Identity of the Axial Ligand of the High-Spin Heme in Cytochrome Oxidase: Spectroscopic Characterization of Mutants in the *bo*-type Oxidase of *Escherichia coli* and the aa_3-type Oxidase of *Rhodobacter sphaeroides*†

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ABSTRACT: Prokaryotic and eukaryotic cytochrome *c* oxidases and several bacterial ubiquinol oxidases compose a superfamily of heme–copper oxidases. These enzymes are terminal components of aerobic respiratory chains, the principal energy-generating systems of aerobic organisms. Two such heme–copper oxidases are the aa_3-type cytochrome *c* oxidase of *Rhodobacter sphaeroides* and the *bo*-type ubiquinol oxidase of *Escherichia coli*. These enzymes catalyze the reduction of oxygen to water at a heme–copper binuclear center. Energy conservation is accomplished by coupling electron transfer through the metals of the oxidases to proton translocation across the cellular membrane. The *Rb. sphaeroides* and *E. coli* enzymes have previously been utilized in site-directed mutagenesis studies which identified two histidines which bind the low-spin heme (heme *a*), as well as additional histidine residues which are probable ligands for copper (Cu_B). However, the histidine that binds the heme of the binuclear center (heme a_3) could not be unequivocally identified between two residues (His284 and His419). Additional characterization by Fourier transform infrared spectroscopy of the CO-bound forms of the *E. coli* enzyme in which His284 is replaced by glycine or leucine demonstrates that these mutations cause only subtle changes to CO bound to the heme of the binuclear center. Resonance Raman spectroscopy of the *Rb. sphaeroides* enzyme in which His284 is replaced by alanine shows that the iron–histidine stretching mode of heme a_3 is maintained, in contrast with the loss of this mode in mutants at His419. These results demonstrate that His284 is not the heme a_3 ligand. Therefore, the remaining conserved histidine within subunit I of the oxidases (His419) is proposed to be the heme a_3 ligand. In this model, the axial ligands of the two hemes are located within a single helix and thus are connected by a pathway of covalent bonds. The implications of this model on the control of electron transfer through the enzyme are discussed.

Prokaryotic and eukaryotic cytochrome *c* oxidases as well as several bacterial ubiquinol oxidases compose a large superfamily of heme–copper oxidases that function in the catalysis of oxygen reduction to water. These oxidases are the terminal components of aerobic respiratory chains, the principal energy-generating systems of aerobic organisms. Energy conservation is accomplished by the coupling of electron transfer through the metal centers of the oxidases to the translocation of protons across the cellular membrane. Two examples of such heme–copper oxidases are the aa_3-type cytochrome *c* oxidase of *Rhodobacter sphaeroides* and the *bo*-type ubiquinol oxidase of *Escherichia coli*.

In addition to the common function of the heme–copper oxidases, comparison of primary amino acid sequences suggests a high degree of structural similarity among these enzymes, particularly within the largest subunit. In all of the heme–copper oxidases, this subunit (subunit I) binds two heme groups and a copper atom (Cu_B). One of the hemes (heme a_3)[1] is closely associated with Cu_B, and these two metals form a binuclear center which is the site of oxygen reduction. Spectroscopic studies of the bovine and yeast cytochrome *c* oxidases, as well as of the *bo*-type ubiquinol oxidase of *E. coli*, have demonstrated that all three metal centers are bound by histidine residues (Eglinton et al., 1980; Stevens et al., 1981; Ingledew et al., 1993; Cheesman et al., 1993).

The results of site-directed mutagenesis of the conserved histidine residues of the aa_3-type oxidase from *Rb. sphaeroides* and the *bo*-type ubiquinol oxidase from *E. coli* have identified the two histidine residues of subunit I which ligate the low-spin, six-coordinate heme (heme *a*)[1] (Shapleigh et al., 1992b; Lemieux et al., 1992; Minagawa et al., 1992). In addition, two other histidines were identified as probable ligands to Cu_B. However, the histidine ligand of the oxygen binding high-spin heme could not be conclusively identified by these

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[1] For simplicity, the nomenclature of the aa_3-type cytochrome *c* oxidases is used. In the *E. coli* oxidase, heme b_{562} is the analogue of heme *a*, the low-spin six-coordinate heme. In *E. coli*, the equivalent to heme a_3 is heme *o*, the site of oxygen reduction to water.

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studies. It was proposed that histidine 284[2] (His284) was the proximal ligand of the heme at the binuclear center, although it was acknowledged that this model had little experimental basis, and ligation by His419 was equally possible. Spectroscopic evidence presented here demonstrates that His284 is not the proximal ligand of the carbon monoxide binding heme and suggests that this residue may be a ligand for Cu_B.

