Site-Directed Mutagenesis of Highly Conserved Residues in Helix VIII of Subunit I of the Cytochrome *bo* Ubiquinol Oxidase from *Escherichia coli*: An Amphipathic Transmembrane Helix That May Be Important in Conveying Protons to the Binuclear Center†

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ABSTRACT: Cytochrome *bo* from *Escherichia coli* is a ubiquinol oxidase which is a member of the superfamily of heme–copper respiratory oxidases. This superfamily, which includes the eukaryotic cytochrome *c* oxidases, has in common a bimetallic center consisting of a high-spin heme component and a copper atom (Cu_B) which is the site where molecular oxygen is reduced to water. Subunit I, which contains all the amino acid ligands to the metal components of the binuclear center, has 15 putative transmembrane spanning helices, of which 12 are common to the entire superfamily. Transmembrane helix VIII has been noted to contain highly conserved polar residues that fall along one face of the helix. These residues could, in principle, be important components of a pathway providing a conduit for protons from the cytoplasm to gain access to the binuclear center. These conserved residues include Thr352, Thr359, and Lys362. In addition, Pro358, in the middle of this transmembrane helix, is totally conserved in the superfamily. Some substitutions for Thr352 (Ala, Asn) result in major perturbations at the binuclear center as judged by the low-temperature Fourier transform infrared (FTIR) absorbance difference spectroscopy of the CO adducts. Whereas Thr352Ala is inactive enzymatically, both Thr352Asn and Thr352Ser have substantial activity. Substitutions for Thr359 (Ala or Ser) also do not perturb the spectroscopic properties of the binuclear metal center, but the Thr359Ala mutant is devoid of enzyme activity. Changing the neighboring Pro358 to Ala has no detectable effect on the properties of the oxidase. However, all substitutions for Lys362 (Leu, Met, Gln, or Arg) are inactive. Whereas the Lys362Met has a wild-type FTIR spectrum of the CO adduct, the Lys362Gln shows substantial perturbation to the metal center. In summary, the data suggest that residues in helix VIII are in the immecdiate vicinity of the binuclear center and show that some of the polar residues within helix VIII are functionally essential, independent of any role in maintaining the structural integrity of the metal centers. A distinct possibility is that these residues are important components of a proton and/or water conducting channel from the cytoplasmic surface to the site where oxygen is reduced to water at the heme–copper center.

The cytochrome *bo* ubiquinol oxidase from *Escherichia coli* is a member of a large superfamily of heme–copper respiratory oxidases that includes the mitochondrial cytochrome *c* oxidases (Hosler et al., 1993; Puustinen et al., 1991). These enzymes are redox-driven proton pumps that couple the reduction of molecular oxygen to water to the vectorial translocation of protons across the membrane (Krab & Wikström, 1978; Solioz et al., 1982; Sone & Hinkle, 1982; Wikström, 1977; Wikström & Krab, 1979), thus generating a transmembrane proton electrochemical gradient (Hosler et al., 1993; Puustinen et al., 1991). The site where oxygen is reduced to water is a bimetallic center consisting of a high-spin heme component and a nearby copper atom (Cu_B) (Alben et al., 1981; Hill et al., 1992; Shapleigh et al., 1992a). The high-spin heme component is heme *o* (Puustinen et al., 1992; Puustinen & Wikström, 1991; Wu et al., 1992) or heme a_3, respectively, for cytochrome *bo* and cytochrome aa_3. All members of this oxidase superfamily also have in common a low-spin heme component, which is heme b_{562} in cytochrome *bo* and heme *a* in the aa_3-type oxidases. Recent studies have identified amino acid ligands to each of these three metals within subunit I (Lemieux et al., 1992; Minagawa et al., 1992; Shapleigh et al., 1992b), the most highly conserved subunit within this oxidase superfamily (Chepuri et al., 1990; Lemieux et al., 1992; Santana et al., 1992; Shapleigh et al., 1992b). Previous experimental work (Chepuri & Gennis, 1990) supports a model of subunit I of cytochrome *bo* with 15 putative transmembrane helices, of which 12 appear to be common to the entire superfamily (Brown et al., 1993; Saraste, 1990). This model provides a useful framework for discussing the effects of various mutations, but it must be recognized that the model is largely speculative.

Residues near the periplasmic side of transmembrane helices II, VI, VII, and X have been implicated as metal ligands or as being critical to maintaining the structural integrity of the metal sites (Hosler et al., 1993; Lemieux et al., 1992; Minagawa et al., 1992; Shapleigh et al., 1992b). Of particular significance is the localization of all the metal centers near the periplasmic side of the membrane. Since the reduction of molecular oxygen to water requires four protons (one per electron) which must originate in the cytoplasm, this topo-

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logical arrangement (Chepuri & Gennis, 1990) requires that the oxidase contain a proton-conducting channel to convey these so-called scalar protons from the cytoplasm to the binuclear center. In addition to the protons required for the oxygen chemistry, the oxidase actively pumps one proton per electron from the cytoplasm to the periplasm (Puustinen et al., 1989). In principle, the channel utilized by the scalar protons to get to the binuclear center could also serve as a portion of the conduit for the pumped protons as well. The pumped protons would, of course, also require a pathway to reach the periplasm. In any event, it is clear that a minimal requirement for these proton-pumping oxidases is at least one proton conducting channel leading from the cytoplasm to the periplasmic side of the membrane.

Studies with the aa_3-type cytoplasm *c* oxidase isolated from *Paracoccus denitrificans* have demonstrated that subunits I and II provide a minimal structural unit, containing everything required both for cytochrome *c* oxidase activity and for proton pumping (Hendler et al., 1991; Solioz et al., 1982). Since subunit II for nearly all members of the oxidase superfamily contains only two transmembrane helices, it is likely that amino acid residues within subunit I provide many of the elements required for proton movement accompanying catalytic turnover. Helix VIII in subunit I contains several highly conserved polar residues which are located in the same side of this proposed amphiphilic helix. Hence, helix VIII is an excellent target in the search for residues that may be important for the movement of protons from the cytoplasm to the binuclear center or to the periplasm.

