# Spectroscopic Characterization of Mutants Supports the Assignment of Histidine-419 as the Axial Ligand of Heme *o* in the Binuclear Center of the Cytochrome *bo* Ubiquinol Oxidase from *Escherichia coli*†

Melissa W. Calhoun,‡ Laura J. Lemieux,‡,§ Jeffrey W. Thomas,‡ John J. Hill,‡ Visala Chepuri Goswitz,‡,∥ James O. Alben,⊥ and Robert B. Gennis*,‡

*School of Chemical Sciences, University of Illinois at Urbana—Champaign, Urbana, Illinois 61801, and Department of Medical Biochemistry, The Ohio State University, Columbus, Ohio 43210*

*Received June 16, 1993; Revised Manuscript Received September 13, 1993*®

ABSTRACT: The *bo*-type ubiquinol oxidase of *Escherichia coli* is a member of the superfamily of heme–copper oxidases which also includes the $aa_3$-type cytochrome *c* oxidases. The oxygen-binding binuclear center of cytochrome *bo* is located in subunit I and consists of a heme (heme *o*; heme $a_3$ in the $aa_3$-type oxidases) and a copper ($Cu_B$). Previous spectroscopic studies have shown that heme *o* is bound to the protein via a single histidine residue. Site-directed mutagenesis of conserved histidine residues in subunit I has identified two residues (H284 and H419) which are candidates for the ligand of heme *o*, while spectroscopic studies of mutants at H284 definitively demonstrated that this residue cannot be the axial ligand. Consequently, the single remaining conserved histidine in subunit I (H419) was assigned as the ligand for the heme of the binuclear center. In this paper, this assignment is tested by characterization of additional mutants in which the putative heme *o* axial ligand, H419, is replaced by other amino acids. All mutations at H419 result in the loss of enzyme activity. Analyses via UV–visible and Fourier transform infrared spectroscopies reveal that substantial perturbation has occurred at the binuclear center as a result of the amino acid substitutions. In contrast with the wild-type enzyme, the mutant enzymes bind very little carbon monoxide. Three other amino acid residues which are potential ligands for heme *o* are shown to be nonessential for enzyme activity. Mutations in these residues do not perturb the UV–visible or FTIR spectroscopic characteristics of the enzyme. These results are consistent with the assignment of H419 as the axial ligand of heme *o* in the *E. coli* enzyme and, by analogy, heme $a_3$ in the $aa_3$-type cytochrome *c* oxidases.

The cytochrome *bo* quinol oxidase from *Escherichia coli* and the $aa_3$-type cytochrome *c* oxidases are members of a superfamily of structurally related heme–copper respiratory oxidases (Chepuri et al., 1990b; Saraste et al., 1989). The heme–copper binuclear center, the site of oxygen reduction to water, is diagnostic of this class of respiratory oxidases, and consists of a high-spin heme magnetically coupled to a copper, denoted $Cu_B$ (Chepuri et al., 1990a; Hill et al., 1992; Hosler et al., 1993; Woodruff, 1993). A second heme, which is six-coordinate and low-spin, is also present in all members of this superfamily. Vectorial proton translocation across the membrane bilayer appears to be another common feature of the heme–copper oxidases (Puustinen et al., 1991; Larson et al., 1992; Babcock & Wikström, 1992; Hosler et al., 1993a).

The metal ligands for both cytochromes as well as for $Cu_B$ are located within subunit I of the heme–copper oxidases (Saraste, 1990). In the $aa_3$-type cytochrome *c* oxidases, hemes *a* and $a_3$ are, respectively, the low-spin and the high-spin heme components. Spectroscopic studies have shown that heme *a* has two histidine axial ligands while heme $a_3$ has one histidine axial ligand (Eglinton et al., 1980; Palmer, 1983; Stevens & Chan, 1981). Similar spectroscopic studies of the *bo*-type oxidase of *E. coli* also demonstrated that the low-spin heme (denoted heme $b_{562}$) has two histidine ligands (Salerno et al., 1990; Cheesman et al., 1993; Ingledew et al., 1993). Analyses of cytochrome *bo* via magnetic circular dichroism and electron paramagnetic resonance have shown that the high-spin heme (heme *o*) has one histidine ligand (Cheesman et al., 1993; Ingledew et al., 1993). $Cu_B$ appears to bind to two or more histidines (Cline et al., 1983; Powers et al., 1987; Scott et al., 1986; Surerus et al., 1992; Ingledew & Bacon, 1991).

The high degree of similarity among the primary sequences of the enzymes (Saraste, 1990), the structural similarities revealed by spectroscopic studies (Salerno & Ingledew, 1991; Calhoun et al., 1992; Hill et al., 1992; Tsubaki et al., 1993; Wang et al., 1993; Watmough et al., 1993), and also the identical function of the members of the heme–copper oxidase superfamily make it likely that the amino acids which form the ligands of the metals are conserved throughout. Mutagenesis studies of the *bo*-type oxidase from *E. coli* and the $aa_3$-type oxidase of *Rhodobacter sphaeroides* are consistent with the proposal of a similar structure (Minagawa et al., 1992; Lemieux et al., 1992; Hosler et al., 1993; Calhoun et al., 1993a). Sequence alignments of all known sequences [approximately 75 in the GenBank database (Bilofsky & Burks, 1988)] of subunits I of the heme–copper oxidases reveal only 6 totally conserved histidines (Saraste, 1990; Calhoun, unpublished results). These six histidines (H106, H284, H333, H334, H419, and H421) are depicted in a two-dimensional model of subunit I (Figure 1). Also shown in Figure 1 is the location of H411, which is conserved in all of the subunit I sequences except the two from *Bradyrhizobium japonicum*,

† This work was supported by grants from the U.S. Department of Energy (R.B.G.) (DEFG02-87ER13716) and the National Science Foundation (J.O.A.) (DMB89-04614).

‡ University of Illinois at Urbana—Champaign.

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

∥ Present address: Department of Chemistry, University of St. Thomas, St. Paul, MN 55105.

⊥ The Ohio State University.

® Abstract published in *Advance ACS Abstracts,* November 15, 1993.