The techniques utilized in this study are particularly effective in probing the structural environment of the heme binding site. Fourier transform infrared (FTIR)[3] spectroscopy of the CO adduct of the bovine cytochrome *c* oxidase has been instrumental in defining the binuclear center which is common to all heme–copper oxidases (Alben et al., 1981; Einarsdóttir et al., 1989; Woodruff et al., 1991; Hill et al., 1992; Shapleigh et al., 1992a). Resonance Raman spectroscopy is a powerful technique for probing the influence of the protein environment on the heme centers of cytochrome oxidase (Babcock, 1988). The particular relevance of resonance Raman spectroscopy in this study is the ability to monitor the specific stretching frequency of the heme a_3 iron–histidine bond.

Evidence from FTIR spectroscopy of carbonmonoxy forms of two mutants at histidine 284 in the *bo*-type oxidase of *E. coli* demonstrates that these mutations cause only minor perturbations to the heme binding site, but large changes at Cu_B. Resonance Raman spectroscopy of the analogous mutant in the oxidase from *Rb. sphaeroides* demonstrates that the iron–histidine (Fe–N_{His}) stretching mode of heme a_3 is present, but to varying degrees depending on the conditions used for sample preparation. These results demonstrate that histidine 284 is not the ligand for heme a_3. A revised model of the catalytic core of the heme–copper oxidases is presented which reflects the assignment of the heme ligand at the binuclear center. The implications of this model on electron transfer within the enzyme are discussed.

MATERIALS 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 enzymes and kits were purchased from U.S. Biochemical Corp. Carbon monoxide (CO) used for FTIR was purchased from Matheson and was 99.5% pure. All other materials used were scientific grade.

Construction of Mutants. The constructions of the *E. coli* His284Leu and His284Gly mutants and the *Rb. sphaeroides* His284Ala mutant have previously been described (Lemieux et al., 1992; Shapleigh et al., 1992b). One M13 bacteriophage vector (M13SEΔH) was constructed for use in this study. This vector contains a portion of the *cyo* operon lacking the second *Hin*dIII site within *cyo*B. The construction of this silent mutation has been previously described (Lemieux et al., 1992). The other *E. coli* mutants described in this work were constructed and confirmed according to previously published methods (Lemieux et al., 1992).

Expression and Spectroscopic Characterization of Mutants. The growth, expression, complementation analysis, and characterization by visible spectroscopy of the *E. coli* mutants were performed as described (Lemieux et al., 1992). All characterization of the *E. coli* mutants was performed with membranes isolated from bacterial strains containing overexpressed plasmid-borne *cyo* operon as well as chromosomal levels of the alternate *E. coli* oxidase, the cytochrome *bd* complex. Levels of the cytochrome *bd* complex were minimized by harvesting cell cultures in the early log phase of growth. Cell growth, isolation of cytoplasmic membranes, and purification of the *Rb. sphaeroides* mutant were as previously outlined (Shapleigh et al., 1992b; Hosler et al., 1992).

The collection of Fourier transform infrared spectra of carbonmonoxy forms of the *E. coli* oxidase was performed as previously described (Hill et al., 1992). Dehydrated, CO-bound samples were pressed to the desired thickness between two CaF_2 windows (Janos Technology, Inc., Townshend, VT). 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 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 radiation from a focused 500-W tungsten lamp, with collection of the "light" spectrum initiated after 10 min of illumination. Heat and UV irradiation were attenuated by passage of the photolyzing beam through glass and water. Subtraction of the least-squares fits of a cubic polynomial to the base-line regions of the spectra was used for base-line correction. There was no further averaging, smoothing, or other correction to the spectra.

Collection of resonance Raman spectra of the *Rb. sphaeroides* enzyme was performed as previously described (Shapleigh et al., 1992b), with the following exceptions. Acquisition of the resonance Raman spectra of the partially purified, lipid-rich form of His284Ala (Figure 5C) required concentration of the mutant oxidase, along with the partial elimination of species that contribute to background fluorescence. To accomplish this, cytoplasmic membranes from cells grown under high oxygen tension (Hosler et al., 1992) were solubilized in 50 mM KH_2PO_4, pH 7.0, 1 mM EDTA, 100 mM KCl, 2.5 μg/mL pepstatin, 2.5 μg/mL leupeptin, 1 mM PMSF, and 3% lauryl maltoside for 20 min at 0 °C. This concentration of detergent dissolves the membranes but is insufficient to completely disperse the respiratory enzymes. The resulting solution was centrifuged at 40 000*g* for 30 min at 4 °C to remove insoluble material, and the supernatant was centrifuged for several hours at 4 °C in a Centricon-100 (Amicon) filtration device to concentrate the cytochromes and remove excess lauryl maltoside. The purified sample of His284Ala (Figure 5B) was prepared with a higher detergent concentration and two column chromatography steps as previously described (Hosler et al., 1992).