In this paper, mutants are examined in which amino acid substitutions are made for five highly conserved residues within helix VIII. Two questions are addressed. The first is whether these residues are important for enzyme turnover. The second question is whether amino acid substitutions for these residues result in structural alterations in the vicinity of the metal centers. Structural perturbations are monitored by optical spectroscopy of the hemes and by low-temperature FTIR spectroscopy of CO bound at the binuclear center (Alben et al., 1981; Fiamingo et al., 1982; Hill et al., 1991, 1992; Shapleigh et al., 1992a). It is concluded that at least one residue in helix VIII, Thr352, appears to be in the immediate vicinity of this site in the oxidase. Furthermore, two other polar residues in helix VIII, Thr359 and Lys362, appear to be important for catalytic function for reasons independent of maintenance of the structure of the binuclear center. It is plausible that these residues may be important for facilitating the movement of protons during enzyme turnover.

MATERIALS AND METHODS

Site-directed mutagenesis and cloning were performed as noted previously (Lemieux et al., 1992). The M13-XE and M13-XE2 (addition of an *Xho*I site at 3.38 kbp) templates were used along with mutagenesis primers made at the University of Illinois Biotechnology Center (Urbana, IL). A 1.3 kbp *Hin*dIII/*Nsi*I fragment containing the mutations was cloned into the plasmid pMC31 or pJT39 (pMC39 with an addition of an *Xho*I site at 3.38 kbp) for all mutant strains except those noted below. The Lys362Met mutant strain was constructed by cloning a 1.3 kbp *Afl*II/*Nsi*I fragment into the plasmid pL3, and Thr352Asn was constructed by cloning a 2.7 kbp *Sma*I/*Eco*RI fragment into the plasmid pJT41 (pMC39 with a *Hin*dIII site at 3.65 kbp). The mutant proteins were expressed in the host strain GL101 (*sdh cyo recA*) (Lemieux et al., 1992).

The base pair change(s) resulting in each mutant was (were) confirmed by sequencing after subcloning into the plasmid expression vector. Either the double-stranded plasmid carrying the mutation or a single-stranded M13 vector with the mutation cloned from the double-stranded plasmid was sequences using the Sequenase kit from United States Biochemical (Cleveland, OH).

Cells were grown for membrane isolation according to the protocol noted in Lemieux et al. (1992). All spectroscopic studies were done using GL101 as the host strain overexpressing the mutant oxidases. This strain is *cyo*$^-$ and, therefore, does not contain the cytochrome *bo* oxidase. However, the strain is *cyd*$^+$ and expresses the *E. coli* alternate oxidase, cytochrome *bd*. As a result, this strain can be grown aerobically even if the cytochrome *bo* mutants cannot function. Growth conditions are used so that the amount of cytochrome *bd* in the membrane is small compared to cytochrome *bo*. The dithionite-reduced plus CO *minus* dithionite-reduced visible spectra were recorded with samples containing 2 mg/mL membrane protein. Dithionite-reduced *minus* air-oxidized visible spectra were recorded at 77 K using samples of 8 mg/mL membrane protein. All visible spectra were obtained using an SLM-Aminco DW-2 spectrophotometer equipped with an Olis data reduction package (Bogart, GA). Membrane protein concentrations were determined using the BCA method (Pierce).

In order to measure complementation, plasmids containing the mutations were transformed into the host strain RG129 (Au et al., 1985). This strain lacks both respiratory oxidases (*cyo cyd recA*) and cannot grow aerobically on nonfermentable substrates in the absence of a plasmid-derived functional oxidase. Complementation of aerobic growth was determined as noted (Chepuri et al., 1990).

The ubiquinol oxidase activity of the membranes containing the mutant proteins was compared to that of wild type by measuring the consumption of oxygen at 25 °C using a YSI (Model 53) oxygen electrode. The solution contained 50 mM Tris-HCl (pH 7.4), 0.5 mM EDTA, 165 μM ubiquinone-1, and 4.5 mM dithiothreitol (DTT). The amount of cytochrome *bo* present varied with each mutant, but was on the order of 10 pmol. Ubiquinone-1 was a gift from Hoffman–LaRoche A. G. (Basel, Switzerland).

The concentration of cytochrome *bo* in membranes was determined spectrophotometrically. The peak-to-trough absorbance difference between 415 and 430 nm was determined in a dithionite-reduced *plus* CO *minus* dithionite-reduced visible difference spectrum using an extinction coefficient of 287 mM^{-1} cm^{-1} for the two-heme enzyme (Puustinen & Wikström, 1991). It is noted, however, that the reported extinction coefficient for the CO difference spectrum varies widely and includes values of 89 mM^{-1} cm^{-1} (Cheesman et al., 1993) and 135 mM^{-1} cm^{-1} (Kita et al., 1984).

Sample preparation for Fourier transform infrared (FTIR) spectroscopy and instrument conditions were similar to those noted in Shapleigh et al. (1992a). Crude membranes (Lemieux et al., 1992) containing cytochrome *bo* in the host strain GL101 were used to prepare the reduced CO adduct for FTIR. One milliliter of isolated membranes from approximatly 1 L of cells was suspended in 14 mL of 50 mM Tris-HCl (pH 7.5) and sealed in a 60Ti centrifuge tube using a rubber septum. The tubes were then made anaerobic by cycles of vacuum and argon flushing. One milliliter of 1.0 M sodium dithionite, previously made anaerobic, was added to the tube followed by additional cycles of vacuum and argon. CO was added by passing 1 atm of CO over the above sample for 10 min. Under a flow of CO, the tube was sealed with the appropriate centrifuge cap, and then the sample was pelleted at 150000*g* for 2 h. Again under a flow of CO, the