0006-2960/93/0432-13254$04.00/0 

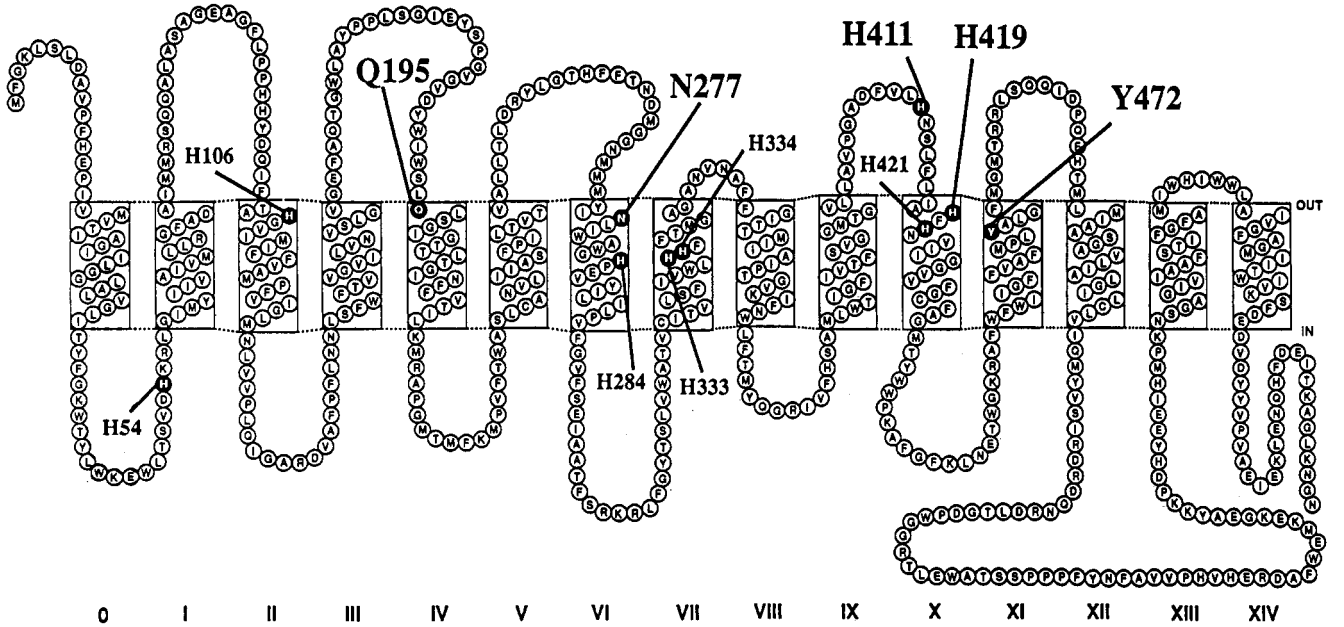


FIGURE 1: Two-dimensional topological model of subunit I of the *E. coli bo*-type oxidase. Topology is based on hydropathy profiling and gene fusions (Chepuri & Gennis, 1990). Highlighted in larger letters are five residues which were mutated in this study. Other histidine residues which have been examined in previous studies (Minagawa et al., 1992; Lemieux et al., 1992) are indicated in smaller letters.

which have a glutamine and a serine at the equivalent position (Bott et al., 1990, 1992; Gabel & Maier, 1990). Another less conserved histidine (H54) which has been studied in *E. coli* is also noted (Minagawa et al., 1992).

Site-directed mutagenesis of the *E. coli* enzyme has been used to determine the residues required for the binding of prosthetic groups. Each of the residues named above has been changed to other amino acids, and the resultant mutant proteins have been characterized by complementation (*in vivo* activity) analysis, as well as visible and, in some cases, electron paramagnetic resonance and Fourier transform infrared (FTIR)[1] spectroscopies (Lemieux et al., 1992; Minagawa et al., 1992; Calhoun et al., 1993a,b). The results of these studies, in combination with similar studies of the $aa_3$-type cytochrome *c* oxidase of *Rb. sphaeroides*, have identified the two histidines (H106 and H421 in the *E. coli* oxidase) which bind the low-spin heme $b_{562}$ (Lemieux et al., 1992; Minagawa et al., 1992; Shapleigh et al., 1992a). Two other residues (H333 and H334) are unnecessary for heme binding, but are essential for the assembly of the $Cu_B$ site (Lemieux et al., 1992; Minagawa et al., 1992; Hosler et al., 1993; Calhoun et al., 1993b). H54 and H411, two less conserved residues, have previously been shown to be nonessential for enzyme activity (Lemieux et al., 1992; Minagawa et al., 1992). Spectroscopic studies of mutants at H284 in both the *E. coli* and the *Rb. sphaeroides* enzymes demonstrated that this residue is not the ligand for the heme component of the binuclear center (Hosler et al., 1993a; Calhoun et al., 1993a).

If the interpretation of spectroscopic studies previously performed with the wild-type cytochrome *bo* and the assumption of similar structures among the heme–copper oxidase superfamily are correct, then the axial ligand of heme *o* must be H419, the only remaining totally conserved histidine in subunit I of the heme–copper oxidases. Despite spectroscopic evidence to the contrary, there is an unlikely possibility that the axial ligand of the high-spin heme component in cytochrome *bo* from *E. coli* is not a histidine. Three histidines are conserved in the vast majority of sequences of subunit I which are not present in the sequence of cytochrome *bo* from *E. coli*. These residues are predicted to be located on the periplasmic end of putative transmembrane helices, and are replaced in the sequence of cytochrome *bo* by a residue that could, in principle, act as a heme ligand (Q195, N277, and Y472). One goal of the work presented in this paper was to test the possibility that one of these residues might be the axial ligand of heme *o*. The results clearly show that this is not the case.

The assignment of H419 as the axial ligand of heme *o* was examined further through the construction of several mutations in which potential heme ligands were substituted for H419. In all cases, the resulting enzyme is inactive, but H419N retains a binuclear center in which the structure is clearly perturbed. It is likely that asparagine can substitute for H419 as the axial ligand for heme *o*, albeit without retention of function. The proposed assignment of H419 as the heme *o* axial ligand predicts that other residues located nearby in the sequence might be important for maintaining the structural integrity of the binuclear center. For example, H411, which was previously shown to be nonessential, might be expected to be near the binuclear center, as it is only eight residues from H419. Although H411G is indistinguishable from the wild-type oxidase, substitution of leucine (H411L) results in the loss of activity and the severe disruption of the binuclear center, with little effect at heme $b_{562}$. This result is consistent with the current model of the structure of the oxidase, in which H419 is assigned as the axial ligand to the oxygen-binding heme *o*.

## 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 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 H411L, H411G, H419L, and H419N mutants have been described

[1] Abbreviations: FTIR, Fourier transform infrared (spectroscopy); $\nu$(Cu—C≡O), stretching frequency of CO bound to copper; $\nu$(Fe—C≡O), stretching frequency of CO bound to iron.

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

*Expression and Spectroscopic Characterization of Mutants.* To identify mutants which maintain enzyme function, complementation analysis was performed in either strain GO105 or strain RG129, as previously outlined (Lemieux et al., 1992). Strain RG129 (*cyo cyd rec*A) lacks both of the terminal oxidases of *E. coli* (Au et al., 1985). Strain GO105 contains a point mutant in the *cyo* gene and a deletion of the *cyd* gene, and it is recombination-defective (*recA*). It was constructed by bacteriophage P1 transduction of the *rec*A allele into strain GO102 (Oden & Gennis, 1991).

Growth and expression of the mutants were performed as described using the host strain GL101 (*cyo sdh rec*A) or the host strain GO105 (*cyo* Δ*cyd rec*A) (Lemieux et al., 1992). Under anaerobic conditions, expression of the *bo*-type oxidase is greatly repressed (Iuchi et al., 1990; Minagawa et al., 1990). To grow *E. coli* aerobically, a functional terminal oxidase must be present (Au et al., 1985). Therefore, to express inactive mutants, it is necessary to have a functional form of the *bd*-type oxidase, which has three heme components, $b_{558}$, $b_{595}$, and *d* (Lorence et al., 1986; Anraku & Gennis, 1987; Anraku, 1988). Therefore, inactive mutants were expressed in the host strain GL101, where overexpression of the cytochrome *bo* complex permits substantial characterization of mutants without significant interference from the *bd*-type oxidase. This allows characterization of the mutants in a near-native environment within the cytoplasmic membrane, circumventing artifacts which might be introduced by detergent solubilization and purification procedures. Active mutants can be expressed in the absence of the *bd*-type oxidase, and for these mutants, the host strain GO105 was employed.

The ubiquinol oxidase activity of the mutants which were able to restore aerobic growth in the complementation test was measured by monitoring the oxidation of NADH in membrane samples. Quantitation of *bo*-type oxidase was based upon the amount of CO binding, as calculated from the CO difference spectrum, using the extinction coefficient 135 $mM^{-1}$ $cm^{-1}$ for the peak (415 nm) to trough (430 nm) difference (Kita et al., 1984). NADH oxidase assays were performed spectrophotometrically as previously described (Matsushita et al., 1987).