RESULTS

A variety of residues were chosen to substitute for the histidine at position 284 in the *E. coli bo*-type oxidase.

[2] The numbering of the equivalent conserved histidine residues in the *E. coli* and the *Rb. sphaeroides* heme–copper oxidases is identical except for the first histidine. In *E. coli*, this is residue H106, while in *Rb. sphaeroides*, this residue is H102.

[3] Abbreviations: CO, carbon monoxide; Cu, copper; Cu-CO, carbonmonoxy adduct of copper; Fe, heme iron; Fe-CO, carbonmonoxy adduct of heme; FTIR, Fourier transform infrared; Fe–N_{His}, iron–histidine (stretching mode); PMSF, phenylmethanesulfonyl fluoride; $\Delta\nu_{1/2}$, full width of stretching frequency band at half-maximal height.

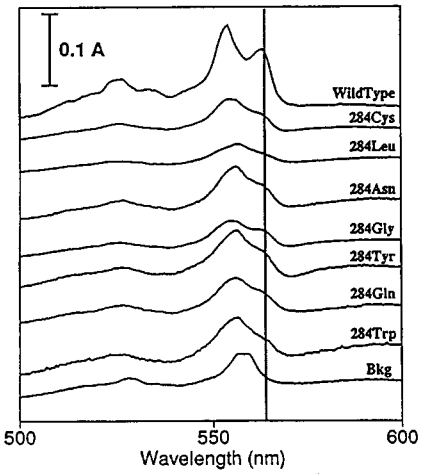

FIGURE 1: Visible spectra of the mutants at histidine 284 (His284) in the *E. coli bo*-type oxidase. Spectra were recorded at 77 K as described under Materials and Methods to maximize the features of the split α-band of cytochrome b_{562}. Each sample contained 8 mg of membrane protein/mL. The absorbance in the background strain (Bkg) arises from the alternate oxidase of *E. coli*, the *bd*-type oxidase. None of the mutants at His284 perturbs the spectrum of low-spin, six-coordinate heme b_{562}.

Previously, Ala, Leu, and Gly had been substituted for this residue (Lemieux et al., 1992; Minagawa et al., 1992). Mutants containing Cys and Tyr, amino acids known to be heme ligands in other proteins (cytochromes P450, catalase), were also constructed (Poulos, 1988). In order to make as subtle a change as possible, the histidine residue was changed to Asn and to Gln. A dramatic change in amino acid volume (from 153 to 227 Å^3; Creighton, 1983)was the goal in making the Trp mutation. None of the mutations at His284 restore aerobic growth to oxidase-deficient *E. coli* strains, suggesting that these mutants are devoid of activity.

To express inactive mutants of the *bo*-type oxidase, the cells must express the alternative oxidase of *E. coli*, the *bd*-type oxidase, which has three heme components, b_{558}, b_{595}, and *d* (Anraku, 1987; Anraku & Gennis, 1988). The overexpression of the *bo*-type oxidase permits substantial characterization of mutants without significant interference from the *bd*-type oxidase. Therefore, the oxidase mutants may be characterized in a near-native environment within the cytoplasmic membrane, circumventing artifacts which might be introduced by detergent solubilization and purification procedures.

Recent work has demonstrated that >90% of the absorbance in the α-band region of the spectrum of the reduced *bo*-type oxidase arises from the low-spin, six-coordinate cytochrome b_{562} (Puustinen et al., 1991; Minghetti et al., 1992). The low-temperature visible spectra of the His284 mutants in the *E. coli* oxidase show that cytochrome b_{562} is unperturbed by the mutations (Figure 1). However, the visible CO difference spectra of the mutants show substantially reduced levels of CO binding, although the signature wavelengths (415-nm peak, 430-nm trough) are not significantly altered (Figure 2). At least in the case of the His284Gly mutant, it is possible to increase the level of bound CO by increasing the concentration of CO in the cuvette at high pressure (D. Lemon, personal communication), suggesting that the high-spin heme is inserted at the binuclear center in these mutants, but that CO binding is perturbed.

The low-temperature FTIR spectra of the CO-bound *E. coli* mutants are shown in Figures 3 and 4. The His284Gly and His284Leu mutants maintain an Fe–CO stretching band which is nearly identical to that of the wild-type Fe–CO band.

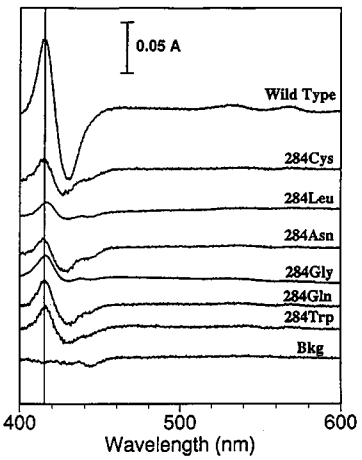

FIGURE 2: CO difference spectra of the mutants at His284 of the *E. coli bo*-type oxidase. Each sample contained 2 mg of membrane protein/mL. The peak at 415 nm and the trough at 430 nm are characteristic of CO binding to the oxidase. All of the mutations at His284 reduce the amount of CO binding. Bkg = background strain which lacks the *bo*-type oxidase.