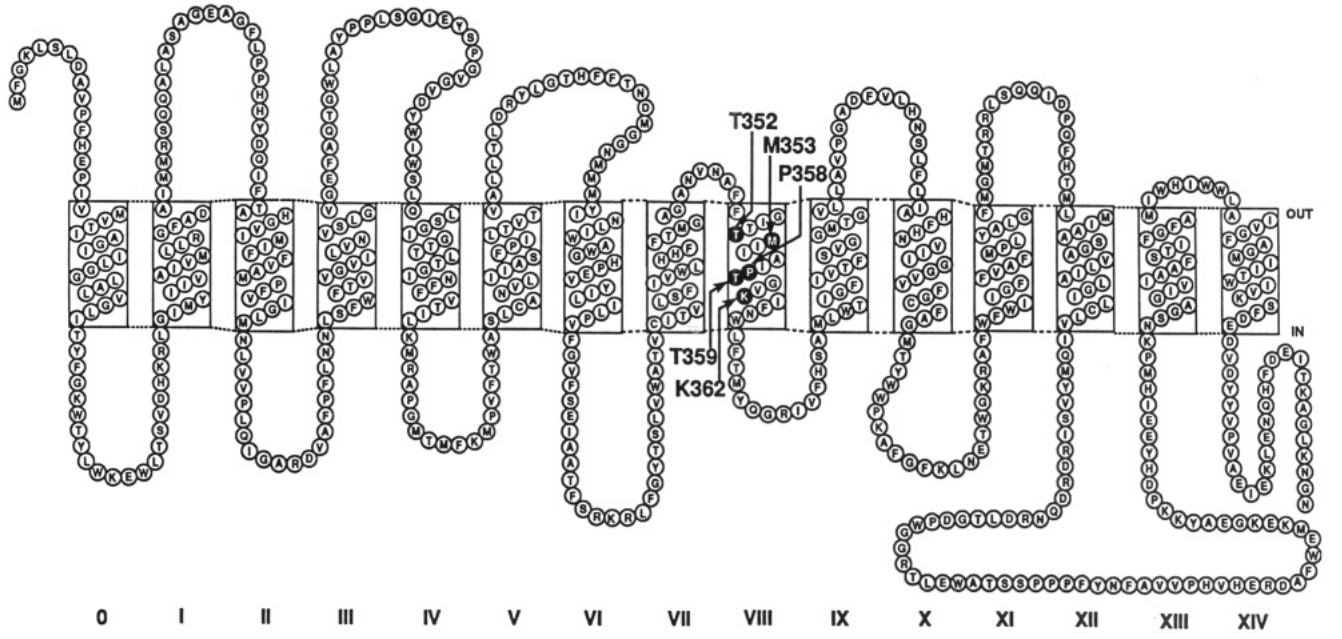

FIGURE 1: Two-dimensional topological model of subunit I of the *bo*-type ubiquinol oxidase from *Escherichia coli*. The highly conserved polar residues in helix VIII are highlighted.

supernatant was decanted and CO-saturated glycerol and added to dehydrate the membrane sample. The tubes were purged with CO and the caps replaced. The sample was kept at 4 °C overnight.

A portion of the sample was placed between two CaF_2 windows (Janos Technology, Inc.) and pressed to an appropriate thickness. A Mattson Sirius FT-IR interferometer equipped with a Lake Shore Crytronics closed-cycle helium refrigerator and a liquid nitrogen cooled indium antimonide detector was used to record the FTIR spectra. Interferograms were detected in the single-beam mode and are presented as a "light" *minus* "dark" absorbance difference spectrum with a resolution of 0.5 cm^{-1}. The "dark" spectrum was recorded before photolysis. The "light" spectrum was recorded during continuous irradiation of the sample using a 500-W tungsten bulb filtered through glass and water. The "light" and "dark" spectra are the average of 512 scans collected at 20 K. Subtraction of the least-squares fits of a cubic polynomial to the base-line regions of the spectra was used for base-line correction of the Thr352Asn and Met353Ala mutants. There was no further averaging, smoothing, or other correction to the spectra.

RESULTS

Amino acid substitutions for five different highly conserved residues within helix VIII of subunit I of cytochrome *bo* were constructed. Figure 1 shows the location of these five residues (Thr352, Met353, Pro358, Thr359, and Lys362) in a two-dimensional schematic of this subunit. The topological arrangement of the subunit in the membrane is based on the results of gene fusion experiments which have been previously reported (Chepuri & Gennis, 1990). Figure 2 is a proposed arrangement of five of the helices of subunit I in relation to the metal centers as viewed from the periplasmic side. The placement of helices II, VI, VII, and X is based on the assignment of the specific amino acids which bind to the metals (Lemieux et al., 1992; Minagawa et al., 1992; Shapleigh et al., 1992b), and the placement of helix VIII in this model is supported by the work presented in the current paper. In the helical wheel projection of helix VIII shown in Figure 2, it can be seen that the three conserved polar residues (Thr352,

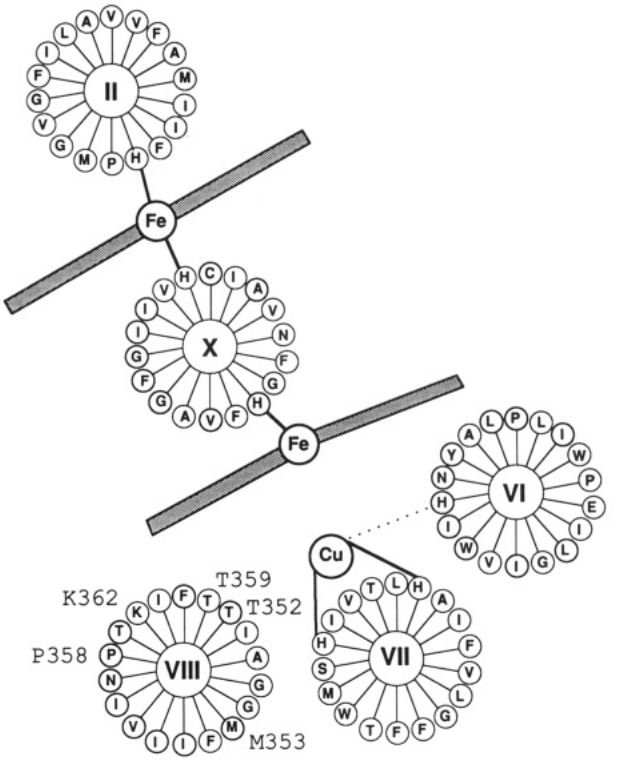

FIGURE 2: Helical wheel representation viewed from the periplasm of five of the putative transmembrane helices of subunit I cytochrome *bo* from *Escherichia coli*. The highly conserved polar residues in helix VIII are highlighted. The dotted line from His284 (helix VI) is to indicate possible ligation to Cu_B, which is postulated to bind to His333 and His334 (helix VII). The low-spin heme b_{562} is shown ligated by histidines in helix II and helix X, and heme *o* is ligated to His419 in helix X.

Thr359, and Lys362) are all on the same face of the putative α helix. Met353 is predicted to be on the opposite face of the helix, which is both less polar in character and less conserved within the oxidase superfamily. It is noted that the helical wheel projection does not take into account any kink or other distortion of the putative helix which could result from the presence of Pro358.