Visible spectroscopy was performed with membrane preparations as previously described (Lemieux et al., 1992). The collection of cryogenic Fourier transform infrared absorption difference spectra of carbonmonoxy adducts of the *E. coli* oxidase was performed as previously described and outlined briefly below (Hill et al., 1992; Calhoun et al., 1993a,b). Infrared spectra were collected with a Mattson Sirius 100 FTIR interferometer at 0.5-$cm^{-1}$ resolution. Cryogenic temperatures of 10–20 K were measured and maintained by a Lake Shore Cryotronics closed-cycle helium refrigerator. The spectra are presented as absorbance 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 (2-in. path length) and glass (0.5-in. path length). Where indicated, 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.

The sample used for collection of the carbonmonoxy spectrum of the alternative oxidase from *E. coli*, the cytochrome *bd* complex, was prepared as described from a strain which overexpresses this oxidase (Hill et al., 1993). The sample used for collection of the FTIR spectrum of the wild-type cytochrome *bo* contains purified cytochrome *bo* prepared according to a published method (Minghetti et al., 1992).

## RESULTS

Previously published experiments have eliminated five of the six totally conserved histidines in subunit I as candidates for the axial ligand of heme *o* (Lemieux et al., 1992; Minagawa et al., 1992; Calhoun et al., 1993a). The primary purpose of this work is to examine the consequences of substituting a variety of amino acid residues for the remaining histidine, H419. Preliminary characterization of alanine, leucine, glycine, and asparagine substitutions for H419 has been reported (Lemieux et al., 1992; Minagawa et al., 1992). In the current work, H419G, H419L, and H419N are examined in more detail, and five additional mutations were also constructed (H419C, H419Y, H419D, H419M, and H419Q). Cysteine, tyrosine, and methionine are heme ligands in other heme proteins (Poulos, 1988; Poulos & Finzel, 1984; Simpkin et al., 1989). None of the mutations at H419 restore aerobic growth to oxidase-deficient *E. coli* strains, demonstrating that H419 is essential for activity of the enzyme.

Visible spectroscopy of the H419 mutants in the *E. coli* membrane shows that the split α band of heme $b_{562}$ is not purturbed by each of the mutations (Figure 2A). However, the CO difference spectra of the mutants show drastically reduced levels of CO binding (Figure 2B), indicating a perturbation of the heme–copper binuclear center. The apparent loss of CO binding could mean that heme *o* is absent or is present in low amounts, or that the affinity for CO is substantially reduced.

The low-temperature FTIR absorbance difference spectra of the CO adducts of the two oxidases from *E. coli* are shown in Figure 3. The sample of the wild-type cytochrome *bo* oxidase is of pure cytochrome *bo* complex, isolated according to previously published methods (Minghetti et al., 1992). As previously reported (Hill et al., 1992), this heme–copper oxidase has an Fe—C≡O band at 1959 $cm^{-1}$ and in the photolyzed state, a Cu—C≡O band with the major peak at 2065 $cm^{-1}$ and a shoulder at 2054 $cm^{-1}$ (Figure 3A). Figure 3B shows the spectrum of the alternate oxidase of *E. coli*, the cytochrome *bd* complex. The Fe—C≡O band in this oxidase is located at 1984 $cm^{-1}$, and the photodissociated state at 2133 $cm^{-1}$ represents CO bound to the surface of the heme pocket. This alternate cytochrome *bd* oxidase is present in the membranes of the mutants at H419.

The low-temperature FTIR absorbance difference spectra of the CO adducts of the H419 mutants are shown in Figure 4. In contrast to the wild-type oxidase (Figure 3A), the CO adducts of H419D, H419G, H419M, and H419L have FTIR absorbance difference spectra which lack features attributable to cytochrome *bo*. These data are consistent with either the loss of the CO-binding heme *o* or the occlusion of the CO-binding site, as is also suggested by the visible spectra shown in Figure 2B. The spectrum of H419L is representative of this group (Figure 4A). The sharp trough at 1984 $cm^{-1}$, which is very apparent in the spectrum of H419L and in the spectra of most of the other mutants, is due to CO bound to the

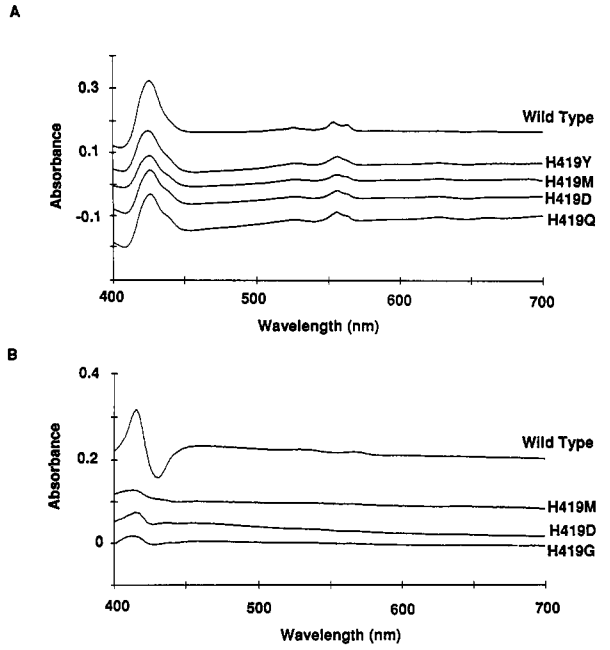


FIGURE 2: UV–visible spectra of a representative set of H419 mutants. (A) Samples contained 8 mg of membrane protein/mL. Spectra were recorded at 77 K and are presented as dithionite-reduced *minus* air-oxidized visible spectra. The presence of the cytochrome *bo* complex is evident from the split $\alpha$-band near 560 nm and particularly the band at 564 nm, which is at lower energy than the other features in these samples. The feature at 650 nm arises from the alternate *E. coli* oxidase, the cytochrome *bd* complex, which also has contributions in the 415- and 560-nm regions. (B) Visible spectra of the CO adducts of a representative set of H419 mutants. Samples contained 2 mg of membrane protein/mL. Spectra are presented as (dithionite-reduced *plus* CO) *minus* dithionite-reduced difference spectra. The lack of the characteristic peak (415 nm) and trough (430 nm) indicates the loss of the CO-binding component of the *bo*-type oxidase resulting from the mutations at H419.

alternate oxidase in *E. coli*, cytochrome *bd* (Figure 3B; Hill et al., 1993). The relative amounts of cytochrome *bo* and cytochrome *bd* can be controlled by the cell growth phase. The ratio of cytochrome *bo* to cytochrome *bd* decreases rapidly over a narrow range between the early and late logarithmic growth phases (Minagawa et al., 1990). This can be seen in the contrast between the small amount of the alternate oxidase in the H419N sample (Figure 4C) compared to the amount in the H419L sample (Figure 4A), which reflects a difference in the cell density at the time of cell harvest. The presence of the alternate oxidase provides an internal control to ensure the preparation of the carbon monoxide complex of the sample.

The H419C enzyme has a unique FTIR spectrum (Figure 4B), in that the "dark" and photodissociated states appear to have the same center frequency (1957 $cm^{-1}$). The trough from the "dark" state appears to be split by a positive peak from the photodissociated state. The spectrum of this mutant suggests that upon photolysis, CO rebinds to the same heme but that, although the new Fe—C≡O adduct has the same center frequency, the bandwidth is smaller. Preliminary results suggest that the relative populations of the two states of the Fe—O≡O adduct of this enzyme vary with temperature (M. W. Calhoun, unpublished results).

