O adduct. In hemoglobin, which does not contain copper, at 120 K, virtually all of the CO rapidly relaxes to the heme (Fiamingo et al., 1986). This difference in relaxation kinetics can be used to distinguish proteins which contain heme–copper binuclear centers from those which do not. To further demonstrate the lack of a Cu—C≡O complex in the photolyzed state in the mutant enzymes, the FTIR spectrum of each of the mutants was collected at 100–120 K (data not shown). In these experiments, a dark spectrum was collected, and the sample was subjected to photolysis for 10 minutes. The sample was allowed to relax for 10 min in the dark, and then a light spectrum was collected. In the wild-type enzyme, similar experiments at 100 K yield Fe—C≡O and Cu—C≡O band areas equivalent to those present at 10–20 K (J. J. Hill, R. B. Gennis, and J. O. Alben, manuscript in preparation). In contrast, the mutant enzymes show Fe—C≡O and protein–C≡O band areas that are less than 10% of those observed at 10–20 K, where no significant relaxation occurs (data not shown).

These experiments indicate that in all of the mutants, upon photolysis of the Fe—C≡O, the CO molecule weakly associates with the protein in the heme distal pocket, and at a temperature of 100–120 K there is no substantial barrier to impede relaxation of the CO back to the heme iron. These results are similar to those seen in hemoglobin, and they demonstrate that, in each of the mutants, the photolyzed CO is not interacting with a copper. The absence of Cu—C≡O bands is not the result of a lack of copper in the growth medium. Identical FTIR spectra were recorded from samples prepared from cells grown in a medium supplemented with 0.3 mM copper sulfate (data not shown).

EPR Spectroscopy. The magnetic coupling between heme *o*, the heme in the binuclear center of the *bo*-type oxidase, and Cu_B prevents the direct observation of Cu_B via EPR spectroscopy. However, the presence of Cu_B can be inferred because the coupling reduces the height of the EPR signal from heme *o* when both centers are in the oxidized state. This interaction results in a bell-shaped curve, which can be seen in titrations of the wild-type enzyme (Salerno et al., 1990; Figure 3). Other heme proteins, such as myoglobin and the *bd*-type oxidase from *E. coli*, contain magnetically isolated hemes. In such heme proteins, EPR analyses of redox titrations show direct correlations of signal height with redox potential, which are dependent only upon the midpoint potential of the heme iron. This type of behavior can be seen with the alternative oxidase from *E. coli*, the cytochrome *bd* complex (Meinhardt et al., 1989; Figure 3).

EPR analysis of redox titrations with membranes containing the H333L mutant is shown in Figure 3. These membranes contain two hemes which exhibit EPR signals at $g = 6$. One of these is the high-spin heme *d* of the alternate *E. coli* oxidase (Meinhardt et al., 1989), which titrates at the higher potentials ($E_m \approx 300$ mV). The lack of oxidase activity from cytochrome *bo* as a result of the H333L mutation induces the production of a higher level of the alternative *bd*-type oxidase than what occurs in the wild-type control. The second high-spin heme

[2] Quantitation of integrated areas is presented in units of wavenumber·milliabsorbance.

[3] Full width of band at half-height.