Met353 and Pro358 Are Nonessential Residues. As shown in Table I, substitution of Met353 by alanine yields an oxidase

Table I: Complementation and Activity Data for the Cytochrome *bo* Mutants from Helix VIII of Subunit I

	complementation[a]	activity (e^-/s)		complementation[a]	activity (e^-/s)
wild type	+	990	T359A	–	NA
T352A	–	NA[b]	T359S	+	950
T352N	+	580	K362L	–	NA
T352S	+	580	K362M	–	NA
M353A	+	730	K362Q	–	NA
P358A	+	520			

[a] Complementation in a mutant is defined as the ability to support aerobic growth as the sole oxidase on a nonfermentable substrate in the RG129 host strain. [b] NA = not applicable.

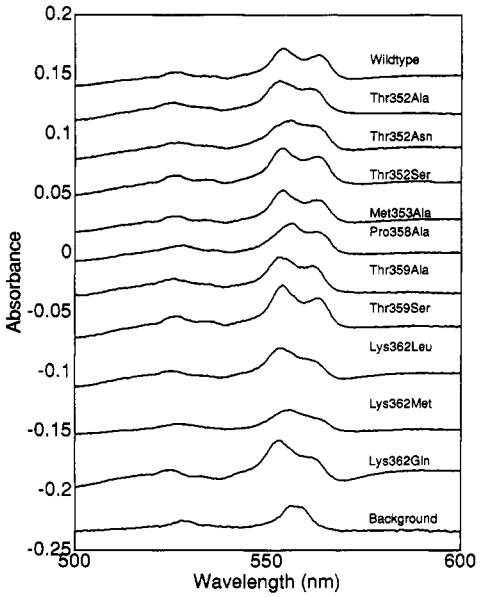

FIGURE 3: α region of the dithionite-reduced *minus* air-oxidized visible spectra at 77 K for the noted mutants in helix VIII. The split α peak at 562 nm is diagnostic for the low-spin heme center. The background spectrum was obtained from the host strain GL101 which expresses cytochrome *bd* alternate oxidase. The concentration of cytochrome *bd* may be estimated from the FTIR spectra.

which can complement an oxidase-deficient strain of *E. coli* and which has a quinol oxidase specific activity which is only slightly diminished in comparison to the wild-type control. Met353 is present in about 80% of the approximately 75 sequences currently available for subunit I from various species (Bilofsky & Burks, 1988), and these data clearly indicate it is not an essential residue. The dithionite-reduced *minus* air-oxidized visible difference spectrum (Figure 3) of membranes containing the overproduced Met358Ala mutant shows the split α band diagnostic of the heme b_{562} component of the oxidase (Lemieux et al., 1992; Minghetti et al., 1992). Hence, there is no evidence for any perturbation of the low-spin heme component of the oxidase resulting from this mutation. Indeed, by this criterion, none of the mutations examined in this work affect the spectroscopic properties of the low-spin heme b_{562}.

Figures 4 and 5 show the low-temperature FTIR absorbance difference spectra of the CO adduct to the wild type and Met353Ala mutant, respectively, in the *E. coli* membrane. In this technique, the FTIR spectrum of a membrane sample containing the CO adduct of the oxidase is taken in the dark and is then subtracted from the spectrum of the same sample during continuous exposure to visible light. In the "dark" spectrum, the CO is bound to the heme Fe (heme *o*), whereas in the "light" spectrum the CO has been photodissociated from the heme and, at the low temperature employed (20 K), is stably bound to Cu_B in the binuclear center (Fiamingo et al., 1982; Hill et al., 1991, 1992; Shapleigh et al., 1992a).

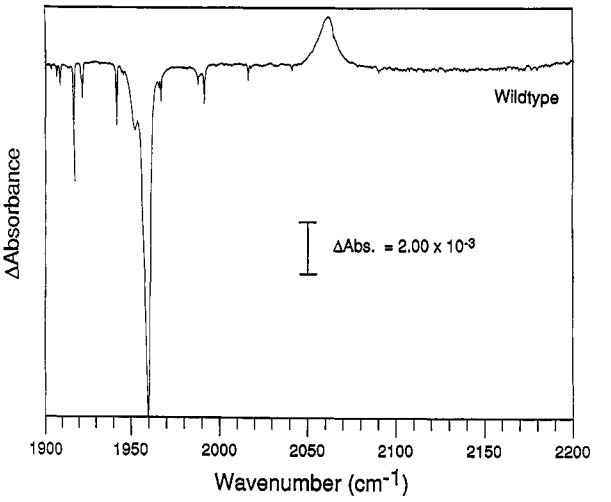

FIGURE 4: FTIR absorbance spectrum of wild-type cytochrome *bo*-type oxidase from *E. coli* at 20K. The Fe—C≡O center frequency is 1959 cm^{-1}, and the Cu—C≡O center frequency is 2063 cm^{-1}. Sample thickness is 27 μm. The Fe—C≡O band of the cytochrome *bd* oxidase is at 1984 cm^{-1}, and the band at 1990 cm^{-1} is attributed to misassembled protein.

Since the CO stretching frequency is different for the Fe—C≡O adduct (1959 cm^{-1}) (wild type, Figure 4) and for the Cu—C≡O (2063 cm^{-1}) (Hill et al., 1991), these two features are prominent in the FTIR absorbance difference spectrum. Furthermore, the CO stretching frequency is sensitive to the electronic environment sensed by the CO molecule. Changes in the center frequency and in the width of the FTIR absorption bands reflect changes in the polarity or in the degree of order in the CO binding pocket within the protein. Hence, local changes in the protein conformation at the binuclear center can be monitored by perturbations of the FTIR absorbance difference spectrum of the CO adduct compared to that from the wild type (Alben, 1987; Alben & Caughey, 1968). In the case of the Met353Ala mutant, the spectrum is very similar to that of the wild-type control, shown in Figure 4. Most notable is the very narrow trough centered at 1959 cm^{-1}, indicative of a very ordered environment sensed by CO bound to the heme Fe.

The mutation of Pro358 to alanine is also active (Table I), and all the spectroscopic features are virtually indistinguishable from those of the wild type (Figures 3–5). Pro358 is present in virtually all the reported sequences of subunit I with the single exception of the unusual oxidase from *Sulfolobus acidocaldarius*. Despite this, the data clearly show it is not essential for the assembly of functional oxidase, and the metal centers appear to be unperturbed in the Pro358Ala mutant.