The spectrum of the H419N mutant (Figure 4C) has a $\nu$(Fe—C≡O)[2] at 1951 $cm^{-1}$, which is 8 $cm^{-1}$ less than that of the wild-type enzyme. This spectrum also contains a $\nu$(Cu—C≡O)[3] with a center frequency of approximately 2045 $cm^{-1}$, about 20 $cm^{-1}$ lower than that of the wild-type enzyme.

[2] Stretching frequency of CO bound to iron.

[3] Stretching frequency of CO bound to copper.

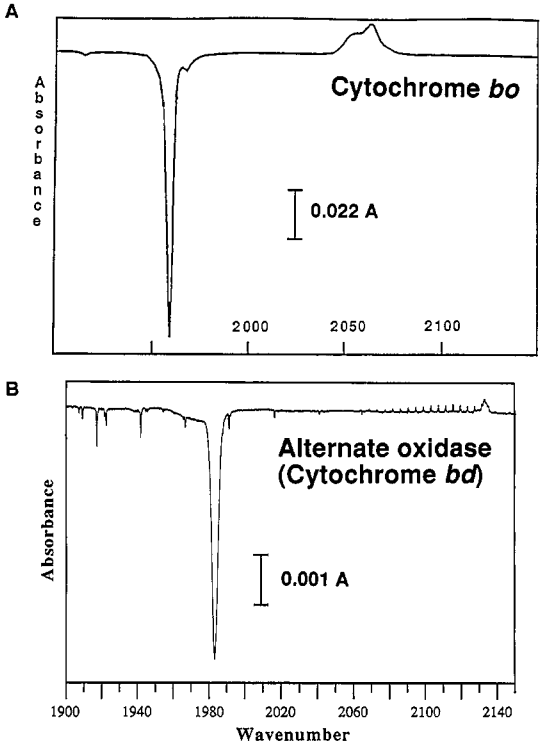


FIGURE 3: FTIR spectra of the two oxidases from *E. coli*. Spectra are presented as "light" *minus* "dark" spectra, as defined in the text. The sample temperature and path length are listed. (A) Wild-type cytochrome *bo*, 12 K, 95 $\mu$m; (B) alternate oxidase, cytochrome *bd*, 15.5 K, 38 $\mu$m.

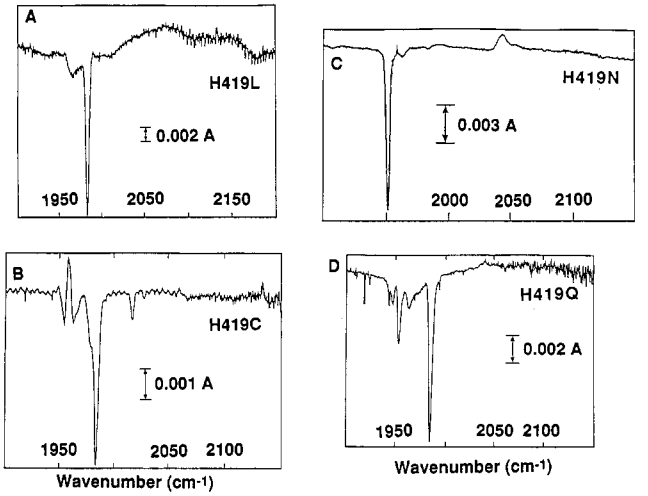


FIGURE 4: FTIR spectra of the H419L, H419C, H419N, and H419Q mutants of the *E. coli bo*-type oxidase. Spectra are presented as "light" *minus* "dark" spectra, as defined in the text. The Fe—C≡O band (trough) at 1984 $cm^{-1}$ in the mutant samples arises from the alternate *E. coli* oxidase, the cytochrome *bd* complex (see Figure 3B; Hill et al., 1993). The sample temperature, the path length, and the frequencies of the Fe—C≡O bands and Cu—C≡O bands attributed to the *bo*-type oxidase are listed. If the spectrum was subjected to base-line correction (see Materials and Methods), the note "b.c." is present. (A) H419L: 12 K, 16 $\mu$m, negligible signal at 1962 $cm^{-1}$ (Fe). (B) H419C; 18 K, 51 $\mu$m, 1957 $cm^{-1}$ (Fe), b.c. (C) H419N: 11 K, 27 $\mu$m, 1951 $cm^{-1}$ (Fe), 2043 $cm^{-1}$ (Cu, major form). (D) H419Q: 22 K, 25 $\mu$m, 1952 $cm^{-1}$ (Fe, major form).

Both the $\nu$(Fe—C≡O) and the $\nu$(Cu—C≡O) have approximately the same bandwidths as the corresponding features in the spectrum of the wild-type enzyme. Overall, these data show that the binuclear center of the H419N mutant is highly ordered, and similar to the wild type, but that the CO, when bound to heme *o* or to $Cu_B$, is sensing a different environment than in the wild type.

Table I: Complementation Analysis and Activity Assays of Mutants

| mutant | complementation[a] | activity[b] |
|---|---|---|
| wild type | + | 100 |
| Q195E | + | 131 |
| N227D | + | 28 |
| N277H | + | 101 |
| N277Y | + | 56 |
| H419[d] | - | ND[c] |
| Y472H | + | 25 |

[a] Complementation was assessed as described under Materials and Methods. [b] NADH oxidase activity was measured spectrophotometrically and is expressed as a percentage of the wild-type activity [5.0 μmol of NADH oxidized $min^{-1}$ (nmol of cytochrome *bo*)$^{-1}$]. [c] ND = not determined. Activity levels of mutants which cannot support aerobic growth (negative result in complementation test) cannot be determined in membrane preparations. These mutants can be expressed only in a strain which also contains the alternate oxidase of *E. coli*, cytochrome *bd*. [d] C, D, G, L, M, N, Q, and Y substitutions for H419.

In the FTIR absorbance difference spectrum of the CO adduct of H419Q, there are very minor modes which are at the same frequency as those of the H419N enzyme, suggesting that species which are similar to the N419N enzyme can be formed in the H419Q mutant, but with much lower yields (Figure 4D).

*Substitutions for Q195, N277, and Y472.* Mutants were made at three other sites in the enzyme (Q195, N277, and Y472) which are occupied by histidines in the majority of members of the heme–copper oxidase family. N277 was replaced with aspartate, histidine, and tyrosine. Q195 was changed to glutamate and Y472 to histidine. All of the mutants at Q195, N277, and Y472 can restore aerobic growth to oxidase-deficient strains of *E. coli*, demonstrating that these residues are not essential for enzyme function. The NADH oxidase activities of membranes containing the mutant enzymes are listed in Table I. All of the mutants at these three positions have significant levels of activity, although the levels vary. The split α-band signature of heme $b_{562}$ in the dithionite-reduced *minus* air-oxidized spectrum and the spectroscopic blue shift of the Soret band due to CO binding to heme *o* are identical to those of the wild-type enzyme (not shown). The Fe—C≡O and Cu—C≡O species in the FTIR absorbance difference spectra of the CO adducts of the mutants at Q195, N277, and Y472 are identical to those of the wild-type enzyme (Figure 5C). These data show that the binuclear center and the low-spin heme $b_{562}$ are not perturbed by the mutations at Q195, N277, or Y472. Hence, the low turnover observed with some of the mutants (e.g., N277D and Y472H) is not due to gross changes at the metal-binding sites, although it does suggest that the regions of subunit I containing these residues may be important in enzyme function.