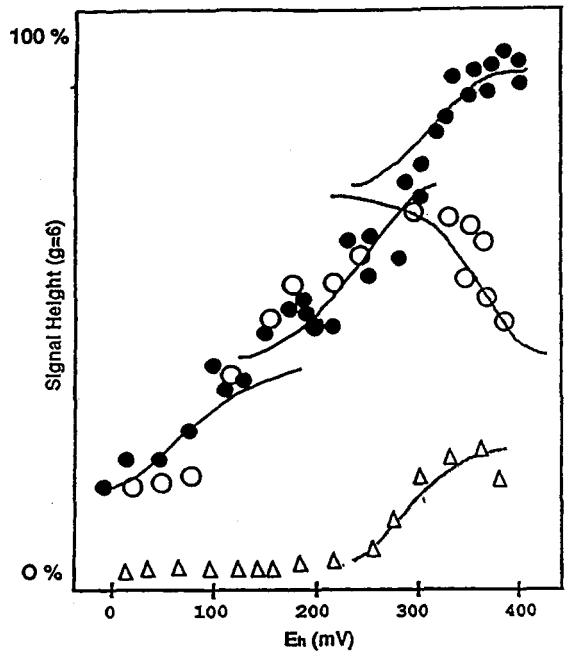

FIGURE 3: Plot of oxidation–reduction titrations of high-spin centers in membranes containing the H333L mutant. Experimental details are described in the text. The relative signal height of the $g = 6$ signal is plotted against the redox potential (millivolts) of the sample. In the membranes from the wild-type (○), this signal is the sum of a small amount of the high-spin heme component from cytochrome *bd* and the signal from heme *o*. Membrane samples containing the H333L mutant, which also contain the cytochrome *bd* oxidase (●), are the average of five individual titrations. Each of the smooth curves approximate a single redox phase of an $n = 1$ component. One curve approximates the titration of the heme *d* of the cytochrome *bd* complex ($E_m \approx 300$ mV). The contribution from cytochrome *bd* can be estimated by the titration of membranes from a strain containing only cytochrome *bd* (△). For the wild-type sample, three Nernst $n = 1$ curves are shown for the heme *o* of the wild-type cytochrome *bo*. One curve reflects the effect of the putative change in redox state of Cu_B on the heme *o* signal at potentials above 300 mV, while two Nernst $n = 1$ curves represent biphasic heme *o* ($E_m \approx 100$ mV and $E_m \approx 225$ mV). The biphasic nature of the heme *o* site (between 0 and 300 mV) reflects the effects on heme *o* of the heme–heme interaction with the low-spin heme b_{562} site (Salerno et al., 1990). For the H333L mutant sample, the two $n = 1$ curves drawn for the wild-type cytochrome *bo* sample approximate the same species in the mutant ($E_m \approx 100$ mV and $E_m \approx 225$ mV), while an additional $n = 1$ curve represents the titration of heme *d* of the cytochrome *bd* complex ($E_m \approx 300$ mV).

at $g = 6$ is heme *o* of the *bo*-type oxidase, which can be modeled using two $n = 1$ components of $E_m \approx 100$ mV and $E_m \approx 225$ mV. This biphasic behavior of the heme *o* center is believed to result from heme–heme interactions within the enzyme. This theory has been supported by optical and EPR spectroscopies (Salerno et al., 1989, 1990; Bolgiano et al., 1991).

The increase in the height of the $g = 6$ signal above 300 mV in the plot of the H333L mutant sample demonstrates that the coupling between heme *o* and Cu_B has been abolished. In addition, no EPR signals that can be attributed to Cu_B were detected during the redox titration (data not shown). A significant increase in the height of the $g = 6$ signal is present in the H334L mutant when compared with membranes containing the wild-type enzyme, suggesting that this mutation also results in the loss of the magnetic coupling.

Unlike membrane preparations containing the wild-type *bo*-type oxidase, heme *o* in the H333L mutant membranes does not appear to equilibrate readily with the solution potential. This behavior results in a slightly less even distribution of sample points for the titration curve of the mutant and may be the result of the absence of Cu_B.

DISCUSSION

Studies utilizing extended X-ray absorption fine structure (EXAFS) and electron–nuclear double-resonance (ENDOR) techniques with the heme–copper oxidases implicate 2–4 histidines as ligands for Cu_B (Cline et al., 1983; Scott et al., 1986; Powers et al., 1987; Surerus et al., 1992). The two histidines that are the focus of this work (H333 and H334) previously were changed to leucine and to alanine. These mutations resulted in only slight modifications of the visible spectra of the hemes, and the purified forms of the alanine mutants have been shown to lack copper (Lemieux et al., 1992; Minagawa et al., 1992). Consequently, H333 and H334 were proposed to be ligands to Cu_B. In this study, each of these residues was replaced with five alternative amino acids (Arg, Asn, Cys, Gln, and Met), and the effects of these mutations on the CO adducts and EPR properties of the enzymes were examined.