Thr352, Thr358, and Lys362 Are Important for Catalytic Function. In contrast to the results described above, amino acid substitutions for Thr352, Thr359, or Lys362 can have dramatic effects on the oxidase structure and function. Replacing Lys362 by methionine, glutamine, or arginine results in an inactive oxidase. The dithionite-reduced *minus* air-oxidized visible difference spectra of all three Lys362 mutants are wild type in character and indicate no perturbation of the low-spine heme b_{562} component of the oxidase (Figure 3). The FTIR absorbance difference spectrum of the CO adduct of the Lys362Met mutant is also wild type in character (Figure 6), and indicates no perturbation in the vicinity of the binuclear center. However, the Lys362Gln mutant exhibits perturbations at the binuclear center. The FTIR spectrum of the Fe—C≡O adduct (Figure 6) has multiple bands at 1950, 1958, and 1966 cm^{-1}, and the Cu—C≡O adduct has a prominent band at 2056 cm^{-1} and a shoulder at 2068 cm^{-1}.

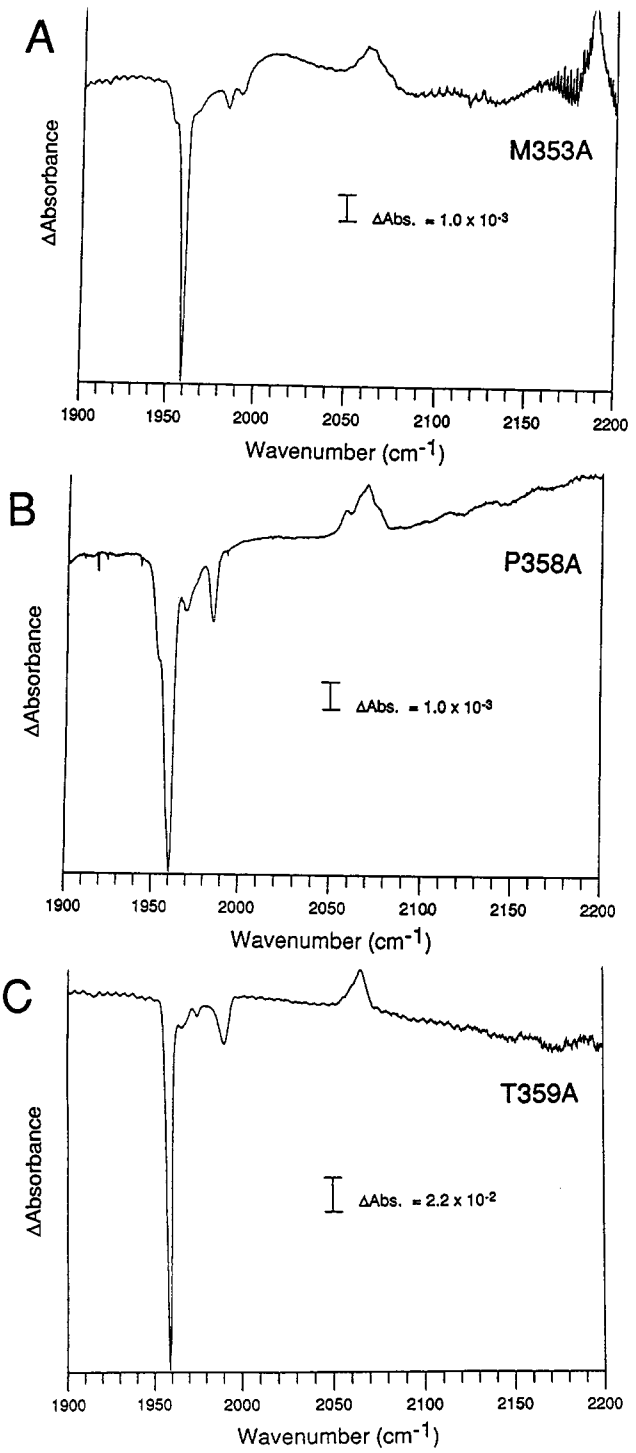

FIGURE 5: FTIR absorbance difference spectra of (A) Met353Ala at 20 K and a path length of 27 μm. The center frequencies of the Fe—C≡O and Cu—C≡O bands are identical to that of wild type. (B) Pro358Ala at 20 K and a path length of 27 μm. The center frequency for Fe—C≡O band is 1960 cm^{-1} and for the Cu—C≡O band is 2068 cm^{-1} with a shoulder at 2056 cm^{-1}. (C) Thr359Ala at 20 K and a path length of 24 μm. The Fe—C≡O peak is 1959 cm^{-1}, and the Cu—C≡O peak is 2064 cm^{-1}. The feature in panel A near 2190 cm^{-1} is background noise.

These data demonstrate that both metal components of the binuclear center are present, but there is evidence for structural perturbation and heterogeneity as a result of the substitution of glutamine for Lys362. The Lys 362Leu mutant also has a perturbed FTIR spectrum (not shown), similar to that observed with Lys362Gln. Substitution of an arginine at this position appears to prevent the assembly of the oxidase. By spectroscopic criteria, cytochrome *bo* is not present in the membranes of the strain expressing this mutant allele.