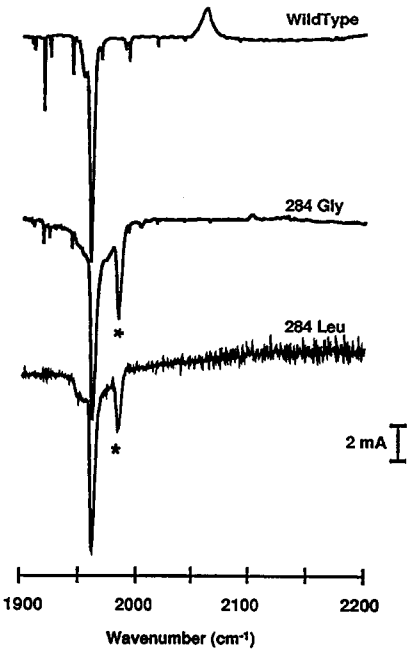

FIGURE 3: Light-minus-dark FTIR spectra of the membranes containing the overexpressed wild-type *E. coli bo*-type oxidase and the His284Gly and His284Leu mutants. Data collection is outlined under Materials and Methods. The Fe–CO band of the wild-type enzyme is at 1959 cm^{-1}, while those of the two mutants are at 1961 cm^{-1}. The feature at 1984 cm^{-1} is the Fe–CO band of the alternate *bd*-type oxidase of *E. coli* [see Hill et al., 1993)] and is indicated by an asterisk. The Cu–CO band in the photolyzed state of the wild-type oxidase is at 2065 cm^{-1}. This feature is absent from the spectra of the mutants. Spectra were collected with a sample path length of 19.5 μm at a temperature of 15 K.

In the His284Gly and His284Leu mutants, the center frequency is 1961 cm^{-1}, while in wild type it is 1959 cm^{-1}. The band width of the Fe–CO in these two mutants is only slightly larger than that of the wild-type enzyme. The Fe–CO band at 1984 cm^{-1} which is present in the mutant samples arises from the *bd*-type oxidase of *E. coli*, which is not a heme–copper oxidase (Hill et al.,1993). The FTIR spectra of the *E. coli* His284Gly mutant are identical at pH 5, 7.5, and 10, as is the case in the wild-type enzyme (data not shown).

Upon photolysis of the Fe–CO in heme–copper oxidases, the CO molecule binds to Cu_B (Alben et al., 1981; Einarsdóttir

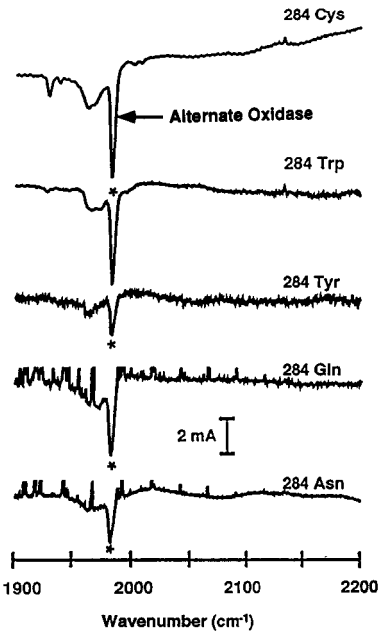

FIGURE 4: Light-minus-dark FTIR spectra of the membranes containing the overexpressed His284 mutants. Data collection is outlined under Materials and Methods. The sharp feature at 1984 cm^{-1} is the Fe–CO band of the alternate *bd*-type oxidase of *E. coli* [see Hill et al., (1993)] and is indicated by an asterisk. The sample path lengths ranged from 24.7 to 51 μm, and the spectra were collected between 15 and 25 K. A spectrum of the wild-type enzyme is shown in Figure 3.