*Substitutions for H411.* Figure 5A,B shows the FTIR spectra of the CO adducts of the H411G and H411L mutants. Previously, the H411G mutant has been shown to maintain enzyme activity, while H411L is inactive (Lemieux et al., 1992). From Figure 5, it can be seen that the lack of activity in the H411L mutant results from dramatic changes that occur at the binuclear center of the enzyme upon substitution of leucine. The spectrum is consistent with the loss of $Cu_B$ and a high degree of disorder and heterogeneity of the CO bound to heme *o*.

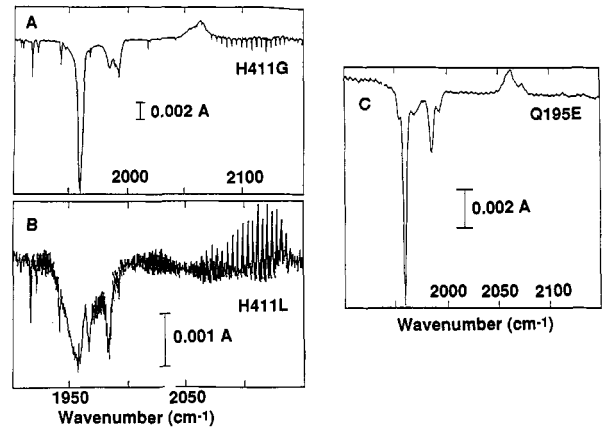


FIGURE 5: FTIR spectra of the H411G, H411L, and Q195E mutants. The spectra are presented as "light" *minus* "dark" spectra, as defined in the text. The sample temperature, the path length, and the frequencies of the bands attributed to the *bo*-type oxidase are listed. If the spectrum was subjected to base-line correction (see Materials and Methods), the note "b.c." is present. (A) H411G: 11 K, 27 μm, 1959 $cm^{-1}$ (Fe), 2065 $cm^{-1}$ (Cu) b.c. (B) H411L: 12.5 K, 27 μm, 1959 $cm^{-1}$ (Fe). (C) Q195E: 20 K, 25 μm, 1959 $cm^{-1}$ (Fe), 2060, 2072 $cm^{-1}$ (Cu). This spectrum is representative of the N277 and the Y472 mutants, all of which are similar to the wild-type enzyme.

## DISCUSSION

Characterization of site-directed mutants in eight highly conserved histidine residues of subunit I of the *bo*-type oxidases has been published previously (Lemieux et al., 1992; Minagawa et al., 1992). These studies demonstrated that two histidines, H54 and H411, are not essential for enzyme activity and identified two other residues (H106 and H421) as the ligands for heme $b_{562}$. Mutants at H333 and H334 have minor effects on the properties of heme *o*, but are essential for the assembly of $Cu_B$ (Lemieux et al., 1992; Minagawa et al., 1992; Brown et al., 1993; Calhoun et al., 1993b).

A previous model, based upon the analysis of mutants in cytochrome *bo*, assigned H284 as the ligand for heme *o* because atomic absorption analysis of the H284A mutant showed the presence of copper, while H419A lacked copper. However, mutagenesis studies of the copper-binding protein azurin demonstrate that removal of copper ligands usually does not result in the loss of copper (Chang et al., 1991; den Blaauwen & Canters, 1991; den Blaauwen et al., 1991; Karlsson et al., 1991; Mizoguchi et al., 1992), although spectroscopic properties of the bound copper may be altered. Therefore, the presence or absence of elemental copper in mutants is not a reliable indicator of the specific role of a residue. In addition, further spectroscopic studies of mutants at H284 have definitively demonstrated that H284 is not the ligand for heme *o*. The iron–histidine stretching mode of heme $a_3$ is present in the H284A mutant in the oxidase from *Rb. sphaeroides*, and only minor perturbations occur to the ν(Fe—C≡O) bandwidth and center frequency in H284G and H284L mutants in the oxidase from *E. coli* (Hosler et al., 1993a; Calhoun et al., 1993a). Combined with the strong spectroscopic evidence that the ligand of heme *o* is a histidine (Cheesman et al., 1993; Ingledew et al., 1993), these findings suggest the assignment of H419 as the ligand of the heme *o*, the CO- and oxygen-binding heme component of the binuclear center.

The negligible levels of CO binding in all of the mutants at H419, as measured by visible spectroscopy, are consistent with the assignment of H419 as the axial ligand of heme *o*. However, the cryogenic FTIR absorbance difference spectra of the CO-bound H419N mutant demonstrate that this enzyme maintains a heme–copper binuclear center. It is unclear why the conditions used for cryogenic FTIR spectroscopy dramatically increase the levels of CO bound to the H419N mutant. Independent studies performed in other laboratories have demonstrated negligible CO binding in flash-photolysis studies of the H419N mutant performed at room temperature (Brown et al., 1993; D. Lemon, personal communication).

The cryogenic temperatures or the sample dehydration by glycerol may provide stabilization of the CO adduct of H419N during collection of the FTIR spectra.

The FTIR absorbance difference spectrum of H419N recorded at cryogenic temperatures shows that asparagine can replace H419 and maintain a mutant enzyme with an intact binuclear center. It has been demonstrated that replacement of the histidine axial ligand of cytochrome *c* peroxidase with glutamine results in an active enzyme (Choudhury et al., 1992; Sundaramoorthy et al., 1991). The X-ray crystal structure of this mutant shows that the oxygen of the glutamine side chain forms a bond to the heme iron (Choudhury et al., 1992; Sundaramoorthy et al., 1991). Therefore, it is reasonable to suggest that an asparagine may substitute for the axial ligand of heme *o* in the H419N mutant. This proposal is supported by the small yield of Fe—C≡O bands in the FTIR spectrum of the H419Q mutant which are similar to those seen in the H419N mutant. In cytochrome *c* peroxidase, glutamine can be positioned with the carbonyl group in nearly the same position as the Nε2 nitrogen of histidine. A comparison of the structures of histidine and asparagine suggests that the carbonyl of the asparagine side chain might mimic the Nδ1 nitrogen of the imidazole ring instead of the Nε2 nitrogen. This suggests that the Nδ1 nitrogen of H419 may form the heme ligand. However, there is no precedent for heme ligation by an Nδ1 nitrogen (Chakrabarti, 1990), and it is questionable that this can occur due to steric interactions.

The center frequency of $\nu$(Fe—C≡O) is lower in the H419N mutant than that of the wild-type enzyme. Such a frequency shift could be explained by the switch of the axial heme ligand, by a change in the polarity of the distal pocket (Oldfield et al., 1991), or by a change in the angle of Fe—C≡O with respect to the heme plane (Ormos et al., 1988). The narrow bandwidth suggests that Fe—C≡O is sampling a very homogeneous environment, which may be similar to the one present in the wild-type enzyme. The $\nu$(Cu—C≡O) in the H419N mutant is also at lower frequency than that of wild type. The band shape of Cu—C≡O is a mirror image of that of the wild type, but the bandwidths are similar. The heme and copper components of the binuclear center are in close proximity (Alben et al., 1981; Scott et al., 1986; Powers et al., 1987), and a change of electron density of the iron atom, which is suggested by the shifted $\nu$(Fe—C≡O), could also, in principle, change the frequeny of $\nu$(Cu—C≡O). Other subtle, but more global, changes in the three-dimensional structure of the protein, and hence the polarity of the binuclear center, might also be responsible for the altered frequency of Cu—C≡O. The difference in size between asparagine and histidine may cause alterations in the relative positions of helix X and heme *o*, and this conformational change may be propagated to $Cu_B$ *via* hydrogen bond interactions to the ligands of $Cu_B$.