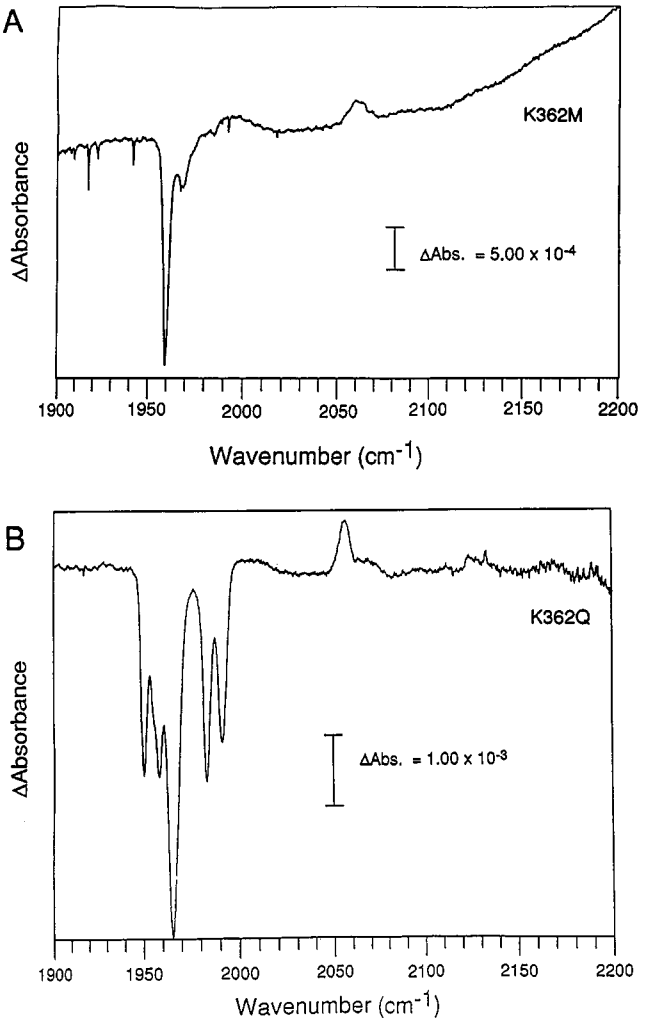

FIGURE 6: FTIR absorbance difference spectra of (A) Lys362Met at 20 K and a path length of 51 μm. The center frequencies are at 1960 and 2059 cm^{-1} for Fe—C≡O and Cu—C≡O, respectively. (B) Lys362Gln at 20 K and a path length of 24 μm. The Fe—C≡O peaks are located at 1950, 1958, and 1966 cm^{-1}. The Cu—C≡O band is a two-component feature at 2056 and 2068 cm^{-1}.

Two amino acid substitutions for Thr359 were examined. A serine at this position yields an oxidase with normal specific activity (Table I) and with a wild-type FTIR spectrum of the CO adduct (not shown). However, an alanine at this position yields an inactive species, unable to complement the oxidase-deficient host strain. The FTIR spectrum of the CO adduct is like that of the wild-type control (Figure 4), indicating that the cause of the dysfunction is not a perturbation to the environment around the heme–copper binuclear center. This is similar to the result obtained with Lys362Met.

Thr352 is the residue predicted to be closest to the periplasmic side of the membrane among the set of residues examined in this work (Figure 1). Three substitutions were examined: alanine, serine, aned asparagine. As shown in Table I, the polar residues, serine and asparagine, yield active oxidase, though with somewhat diminished specific activity. The nonpolar alanine, however, yields an inactive species, similar to the results obtained with Thr359Ala. The FTIR spectrum of the CO adduct of Thr352Ser is indistinguishable from the wild type (not shown). However, the Thr352Asn mutant has a substantially altered FTIR spectrum compared to that of the wild type (Figure 7). The Fe—C≡O clearly has a broad absorbance centered around 1960 cm^{-1}, and there is a distinct feature in the FTIR spectrum centered near 2130 cm^{-1}, which is either partially or entirely from the photolyzed CO in the alternate cytochrome *bd*, as previously observed

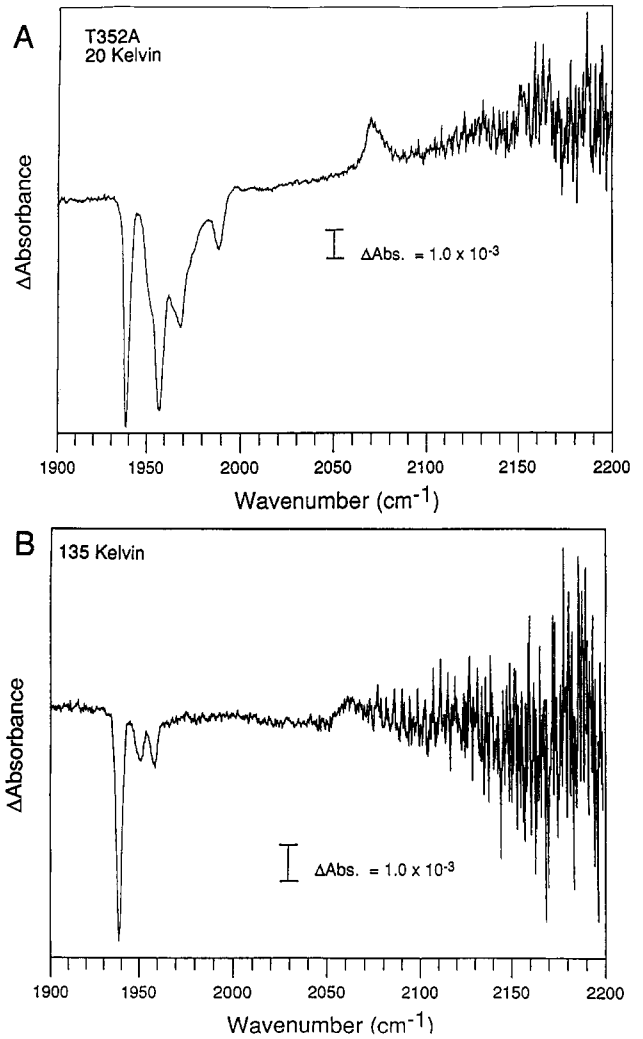

FIGURE 7: FTIR difference spectra obtained from the same sample of Thr352Ala (path length 24 μm). (A) At 20 K. The Fe—C≡O center frequencies were at 1938, 1956, and 1968 cm^{-1}, and the Cu—C≡O band was at 2070 cm^{-1}. (B) At 135 K. The center frequencies for the Fe—C≡O bands are at 1938, 1950, and 1958 cm^{-1}. The Cu—Cu≡O band is centered at 2061 cm^{-1}.