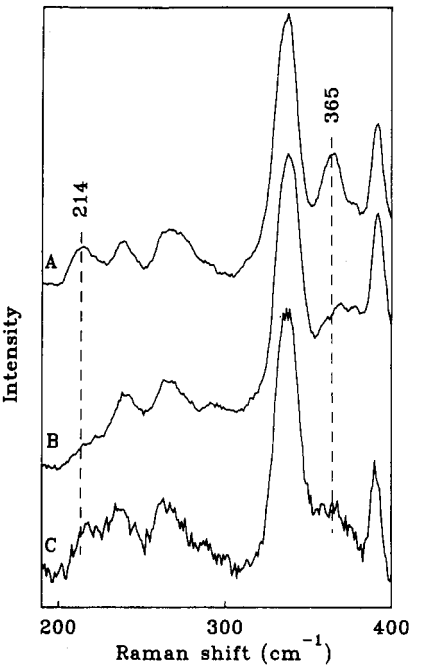

FIGURE 5: Resonance Raman spectra of fully reduced cytochrome *c* oxidase from *Rb. sphaeroides*. Shown are (A) the purified, wild-type enzyme (35 μM), (B) the purified, His284Ala mutant (35 μM), and (C) the lipid-rich form of the His284Ala mutant (14 μM). Indicated are the ring bending mode (365 cm^{-1}) and the Fe–N_{His} stretching mode (214 cm^{-1}) of heme a_3 (Shapleigh et al., 1992b). Acquisition of the resonance Raman spectra of the lipid-rich form of His284Ala (C) required concentration of the mutant oxidase, along with partial elimination of species that contribute to background fluorescence. Details of the preparation are outlined under Materials and Methods.

et al., 1989; Shapleigh et al., 1992a). This interaction must be monitored by time-resolved techniques at room temperature (Woodruff et al., 1991), but can be observed by standard FTIR spectroscopy of samples at low temperatures (<150 K). As described above, the Fe–CO bands of the His284Gly and His284Leu mutants in the *E. coli bo*-type oxidase are similar to the band present in the wild-type enzyme. However, the Cu–CO band, normally seen at 2065 cm^{-1}, appears to be absent from the "light" spectra of the His284Gly and His284Leu mutants. Under ideal instrumental conditions and with very thin samples (15–20 μm), multiple, small bands are evident at higher frequencies (above 2100 cm^{-1}) in the photolyzed state in the His284Gly and His284Leu samples. The band at 2133 cm^{-1} represents the photolyzed state of the *bd*-type oxidase (Hill et al., 1993), but other bands, such as the one at 2103 cm^{-1}, are the photolyzed states of the mutant *bo*-type oxidase.

The integrated area of the Fe–CO band (1961 cm^{-1}) can be used to predict the integrated band area occupied by the sum of the photolyzed states (Alben et al., 1981). The Fe–CO band in the His284Gly sample occupies 65 mA·cm^{-1} (milliabsorbance per centimeter). Accounting for the linear increase in extinction coefficient which occurs with frequency (Alben et al., 1981), the photolyzed state in the His284Gly sample should occupy 42 mA·cm^{-1} at 2030 cm^{-1} or 26 mA·cm^{-1} at 2080 cm^{-1}. With the signal-to-noise level of the spectrum of the His284Gly sample, if present, such a Cu–CO band centered at 2080 cm^{-1} might not be identifiable if the $\Delta\nu_{1/2}$ (full width at half-maximal absorbance) was greater than 50 cm^{-1}. This bandwidth is roughly 5 times the $\Delta\nu_{1/2}$ of the Cu–CO band in the wild type enzyme.

Two major differences have been identified between the CO adducts of heme–copper oxidases when compared with other CO binding heme proteins (*e.g.*, the globins) which do not contain copper. The first difference is the presence of Cu–CO adducts in the IR spectra of the photolyzed states of the heme–copper oxidases when recorded at low temperatures (Alben et al., 1981). The second difference is in the rate of relaxation of the CO to the heme iron as a function of temperature (Alben et al., 1981). The Cu–CO adduct of the heme–copper oxidases is significantly more stable to thermal dissociation of the CO than is the protein-bound CO adduct formed in other heme proteins upon photolysis of the heme–CO species. For example, at 120 K the photolyzed CO in the bovine cytochrome *c* oxidase forms a stable coordination bond with the Cu, while at this temperature the photolyzed CO in myoglobin relaxes back to the heme almost as fast as it is formed (Alben et al., 1981).

The apparent absence of a Cu–CO form in the photolyzed state of the His284Gly and His284Leu CO adducts suggests that these mutations might increase the rate of relaxation of the CO species following the photolysis event. Preliminary analysis of the kinetics of CO relaxation suggests that in contrast to the expected increase in rate, the rate actually decreases by 1–2 orders of magnitude (data not shown) from that of the wild-type enzyme (Hill, unpublished data).

The Fe–CO stretching frequency band in all of the other *E. coli* H284 mutants is broad and, in some cases, rather undefined (Figure 4). As in the His284Gly and His284Leu mutants, there are no obvious Cu–CO species in the photolyzed states. For example, prediction of the expected integrated area of a Cu–CO species in His284Trp suggests that a signal with a $\Delta\nu_{1/2}$ of greater than 20 at 2080 cm^{-1} might not be recognized.