The FTIR absorbance difference spectra of the CO adduct of the bovine, rat, and *Rb. sphaeroides* $aa_3$-type oxidases contain two pairs of Fe—C≡O and Cu—C≡O bands, referred to as the α and β forms (Alben et al., 1981; Fiamingo et al., 1986, 1990; Shapleigh et al., 1992b). The significance of these two forms is not clear. The wild-type *E. coli* cytochrome *bo* does not exhibit α and β forms of the CO adduct. It is interesting to note that the frequency differences between the bands in the wild type and the H419N mutant are similar to those separating the α and β forms in the other enzymes. Multiplicity of forms is also observed with CO adducts of myoglobin (Ormos et al., 1988; Oldfield et al., 1991; Park et al., 1991). Hence, it is clear that such frequency shifts in the oxidases can be explained by subtle conformational differences at the binuclear center.

The FTIR absorbance difference spectrum of the $\nu$(Fe—C≡O) in the H419C mutant suggests that in this enzyme the photolyzed CO rebinds to the same binding site, but in a slightly more homogeneous environment. It is unlikely that the photolyzed state represents CO bound to a copper. The $\nu$(Cu—C≡O) of a large variety of Cu—C≡O complexes, both in mode compounds and in proteins, shows that the only Cu—C≡O species which yield $\nu$(Cu—C≡O) in this region (1955 $cm^{-1}$) are those where the CO molecule bridges between two Cu atoms (S. Kim, personal communication). In this case, it is possible that the CO in the photolyzed state could bridge between the heme Fe and $Cu_B$. However, no model compounds of this state are known.

The axial ligation of heme *o* in the H419C mutant is not known. In cytochrome P-450, the axial heme ligand is clearly a cysteine residue (Dawson & Sono, 1987), and the absorbance maximum of the Soret band of CO-bound enzyme is at 450 nm, which is shifted from the usual maximum of about 420 nm for other CO-binding *b*-type heme proteins. This red shift is believed to arise from the cysteine ligation (Hanson et al., 1976; Stern & Peisach, 1974). The extremely low level of CO- binding in the H419C mutant of cytochrome *bo* prevents such a spectroscopic analysis.

In sum, the results from analysis of the H419 mutants are consistent with the assignment of H419 as the axial ligand to heme *o*, although on their own these data do not rule out other alternatives. In conjunction with previous studies of the other totally conserved histidines in subunit I, these data strengthen the case for the ligation of heme *o* by H419.

A search of the sequence alignments of subunit I of the heme–copper oxidases reveals three residues (Q195, N277, and Y472) which, although not histidines in the *E. coli* oxidase, are histidines in a large majority of the sequences. At the positions corresponding to Q195, N277, and Y472, histidines occur in 60, 71, and 66 out of the 75 sequences of subunit I, respectively. All three of the residues are predicted to be located within putative transmembrane helices near the periplasmic side (Figure 1), which is the proposed location for the binding site of heme *o* (Hosler et al., 1993a). The characterization of mutational substitutions for each of these residues shows that none are essential for enzyme activity. It is unlikely that any of these substituting residues is forming a heme ligand, since no perturbations are seen in either the visible or the FTIR absorbance difference spectra, in contrast with the dramatic effects seen in the H419 mutants. Mutations in these three sites represent reasonable negative controls for the mutations in H419. Clearly, the lack of phylogenetic conservation correlates with the ability of cytochrome *bo* to tolerate substitutions for these three residues.

In the absence of a high-resolution structure of the oxidase, site-directed mutagenesis cannot provide unequivocal assignment of the amino acids which form the ligation spheres of the metals. However, alternative explanations of the data are less satisfying than the assignment of H419 as the ligand of heme *o*. If perturbations from the mutations at H419 result from effects other than the direct loss of a heme ligand, another residue must serve as the heme ligand.

Characterization of the EPR spectrum of NO coordinated to heme *o* is inconsistent with nonnitrogenous ligation, and the characteristics of this spectrum make ligands such as lysine and arginine unlikely candidates (Cheesman et al., 1993). Magnetic circular dichroism and EPR studies with other ligands are also consistent with histidine ligation (Cheesman et al., 1993; Ingledew et al., 1993). If the interpretation of

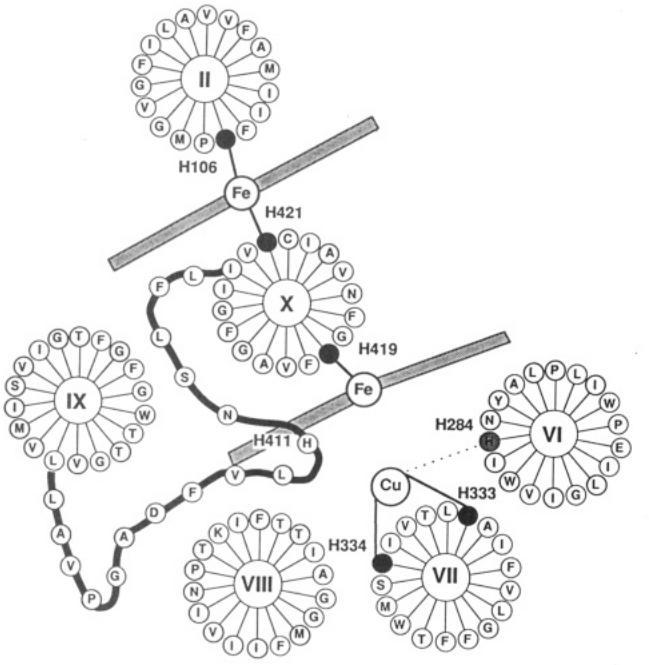


FIGURE 6: Model of the ligation of heme $b_{562}$, heme *o*, and $Cu_B$ by the six conserved histidines of subunit I of the *bo*-type ubiquinol oxidase of *E. coli*. The analogous centers of the $aa_3$-type cytochrome *c* oxidases are, respectively, heme *a*, heme $a_3$, and $Cu_B$. Helical wheel representations of helices II, VI, VII, VIII, IX, and X are shown as viewed from the periplasm. The histidine ligands are in black circles, using the sequence of subunit I of the oxidase from *E. coli*. The ligation of $Cu_B$ by His-284 (gray circle) is shown in the dashed lines to indicate the uncertainty of its function in the ligation of this center (Calhoun et al., 1993a). The location of helix VIII near the binuclear center is the result of mutagenesis studies of several residues in this putative transmembrane helix (Thomas et al., 1993). The proximity of the periplasmic loop between putative transmembrane helices IX and X and the binuclear center is indicated. A counterclockwise progression of helices VI through X is equally consistent with the present knowledge.

the spectroscopic characterization of previous mutagenesis studies is correct, the seven other evolutionarily conserved histidines have been eliminated as candidates (54, 106, 284, 333, 334, 411, and 421). The use of an evolutionarily unconserved histidine residue as the ligand of heme *o* would require substantial differences between the structures of the $aa_3$-type oxidase and the *bo*-type oxidase. The degree of conservation among the primary sequences, the proposed secondary structures of the heme–copper oxidases, and the spectroscopic similarities argue against major differences due to rearrangements in the basic structures of the enzymes. In addition, mutational analysis of the $aa_3$-type oxidase of *Rb. sphaeroides* has also demonstrated that H419 is important for the integrity of the binuclear center, more specifically that of heme $a_3$. Analysis of mutants in the enzyme from *Rb. sphaeroides* via resonance Raman spectroscopy demonstrates the maintenance of the iron–histidine stretch assigned to heme $a_3$ and its histidine ligand in the H284A mutant, but the loss of this band in the H419N mutant (Calhoun et al., 1993a).