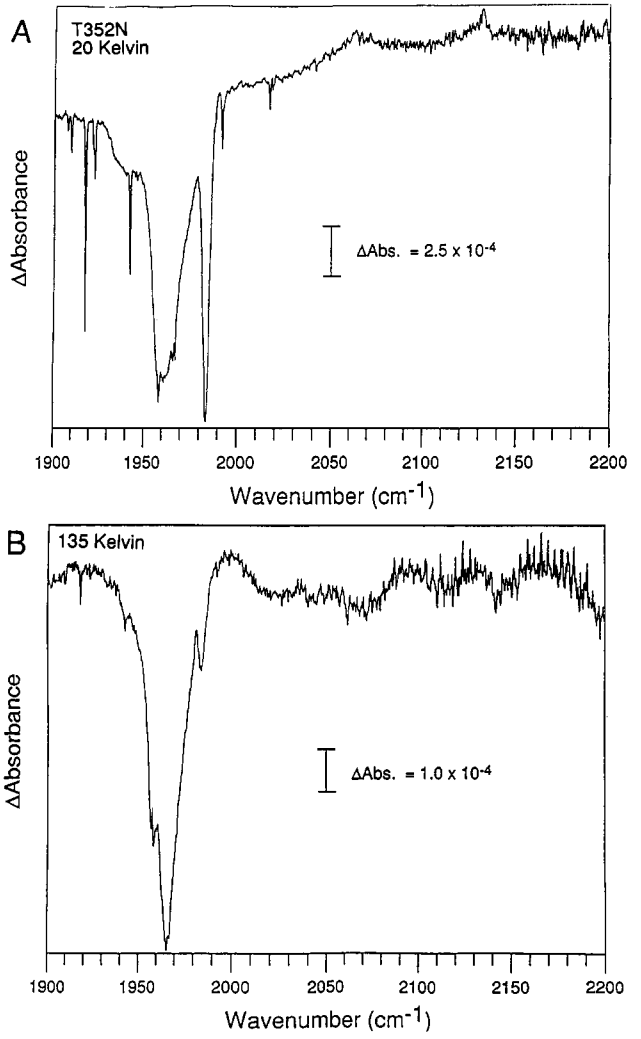

FIGURE 8: FTIR absorbance difference spectra obtained from the same sample of Thr352Asn (path length 27 μm). (A) At 20 K. The center frequencies are at 1960 and 2064 cm^{-1}, respectively, for the Fe—C≡O and Cu—C≡O bands. (B) At 135 K. The Fe—C≡O peak is centered at 1966 cm^{-1}, and the Cu—C≡O peak is sufficiently broadened to make a center frequency determination difficult.

(Hill et al., 1991, 1993). In principle, these features could result from a mixed population of the cytochrome *bo* mutant, in which a portion of the enzyme is similar to the wild type, but with a substantial population where Cu_B either is in a perturbed environment or may even be missing (Hill et al., 1991). To test this possibility, the FTIR absorbance difference spectrum was obtained at 135 K (Figure 7). At this higher temperature, it is expected that photolyzed CO species that are not bound to Cu_B will relax rapidly back to the heme Fe (Fiamingo et al., 1982). This will result in the loss of the Fe—C≡O bands in the absorbance difference spectrum which are associated with the copper-deficient species. The FTIR absorbance difference spectrum of the Thr352Asn mutant taken at 135 K is, however, similar to that observed at 20 K (Figure 7). This indicates that the photolyzed CO is bound to Cu_B in the entire population of molecules.

The substitution of alanine at position 352 causes a very substantial perturbation to the FTIR spectrum of the CO adduct. The Fe—C≡O absorbance is heterogeneous with major peaks centered at 1938, 1956, and 1968 cm^{-1} (Figure 8). The absorbance due to the Cu—C≡O is shifted to higher frequency (2070 cm^{-1}) compared to that of the wild type. Hence, both the heme and copper components of the binuclear center are present, but CO bound to either metal is reporting an altered environment due to the alanine substitution for Thr352. However, the FTIR spectrum obtained at 135 K (Figure 8) indicates that the major 1938-cm^{-1} Fe—C≡O band is associated with slowly relaxing CO (i.e., bound to Cu_B), suggesting that a portion of the oxidase is lacking Cu_B. The narrow band at 1938 cm^{-1} indicates, however, that the environment around Fe—C≡O in the Cu_B-containing species remains highly ordered, despite the large shift in the center frequency from that observed with the wild type (1959 cm^{-1}).

DISCUSSION

The structural model for the cytochrome *bo* oxidase that has emerged recently from mutagenesis studies clearly places the heme *o*–Cu_B binuclear center, the site where the oxygen chemistry occurs, near the periplasmic side of the membrane. The same holds for the heme a_3–Cu_B center in the eukaryotic cytochrome *c* oxidases, which is localized near the intermembrane space in the mitochondrion. During catalytic turnover of either of these enzymes, protons which originate from the opposite side of the membrane from the binuclear center are required both for the formation of water from molecular oxygen (four protons per O_2) and for proton pumping (four protons per O_2). It is evident that the protein must provide one or more pathways for protons to get to the binuclear center, and must also provide a pathway for water (possibly as hydroxide

or hydronium) to exit from the site. The work presented in this paper is motivated in large part by the desire to locate residues within the enzyme which may be important for these functions.

Previous mutagenesis studies have identified His106 (helix II) and His421 (helix X) as the ligands for the low-spin heme b_{562} component of the oxidase (Lemieux et al., 1992; Minagawa et al., 1992; Shapleigh et al., 1992b), His419 (helix X) as the axial ligand for heme *o* (Calhoun et al., in press), and His333 and His334 (helix VII) as Cu_B ligands (Calhoun et al., in press). In addition, both His284 (Calhoun et al., in press) and Tyr288 (Thomas et al., unpublished results) (helix VI) appear to be in the immediate vicinity of Cu_B and are potential ligands to this metal. The arrangement of helices II, VI, VII, and X that follows from these data is shown in Figure 2. The interhelical loop in the cytoplasmic side of the membrane connecting VII and helix VIII is proposed to be quite short (Figure 1), so it is reasonable to place helix VIII in the model, as shown in Figure 2, adjacent to helix VII. This would predict that residues near the periplasmic side of helix VIII should be acjacent to the binuclear center, as is the case with residues in both helix VI and helix VII. Furthermore, it has already been pointed out that helix VIII has a highly conserved face containing several polar residues which could, in principle, play a role in facilitating proton and/or water movement during turnover of the oxidase. Although the data presented in this paper do not prove this hypothesis, they are consistent with this speculation. Mutations in each of three highly conserved polar residues in helix VIII (Thr352, Thr359, and Lys362(were found to eliminate oxidase activity. Two other residues examined in helix VIII (Met353 and Pro358) were found not to be critical for maintaining either the structure or the function of the oxidase.

Methionine-353. This is the only residue in the current set which is neither highly conserved nor part of the polar side of helix VIII. Out of the 75 sequences of subunit I which were aligned, this residue is present in 56, and occupied by a nonpolar residue (Leu, Val, Ile, or Ala) in the other 19 sequences. Replacement of Met353 by alanine in cytochrome *bo* does not significantly alter the oxidase other than a rather modest lowering of the quinol oxidase specific activity. In the model shown in Figure 2, Met353 is proposed to face away from the binuclear center.