Resonance Raman spectra of the wild type and the His284Ala mutant of the aa_3-type oxidase of *Rb. sphaeroides* are shown in Figure 5. As previously noted (Shapleigh et al., 1992b), two modes specific for the interaction of heme a_3 with

the protein, the Fe–N_{His} stretch at 214 cm^{-1} and a ring bending mode at 365 cm^{-1}, are greatly diminished in the spectrum of the purified His284Ala mutant, presumably due to broadening of these signals as the heme assumes multiple orientations within its binding pocket. The same is true for the formyl stretching mode of heme a_3 at 1662 cm^{-1} (Shapleigh et al., 1992b). Careful inspection, however, shows that a 214-cm^{-1} mode can be distinguished in the spectrum of the purified mutant oxidase (Figure 5B). This observation prompted the determination of the spectrum in a sample that was not depleted of bacterial lipids, which occurs upon purification of the enzyme. Such samples were prepared by detergent solubilization of the membranes, but omission of any chromatography. The spectrum of this more native "lipid-rich" form (Figure 5C) clearly shows the presence of the Fe–N_{His} stretching mode which has a slightly shifted frequency. Consistent with this, resonance Raman spectra (not shown) of His284Ala partially purified by chromatography also show an essentially native Fe–N_{His} stretch. In both the lipid-rich and partially purified forms of the mutant enzyme, the 365- and the 1662-cm^{-1} signals are significantly smaller than in the wild-type sample. These data suggest that conformers of His284Ala exist in which the normal ligation of heme a_3 is retained, even though other aspects of the heme environment are altered.

In order to assess the role of the other possible a_3 ligand, His419 (Shapleigh et al., 1992b; Hosler et al., 1993), lipid-rich samples of the solubilized *Rb. sphaeroides* His419Asn enzyme were prepared as described for His284Ala. The resonance Raman spectra (not shown) of these samples show no indication of the Fe–N_{His} stretch, consistent with the previously reported absence of this band in the purified form of this mutant oxidase (Shapleigh et al., 1992b).

DISCUSSION

Previous analyses of site-directed mutants in the heme–copper oxidases from *E. coli* and from *Rb. sphaeroides* identified two conserved histidine residues (His284 and His419) which might form the axial ligand to the oxygen binding heme (Lemieux et al, 1992; Minagawa et al., 1992; Shapleigh et al., 1992b). Further analyses of mutants at His284 are presented here which demonstrate that this residue is not the ligand of heme a_3, leading to the conclusion that His419 is the ligand of heme a_3.

Carbon monoxide is a useful probe because of its strong infrared absorption and its high affinity for oxidases. In addition, the center frequency and the band shape of the CO stretching mode are very sensitive to the coordination state and surrounding protein "solvation shell" of the CO molecule (Alben & Fiamingo, 1984). The stretching frequency of the CO molecule is directly related to the strength of the carbon–oxygen bond. In CO bound to heme proteins, it has been shown that the stretching frequency of the iron–carbon bond is inversely proportional to the stretching frequency of the carbon–oxygen bond [see Rousseau et al., (1993) and references cited therein]. Since the protein ligand in the fifth coordination site of the heme iron makes a large contribution to the electron density on the iron, a change in the axial ligation would be expected to have a significant effect on the stretching frequency of the bound CO.

The experimentally-measured range of the stretching frequency of CO bound to heme proteins is approximately 1905–1984 cm^{-1} (Caughey, 1980; Hill et al., 1993; Einarsdóttir et al., 1989). Although predictions of environment resulting in any given frequency are not yet possible, the frequency is known to reflect the combined effects of the electronic character of the iron, the angle of the bound CO with respect to the heme plane, and the specific interactions of CO with the distal heme pocket. It has been demonstrated that the CO adduct of myoglobin exists in several states, which can be characterized by the angle of the Fe—C═O vector. These states have center frequencies of 1966, 1945, and 1929 cm^{-1}, which correspond to deviations of the bound CO from the perpendicular by 15, 28, and 33°, respectively (Ormos et al., 1988). In the case of the His284Gly and His284Leu mutants of the *bo*-type oxidase which are presented here, only small (<2 cm^{-1}) deviations occur from the CO stretching frequency recorded for the wild-type enzyme. This suggests that the axial ligation of heme *o* in the His284Gly and the His284Leu mutants is identical to that of the wild-type enzyme.

The environment of the CO molecule also determines the bandwidth of its stretching frequency (Alben & Fiamingo, 1984; Caughey, 1980). When bound to a heme protein, the environment surrounding the CO molecule consists of the distal heme pocket. The bandwidth of the CO stretch in the wild-type *bo*-type oxidase (about 4 cm^{-1}; Hill et al., 1992) is small in comparison with most CO-bound heme proteins. For example, the bandwidth of the CO stretch in hemoglobin is 8 cm^{-1}, while that of cytochrome $P450_{cam}$ in the absence of substrate is 23 cm^{-1} (Caughey, 1980). A small bandwidth is indicative of a highly ordered environment. The bandwidths of the CO stretches in the His284Gly and His284Leu mutants are nearly the same as that of the wild-type complex, indicating that the distal heme pocket in the mutants remains very ordered and very similar to that of the wild-type enzyme. This contrasts with the large bandwidths (on the order of 7–25 cm^{-1}) found with the mutants of His333 and His334 of the *E. coli* oxidase (Calhoun et al., 1993).