The proposed assignment of H419 as the axial ligand of heme *o* places the two hemes of the enzyme on opposite sides of putative transmembrane helix X (Figure 6). The implications of such a structure on enzyme mechanism have been discussed previously (Hosler et al., 1993a). The covalent bond network between the two hemes provides a structural explanation of the heme–heme interactions which have been observed in the heme–copper oxidases (Leigh et al., 1974; Wilson et al., 1972; Salerno et al., 1990) and for rapid heme–heme electron transfer (Woodruff, 1993).

The ligation of heme *o* to H419 in helix X also provides a reasonable rationale for the dramatic effects observed in the H411L mutant. FTIR analysis of the CO adduct of this mutant suggests that H411 is important for the integrity of the binuclear center. Although glycine and alanine are tolerated at this position (Lemieux et al., 1992; Minagawa et al., 1992), the larger, hydrophobic leucine is not, resulting in severe damage to the binuclear center. Studies of the kinetics of CO relaxation upon photolysis suggest that the H411L mutant lacks $Cu_B$ (Brown et al., 1993). The FTIR absorbance difference spectrum presented here supports the proposal that $Cu_B$ is absent in H411L (Figure 5B), and suggests possibly important interactions between the interhelical IX–X periplasmic loop and the binuclear center. Other experiments on the $aa_3$-type oxidase from *Rb. sphaeroides* also implicate such interactions (Hosler et al., 1993a,b). In addition, analysis of second-site revertants isolated in yeast cytochrome *c* oxidase have also identified a potential interaction between these two regions of the protein (Meunier et al., 1993).

*Conclusions.* H419 has been shown to be a critical residue to both the function and the structure of the heme–copper oxidases (Minagawa et al., 1992; Lemieux et al., 1992; Shapleigh et al., 1992a; Hosler et al., 1993). The aggregate of the experimental data argues for the assignment of H419 as the ligand for heme *o*. It is almost certain that the structure of the binuclear center among the heme–copper oxidases is conserved, and undisputed evidence demonstrates that the heme $a_3$ (heme *o* equivalent) ligand in the NO-bound form of yeast cytochrome *c* oxidase is a histidine (Stevens & Chan, 1981). Although ligand switching at heme $a_3$ upon binding of CO or during turnover has been proposed (Woodruff, 1993; Rousseau et al., 1993), this does not negate the requirement of a histidine as a high-spin heme axial ligand. Mutagenesis studies of eight highly or totally conserved histidines in subunit I (54, 106, 284, 333, 334, 411, 419, and 421; see Figure 1) of the heme–copper oxidases show that none, with the exception of H419, can be the ligand for heme *o*.

In contrast, the data in this paper show that heme *o* does not bind carbon monoxide in the membranes containing the H419G, -L, -D, -M, or -Y mutants but that an intact, although altered, binuclear center which can bind to CO is observed with the H419N mutant. This totality of evidence supports the proposal that H419 is the axial ligand of heme *o* in cytochrome *bo* from *E. coli* and, by analogy, of heme $a_3$ in the cytochrome *c* oxidases, although a definitive assignment awaits the solution of the high-resolution structure of the enzymes.

## REFERENCES

Alben, J. O., Moh, P. P., Fiamingo, F. G., & Altschuld, R. A. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 234–237.

Anraku, Y. (1988) *Annu. Rev. Biochem. 57*, 101–132.

Anraku, Y., & Gennis, R. B. (1987) *Trends Biochem. Sci. (Pers. Ed.) 12*, 262–266.

Au, D. C.-T., Lorence, R. M., & Gennis, R. B. (1985) *J. Bacteriol. 161*, 123–127.

Babcock, G. T., & Wikström, M. (1992) *Nature*, 301–309.

Bilofsky, H. S., & Burks, C. (1988) *Nucleic Acids Res. 16*, 1861–1864.

Bott, M., Bolliger, M., & Hennecke, H. (1990) *Mol. Microbiol. 4*, 2147–2157.

Bott, M., Preisig, O., & Hennecke, H. (1992) *Arch. Microbiol. 158*, 335–343.

Brown, S., Rumbly, J. N., Moody, A. J., Thomas, J. W., Gennis, R. B., & Rich, P. R. (1993) *Biochim. Biophys. Acta* (in press).

Calhoun, M. W., Gennis, R. B., & Salerno, J. C. (1992) *FEBS Lett. 309*, 127–129.