Proline-358. This residue is very highly conserved and is absent only in 1 out of the 75 sequences which were aligned, that from *Sulfolobus acidocaldarius* (Lübben et al., 1992). It is expected that the proline in the middle of helix VIII might create a kink by as much as 20–30° (Henderson et al., 1990). Prolines within putative transmembrane helices are relatively common and are, for example, in the observed structure of bacteriorhodopsin. It has been suggested that the kink introduced by a proline is necessary to keep the helix rigid, and also to position the resulting convex side of the helix for interhelical connections (Williams & Deber, 1991). Furthermore, the presence of a proline within a helix will free a backbone carbonyl to act as a hydrogen bond acceptor, either to another protein group or to water. Nevertheless, it has been shown in several instances, including bacteriorhodopsin (Ahl et al., 1988) and the *E. coli* lactose permease (Consler et al., 1991), that proline residues within transmembrane helices can be replaced by other residues without loss of function. This is what is observed for the Pro358Ala mutant examined in this work. The enzyme is functional (Table I), and, in addition, the FTIR analysis indicates no structural perturbation in the vicinity of the binuclear center (Figure 5).

Lysine-362. This is the only conserved basic amino acid which can reasonably be modeled within a transmembrane span of subunit I in the oxidase. This lysine is present in all reported sequences of subunit I with the exception of those from *S. acidocaldarius* (Lübben et al., 1992) and the ba_3-type oxidase from *Thermus thermophilus* (Genebank, LO9121, J. Fee, personal communication) (Bilofsky & Burks, 1988). The most interesting result obtained from this work is that the Lys362Met mutant is enzymatically inactive, as judged by the genetic complementation assay, yet the metal centers are unperturbed as determined spectroscopically (Figures 3 and 6). Of course, subtle changes at the metal centers cannot be ruled out, but clearly there is no major alteration in the vicinity of the binuclear center in this mutant. This phenotype is consistent with a lesion which prevents the timely delivery of protons to the binuclear center at one or more steps during the reaction. The Lys362Asn and Lys362Leu mutants are also inactive, but in these cases the FTIR spectra of the CO adducts reveal significant perturbation at the binuclear center (Figure 6). The fact that the FTIR absorbance difference spectrum at 135 K is similar to that obtained at 20 K (not shown) suggests that Cu_B is present in the entire enzyme population. The multiple bands associated with the Fe—C≡O absorbance suggest multiple conformations of the pocket, but that it is still substantially intact. It is likely that this is a result of conformational changes in the protein that are transmitted along helix VIII to the binuclear center.

Threonine-359. This residue is also highly conserved, but is replaced by a serine in three species and reportedly by alanine in three species (*Paramecium tetraurelia, Paramecium aurelia, and Tetrahymena pyriformis*) (Bilofsky & Burks, 1988). Replacement of this Thr359 by a serine in cytochrome *bo* has no significant effect on the oxidase, indicating that serine or threonine at this position works equally well. An alanine at this position, however, results in elimination of enzyme activity. The FTIR analysis of the binuclear center (Figure 5) indicates no structural perturbation to correlate with the loss of enzyme activity. The data are consistent with a role for Thr359 as part of a relay system, including Lys362, to facilitate proton and/or water movement during catalysis.

Threonine-352. This residue is highly conserved in all the reported sequences of subunit I with only four exceptions. In three species, a serine is found at the same position (*Halobacterium halobium, Leishmania*, and *Sauroleishmania tarentolae*), and a leucine is reportedly in the oxidase from *Saccharomyces douglasii*. The Thr352Ser mutant in the *E. coli* oxidase has a somewhat reduced quinol oxidase activity, but otherwise appears to be wild type. The replacement by another polar residue, asparagine, also results in an active species, but in this case the FTIR spectrum (Figure 7) suggests some disorder within the binuclear center. The absorbance bands associated with both the Fe—C≡O and Cu—C≡O bands are each broadened significantly beyond that observed with the wild-type control (Figure 4). The structural perturbation caused by Thr352Asn is noticeable, but it is clear that asparagine can substitute functionally for threonine at this locus. This is not the case for alanine, as the Thr352Ala mutant not only is inactive but also has a perturbed binuclear center as evidenced by the FTIR analysis (Figure 8). The FTIR absorbance difference spectrum of the CO adduct of the Thr352Ala adduct at 135 K, when compared to that obtained at 20 K, indicates that part of the population of the mutant oxidase is missing Cu_B (Figure 8). That portion of the Thr352Ala that appears to retain Cu_B has a narrow absorbance band of the Fe—C≡O at 1938 cm^{-1}, indicating

an ordered, albeit substantially altered, environment, compared to the wild type.

The fact that even the functionally active Thr352Asn mutant exhibits a perturbed environment around the binuclear center is consistent with the model placing this residue in the immediate vicinity of the site where oxygen is reduced to water. It is of interest that a threonine has been proposed to stabilize oxygen intermediates in the active site of cytochrome $P450_{cam}$ prior to the cleavage of diatomic oxygen (Gerber & Sligar, 1992; Martinis et al., 1989; Raag et al., 1991). Possibly, Thr352 could play a similar role in the heme–copper oxidases, since the current model places this residue near the site of oxygen reduction. The current work suggests that a polar residue is important at this position, but further studies will be required to investigate the range of substitutions that maintain enzymatic activity. In addition, studies are currently underway to determine if Thr352 might be involved in direct proton donation to an oxygenated intermediate during catalysis.

In summary, the data presented in this paper allow the structural model of subunit I of the heme–copper oxidase to be further elaborated to include helix VIII (Figure 2). It seems reasonable to conclude that some residues on the periplasmic side of helix VIII, Thr352 in particular, may be in direct contact with the binding pocket encompassing the heme–copper binuclear center. The highly conserved polar face of helix VIII would then lead from the metal centers to the cytoplasmic side of the membrane, and could be important in facilitating proton and/or water movement during catalysis. Aspartate-135, located in the interhelical loop between transmembrane spans II and III (Figure 1), is the only other residue which has been identified to date as having a possible role in proton movements, and this residue has been proposed to reside at the mouth of the putative proton-conducting channel (Thomas et al., in press). It is evident that the interpretation of all these data must be considered to be highly speculative in the absence of detailed mechanistic studies to examine the kinetic steps during turnover that are influenced by the various mutations. Nevertheless, a small number of polar residues have now been identified that are essential for the proper function of the oxidases, and the future challenge to explore the roles of these residues is clear.

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