The FTIR spectra of the photolyzed CO state demonstrate that the wild-type binuclear center is perturbed in the His284Gly and Leu mutants. The copper–CO band at 2065 cm^{-1} appears to be absent in all of the mutants at His284, and no CO stretching frequencies arising from CO bound to copper are identifiable in the "light" spectra of the His284Gly and His284Leu mutants. In the His284Gly and the His284Leu mutants, a small band is present in the photolyzed state at 2103 cm^{-1}, but at this time, it cannot be conclusively determined whether this band arises from a weak interaction of CO with copper or from a strong, noncovalent interaction of the CO molecule with polar protein residues. The integrated area of the 2103-cm^{-1} band is too small to account for all of the CO photolyzed from the heme iron. Several other small bands appear between 2103 and 2143[4] cm^{-1}. The bands in this region probably arise from interactions between the CO molecule and the protein surface of the heme's distal pocket. The low temperature maintained during the FTIR experiments (<200 K) precludes the loss of CO from the protein in the frozen glass state (Steinbach et al., 1991).

The relaxation kinetics of the photolyzed state of the CO in the mammalian oxidases have been extensively characterized (Fiamingo et al., 1982, 1986). Similar studies of the CO relaxation kinetics in the *E. coli bo*-type oxidase reveal characteristics quite similar to those of the mammalian enzymes (Hill, Gennis, and Alben, manuscript in preparation). The relaxation rate of the CO from the Cu to the heme iron

[4] CO stretching frequencies above 2143 cm^{-1} are not expected, as this is the stretching frequency of the free gas state (Jiang et al., 1975).

in the His284Gly mutant is dramatically slower than in the wild-type enzyme (data not shown). This reduction in rate also occurs at room temperature where measurements by visible spectroscopy also demonstrate that the relaxation occurs about 2 orders of magnitude more slowly than in the wild-type enzyme (D. Lemon, Calhoun, Gennis, and W. Woodruff, manuscript in preparation; S. Brown, J. Rumbley, Gennis, Thomas, and P. Rich, submitted for publication). At least two alternative explanations of this change exist. If the copper is present, it must bind the CO in a much more disordered state, such that no IR band is evident. This proposed disorder, in addition to the increase in stability of the hypothetical Cu–CO suggests a major change in the ligation sphere of Cu_B. A corollary to this first proposal may be the assignment of His284 as a ligand of Cu_B.

A second alternative explanation for the change in the rate of CO relaxation may not directly involve Cu_B. In this proposed mechanism, the CO is allowed to move out of the heme binding pocket due to the increased protein motions as a result of the heat input during the photolysis of the heme Fe–CO bond (Henry et al., 1986). Following photolysis, the heat dissipates, and the protein components, which have less mobility at the lower temperature, form what can be termed a "van der Waals trap", which allows only slow relaxation of the CO to the heme iron.

The differences between the CO stretching frequencies of the His284Gly and Leu mutants and the other His284 mutants (Asn, Cys, Gln, Tyr, Trp) suggest that the hydrophobic nature of Gly and Leu may be key to maintenance of an intact heme pocket in the absence of the native amino acid. Although Trp is often considered a hydrophobic amino acid, it contains an imino group which has some polar character.

The results presented here indicate that a near-normal environment for CO bound to heme a_3 can coexist with disruptions in the Cu_B environment in the reduced enzyme. Thus, in contrast to the oxidized form where the two metals closely interact (van Gelder & Beinert, 1969; Scott, 1989; Powers & Kincaid, 1989), in the reduced binuclear center the metals would appear to be distinctly separated, as has previously been proposed (Powers et al., 1981; Alben et al., 1982; Scott, 1989; Oertling et al., 1990).

The identification of the $Fe–N_{His}$ mode in the lipid-rich and partially purified forms of the His284Ala mutant of *Rb. sphaeroides*, as well as the lack of this mode in the spectra of His419Asn, provides evidence that His284 is not the axial ligand of heme a_3. Since alanine cannot function as a metal ligand, the only alternate interpretation of these results is that another residue of the heme pocket replaces His284 as the axial ligand. However, it is highly unlikely that such a ligand replacement would yield the same bond strength, as measured by the frequency of the $Fe–N_{His}$ mode. Attenuation of the 214-cm^{-1} mode of His284Ala does occur during the purification process, which may be the result of loss of the bacterial lipids. Presumably the signal is broadened due to rearrangements of the heme a_3-Cu_B pocket. This reveals that His284 plays an important role in the maintenance of the structure of the binuclear center, although it does not bind the iron of heme a_3.