Calhoun, M. W., Thomas, J. W., Hill, J. J., Hosler, J. P., Shapleigh, J. P., Tecklenburg, M. M. J., Ferguson-Miller, S., Babcock, G. T., Alben, J. O., & Gennis, R. B. (1993a) *Biochemistry 32*, 10905–10911.
Calhoun, M. W., Lemieux, L. J., Hill, J. J., Ingledew, W. J., Alben, J. O., & Gennis, R. B. (1993b) *Biochemistry* (in press).
Chakrabarti, P. (1990) *Protein Eng. 4*, 57–63.
Chang, T. K., Iverson, S. A., Rodrigues, C. G., Kiser, C. N., Lew, A. Y. C., Germanas, J. P., & Richards, J. H. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 1325–1329.
Cheesman, M. R., Watmough, N. J., Pires, C. A., Turner, R., Brittain, T., Gennis, R. B., Greenwood, C., & Thomson, A. J. (1993) *Biochem. J. 289*, 709–718.
Chepuri, V., & Gennis, R. B. (1990) *J. Biol. Chem. 265*, 12978–12986.
Chepuri, V., Lemieux, L., Hill, J., Alben, J. O., & Gennis, R. B. (1990a) *Biochim. Biophys. Acta 1018*, 124–127.
Chepuri, V., Lemieux, L. J., Au, D. C.-T., & Gennis, R. B. (1990b) *J. Biol. Chem. 265*, 11185–11192.
Choudhury, K., Sundaramoorthy, M., Mauro, J. M., & Poulos, T. L. (1992) *J. Biol. Chem. 267*, 25656–25659.
Cline, J., Reinhammar, B., Jensen, P., Venters, R., & Hoffman, B. M. (1983) *J. Biol. Chem. 258*, 5124–5128.
Dawson, J. H., & Sono, M. (1987) *Chem. Rev. 87*, 1255–1276.
den Blaauwen, T., & Canters, G. W. (1991) *J. Inorg. Chem. 43*, 177.
den Blaauwen, T., van de Kamp, M., & Canters, G. W. (1991) *J. Am. Chem. Soc. 113*, 5050–5052.
Eglinton, D. G., Johnson, M. K., Thomson, A. J., Gooding, P. E., & Greenwood, C. (1980) *Biochem. J. 191*, 319–331.
Fiamingo, F. G., Altschuld, R. A., & Alben, J. O. (1986) *J. Biol. Chem. 261*, 12976–12987.
Fiamingo, F. G., Jung, D. W., & Alben, J. O. (1990) *Biochemistry 29*, 4627–4633.
Gabel, C., & Maier, R. J. (1990) *Nucleic Acids Res. 18*, 6143.
Hanson, L. K., Eaton, W. A., Sligar, S. G., Gunsalus, I. C., Gouterman, M., & Connell, C. R. (1976) *J. Am. Chem. Soc. 98*, 2672–2674.
Hill, J., Goswitz, V. C., Calhoun, M., Garcia-Horsman, J. A., Lemieux, L., Alben, J. O., & Gennis, R. B. (1992) *Biochemistry 31*, 11435–11440.
Hill, J. J., Alben, J. O., & Gennis, R. B. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 5863–5867.
Hosler, J. P., Ferguson-Miller, S., Calhoun, M. W., Thomas, J. W., Hill, J., Lemieux, L., Ma, J., Georgiou, C., Fetter, J., Shapleigh, J., Tecklenburg, M. M. J., Babcock, G. T., & Gennis, R. B. (1993a) *J. Bioenerg. Biomembr. 25*, 121–136.
Hosler, J. P., Shapleigh, J. P., Tecklenburg, M. M. J., Thomas, J. W., Kim, Y., Espe, M., Fetter, J., Babcock, G. T., Alben, J. O., Gennis, R. B., & Ferguson-Miller, S. (1993b) *Biochemistry* (in press).
Ingledew, W. J., & Bacon, M. (1991) *Biochem. Soc. Trans. 19*, 613–616.
Ingledew, W. J., Horrocks, J., & Salerno, J. C. (1993) *Eur. J. Biochem. 212*, 657–664.
Iuchi, S., Chepuri, V., Fu, H.-A., Gennis, R. B., & Lin, E. C. C. (1990) *J. Bacteriol. 172*, 6020–6025.
Karlsson, B. G., Nordling, M., Pascher, T., Tsai, L.-C., Sjölin, L., & Lundberg, L. G. (1991) *Protein Eng. 4*, 343–349.
Kita, K., Konishi, K., & Anraku, Y. (1984) *J. Biol. Chem. 259*, 3368–3374.
Larsen, R. W., Pan, L.-P., Musser, S. M., Li, Z., & Chan, S. I. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 723–727.
Leigh, J. S., Wilson, D. F., & Owen, C. S. (1974) *Arch. Biochem. Biophys. 160*, 476–486.
Lemieux, L. J., Calhoun, M. W., Thomas, J. W., Ingledew, W. J., & Gennis, R. B. (1992) *J. Biol. Chem. 267*, 2105–2113.
Lorence, R. M., Koland, J. G., & Gennis, R. B. (1986) *Biochemistry 25*, 2314–2321.
Matsushita, K., Ohnishi, T., & Kaback, H. R. (1987) *Biochemistry 26*, 7732–7737.
Meunier, B., Coster, F., Lemarre, P., & Colson, A. M. (1993) *FEBS Lett. 321*, 159–162.
Minagawa, J., Nakamura, H., Yamoto, I., Mogi, T., & Anraku, Y. (1991) *J. Biol. Chem. 265*, 11198–11203.
Minagawa, J., Mogi, T., Gennis, R. B., & Anraku, Y. (1992) *J. Biol. Chem. 267*, 2096–2104.
Minghetti, K. C., Goswitz, V. C., Gabriel, N. E., Hill, J. J., Barassi, C., Georgiou, C. D., Chan, S. I., & Gennis, R. B. (1992) *Biochemistry 31*, 6917–6924.
Mizoguchi, T. J., di Bilio, A. J., Gray, H. B., & Richards, J. H. (1992) *J. Am. Chem. Soc. 114*, 10076–10078.
Oden, K. L., & Gennis, R. B. (1991) *J. Bacteriol. 173*, 6174–6183.
Oldfield, E., Guo, K., Augspurger, J. D., & Dykstra, C. E. (1991) *J. Am. Chem. Soc. 113*, 7537–7541.
Ormos, P., Braunstein, D., Frauenfelder, H., Hong, M. K., Lin, S.-L., Sauke, T. B., & Young, R. D. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 8492–8496.
Palmer, G. (1983) in *Electron Paramagnetic Resonance of Hemoproteins*, pp 43–88, Addison-Wesley Publish Co., Reading, MA.
Park, K. D., Guo, K., Adebodun, F., Chiu, M. L., Sligar, S. G., & Oldfield, E. (1991) *Biochemistry 30*, 2333–2347.
Poulos, T. L. (1988) in *Heme Enzyme Crystal Structures* (Eichhorn, G. L., & Marzilli, L. G., Eds.) pp 1–36, Elsevier, New York.
Poulos, T. L., & Finzel, B. C. (1984) *Pept. Protein Rev. 4*, 115–171.
Powers, L., Chance, B., Ching, Y.-C., & Lee, C.-P. (1987) *J. Biol. Chem. 262*, 3160–3164.
Puustinen, A., Finel, M., Haltia, T., Gennis, R. B., & Wikström, M. (1991) *Biochemistry 30*, 3936–3942.
Rousseau, D. L., Ching, Y., & Wang, J. (1993) *J. Bioenerg. Biomembr. 25*, 165–176.
Salerno, J. C., & Ingledew, W. J. (1991) *Eur. J. Biochem. 198*, 789–792.
Salerno, J. C., Bolgiano, B., Poole, R. K., Gennis, R. B., & Ingledew, W. J. (1990) *J. Biol. Chem. 265*, 4364–4368.
Saraste, M. (1990) *Q. Rev. Biophys. 23*, 331–366.
Saraste, M., Raito, M., Jalli, T., Chepuri, V., Lemieux, L., & Gennis, R. B. (1989) *Ann. N.Y. Acad. Sci. 550*, 314–324.
Scott, R. A., Schwartz, J. R., & Cramer, S. (1986) *Biochemistry 25*, 5546–5555.
Shapleigh, J. P., Hosler, J. P., Tecklenburg, M. M. J., Kim, Y., Babcock, G. T., Gennis, R. B., & Furguson-Miller, S. (1992a) *Proc. Natl. Acad. Sci. U.S.A. 89*, 4786–4790.
Shapleigh, J. P., Hill, J. J., Alben, J. O., & Gennis, R. B. (1992b) *J. Bacteriol. 174*, 2338–2343.
Simpkin, D., Palmer, G., Devlin, F. J., McKenna, M. C., Jensen, G. M., & Stephens, P. J. (1989) *Biochemistry 28*, 8033–8039.
Stern, J. O., & Peisach, J. (1974) *J. Biol. Chem. 249*, 7495–7498.
Stevens, T. H., & Chan, S. I. (1981) *J. Biol. Chem. 256*, 1069–1071.
Sundaramoorthy, M., Choudhury, K., Edwards, S. L., & Poulos, T. L. (1991) *J. Am. Chem. Soc. 113*, 7755–7757.
Surerus, K. K., Oertling, W. A., Fan, C., Gurbiel, R. J., Einarsdóttir, O., Antholine, W. E., Dyer, R. B., Hoffman, B. M., Woodruff, W. H., & Fee, J. A. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 3195–3199.
Thomas, J. W., Lemieux, L. J., Alben, J. O., & Gennis, R. B. (1993) *Biochemistry* (in press).
Tsubaki, M., Mogi, T., Anraku, Y., & Hori, H. (1993) *Biochemistry 32*, 6065–6072.
Wang, J., Ching, Y.-C., Rousseau, D. L., Hill, J. J., Rumbley, J., & Gennis, R. B. (1993) *J. Am. Chem. Soc. 115*, 3390–3391.
Watmough, N. J., Cheesman, M. R., Gennis, R. B., Greenwood, C. & Thomson, A. J. (1993) *FEBS Lett. 319*, 151–154.
Wilson, D. F., & Leigh, J. S. (1972) *Arch. Biochem. Biophys. 150*, 154–163.
Woodruff, W. H. (1993) *J. Bioenerg. Biomembr. 25*, 177–188.