In all cases, the replacement of H333 or H334 with another residue resulted in a nonfunctional enzyme, on the basis of the *in vivo* complementation assay. Most of the mutations have little effect on the amount of enzyme present in the membrane or the assembly of the two heme components, as demonstrated by visible spectroscopy. Of the mutants examined here, the arginine substitutions appear to be the most destabilizing.

Low-temperature FTIR spectroscopy of the CO adducts of the mutant enzymes reveals that the loss of activity is most likely due to a disruption in the binuclear center. Upon photolysis of the Fe—C≡O of the wild-type enzyme at low temperature, the CO stably binds to Cu_B. In the mutants, the CO does not appear to form a complex with Cu, as evidenced by the absence of Cu—C≡O stretching modes in the photolyzed state as well by the rapid relaxation of CO to the heme iron at temperatures above 100 K. In effect, the mutations have changed the active site of the oxidase into a CO-binding heme pocket which appears to have superficial similarities to the heme distal pocket in myoglobin and hemoglobin. Measurements by visible spectroscopy of the rate of CO relaxation to the heme following photolysis show that the H333L and H334L mutants do indeed have a rate of recombination on the order of that seen in the globins (Woodruff, 1993; Lemon et al., 1993; Brown et al., 1993). These rates are much faster than the rate of recombination of CO to the heme in the wild-type cytochrome *bo* complex (Woodruff, 1993; Lemon et al., 1993; Brown et al., 1993).

Asparagine and glutamine seem to maintain a somewhat more ordered heme distal pocket, as the band width of the stretching frequency of the heme-bound CO is small in comparison with those of the other mutants. The heme iron and Cu_B have been proposed to be less than 5 Å apart (Alben et al., 1981), and it is reasonable to assume that a change of the Cu_B ligands might disorder the heme-bound CO molecule. Effects on the Fe—C≡O stretching frequency are seen in mutants in the distal histidine residue of myoglobin (Braunstein et al., 1988; Balasubramanian et al., 1993).

Although predictions of the environment resulting in any given CO stretching frequency are not yet possible, the CO stretching frequency is known to reflect the combined effects of the electronic character of the heme and the specific interactions of CO with the distal heme pocket. An increase in frequency can be induced by a decrease in the polarity of the CO ligand resulting from changes in the local electrical field caused by changes in hydrogen bonding or interactions with amino acid residues in the distal binding site of the heme (Oldfield et al., 1991; Park et al., 1991; Braunstein et al., 1988; Balasubramanian et al., 1993). This suggests that all

of the changes noted in the FTIR spectra may be attributed to effects distal to the heme, in the region believed to be the Cu_B-binding site. The ranges of FTIR band widths and center frequencies exhibited by the CO-bound forms of each of the oxidase mutants suggest that both H333 and H334 may be located in the vicinity of the CO molecule when it is bound to the heme. The FTIR spectra of the Fe—C≡O species are not identical for all of the mutants, indicating that simply the lack of Cu_B is insufficient to describe the effect of each amino acid substitution. The absence of the Cu_B—C≡O adduct in the mutants at H333 and H334 could be explained either by the absence of copper from the enzyme or by the occlusion of the pathway between the heme and the Cu_B in the binuclear center. The results of EPR analysis suggest that Cu_B is absent in the H333 and H334 mutants.

The mutations at H333 and H334 remove the magnetic coupling between heme *o* and Cu_B. This is apparently due to the loss of Cu_B, as no EPR-detectable copper is found in potentiometric titrations of the H333L mutant. In H333L, heme *o* behaves as a magnetically isolated heme, in contrast with the wild-type enzyme in which heme *o* is magnetically coupled to Cu_B. The H334L mutation also appears to remove the magnetic coupling between heme *o* and Cu_B. The data presented here are entirely consistent with the assignment of H333 and H334 as Cu_B ligands in the *bo*-type oxidase of *E. coli* and, by extrapolation, in the other members of the heme–copper oxidase family.

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