It is not clear from the results presented herein whether His284 is a ligand for Cu_B, although it is apparent that mutations in this residue must alter either the ligation sphere of Cu_B or the pathway between the heme and the Cu. Atomic absorption analysis of the His284Ala mutant of the *bo*-type oxidase has demonstrated that copper is present in this mutant (Minagawa et al., 1992), although the ligation sphere of the copper in this mutant is not known. Electron paramagnetic resonance studies also suggest that the His284Ala mutant of the *Rb. sphaeroides* enzyme contains Cu_B (Hosler et al., unpublished results). Analysis of EXAFS data obtained with both the aa_3-type enzyme and also the *E. coli bo*-type oxidase suggests that as few as two histidines may ligate to Cu_B (Scott et al., 1986; Ingledew & Bacon, 1991). Additional reports propose that the number of histidines bound to Cu_B is three or four (Reinhammar et al., 1980; Surerus et al., 1992). Clearly, His333 and His334 could fulfill the requirement for two residues, as shown in Figure 6.

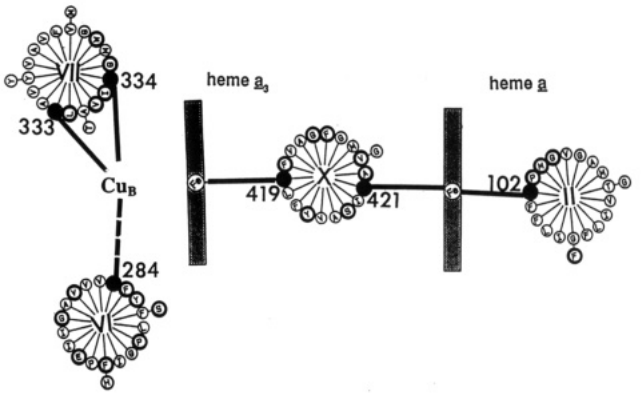

FIGURE 6: Model of the ligation of heme *a*, heme a_3, and Cu_B by the six conserved histidines of subunit I of the heme–copper oxidases. The analogous centers of the *bo*-type oxidase are, respectively, heme b_{562}, heme *o*, and Cu_B. Helical wheel representations of helices II, VI, VII, and X are shown, using the sequence of the aa_3-type oxidase of *Rb. sphaeroides*. The histidine ligands are in filled circles and are numbered according to the sequence of the *Rb. sphaeroides* enzyme. Other highly conserved residues among the heme–copper oxidases are indicated with bold circles. The ligation of Cu_B by His284 is shown in the dashed lines to indicate the uncertainty of its function in the ligation of this center.

The studies presented here demonstrate that His284 is not a candidate for ligation of the oxygen binding heme of the heme–copper oxidases. Previous studies suggested only one other conserved histidine residue which might fulfill this role. In the primary amino acid sequence, this residue, His419, is separated by only one residue from His421, which has been identified as a ligand for the low-spin six-coordinate heme (Lemieux et al., 1992; Minagawa et al., 1992; Shapleigh et al., 1992b). This region of the protein has been proposed to be in a membrane-spanning α-helix. Therefore, these two heme binding residues would be located on approximately opposite faces of a single helix (Figure 6).

Previous models of the structure of heme binding in the heme–copper oxidases have placed Cu_B between the two heme groups, while this model places the oxygen binding heme between the low-spin, six-coordinate heme and Cu_B [Holm et al., 1987; Lemieux et al., 1992; Minagawa et al., 1992; Shapleigh et al., 1992b; but see Brown et al., (1993) and Hosler et al., (1993)]. In addition, previously proposed models for the catalytic activity of the oxidase have often suggested electron transfer from the low-spin heme *a* to Cu_B prior to transfer to the heme of the binuclear center (heme a_3) (Babcock & Wikström, 1992). However, the relative placement of the metal centers defined by the mutant analysis suggests that electron transfer is unlikely to be more rapid between heme *a* and Cu_B than between heme *a* and heme a_3. Consequently, the role of Cu_B in enzyme function might be different than has been previously proposed in electron transfer. However, ligand changes during redox cycling at heme a_3 or Cu_B could affect the midpoint potentials of these metals, allowing control of the pathway of electron transfer (Hosler et al., 1993;

Woodruff, 1993; Rousseau et al., 1993; Larsen et al., 1992). The proposal that Cu_B serves as a CO and oxygen shuttle to the binuclear center is not affected by the results presented herein (Alben et al., 1981; Woodruff et al., 1991; Blackmore et al., 1991).

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