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Polar Residues in Helix VIII of Subunit I of Cytochrome *c* Oxidase Influence the Activity and the Structure of the Active Site†

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ABSTRACT: The aa_3-type cytochrome *c* oxidase from *Rhodobacter sphaeroides* is closely related to eukaryotic cytochrome *c* oxidases. Analysis of site-directed mutants identified the ligands of heme *a*, heme a_3, and Cu_B [Hosler et al. (1993) *J. Bioenerg. Biomembr. 25*, 121−133], which have been confirmed by high-resolution structures of homologous oxidases [Iwata et al. (1995) *Nature 376*, 660; Tsukihara et al. (1995) *Science 269*, 1069; (1996) *272*, 1136]. Since the protons used to form water originate from the inner side of the membrane, and the heme a_3−Cu_B center is located near the outer surface, the protein must convey these substrate protons to the oxygen reduction site. Transmembrane helix VIII in subunit I is close to this site and contains several conserved polar residues that could function in a rate-determining proton relay system. To test this role, apolar residues were substituted for T352, T359, and K362 in helix VIII and the mutants were characterized in terms of activity and structure. Mutation of T352, near Cu_B, strongly decreases enzyme activity and disrupts the spectral properties of the heme a_3−Cu_B center. Mutation of T359, below heme a_3, substantially reduces oxidase activity with only minor effects on metal center structure. Two mutations of K362, ∼15 Å below the axial ligand of heme a_3, are inactive, make heme a_3 difficult to reduce, and cause changes in the resonance Raman signal specific for the iron−histidine bond to heme a_3. The results are consistent with a key role for T352, T359, and K362 in oxidase activity and with the involvement of T359 and K362 in proton transfer through a relay system now plausibly identified in the crystal structure. However, the characteristics of the K362 mutants raise some questions about the assignment of this as the substrate proton channel.

The aa_3-type cytochrome *c* oxidases are metalloenzymes that catalyze the four-electron reduction of oxygen to water and function as the terminal members of the energy-transducing respiratory chain in mitochondria and many aerobic bacteria. These oxidases are members of a superfamily of enzymes that have in common a heme−copper binuclear center where oxygen chemistry is catalyzed (Saraste, 1990; Calhoun et al., 1994; Garcia-Horsman et al., 1994a). During the four-electron reduction of molecular oxygen, four protons are consumed at the active site to form water, while another four protons are translocated through the protein, across the membrane (Wikström et al., 1985). The mechanism by which both "substrate" and "pumped" protons move through the protein is of primary importance in understanding the activity and regulation of cytochrome oxidase.

Subunits I and II of cytochrome oxidase alone are sufficient for both electron transfer activity and for proton pumping (Haltia et al., 1991; Solioz et al., 1982), indicating that the majority of amino acid residues that are directly involved in the movement of protons are likely to be members of these subunits. Subunit I of the aa_3-type oxidases contains 12 transmembrane spans (Iwata et al., 1995) and a number of amino acid residues capable of hydrogen bonding or proton dissociation that are highly conserved in an alignment of 75 subunit I sequences from the oxidase superfamily [Calhoun, 1993; see Figure 1 of Hosler et al. (1993)]. Subunit II contains only two membrane-spanning helices (Iwata et al., 1995), and these are less conserved in species comparisons (Calhoun, 1993; Holm et al., 1987; Saraste, 1990). Thus, it is reasonable to suggest that subunit I contains most of the residues that are important for facilitating proton translocation in the membrane domain of the protein.

Studies of site-directed mutants of the aa_3-type oxidase of *Rhodobacter sphaeroides* have identified the histidine residues that ligate heme *a*, heme a_3, and Cu_B in subunit I of this enzyme (Hosler et al., 1993; Calhoun et al., 1993). Recently these ligand assignments, as well as the prediction that the binuclear center is located near the outer side of the membrane, have been confirmed by high-resolution structures of the closely related aa_3-type oxidases of *Paracoccus*

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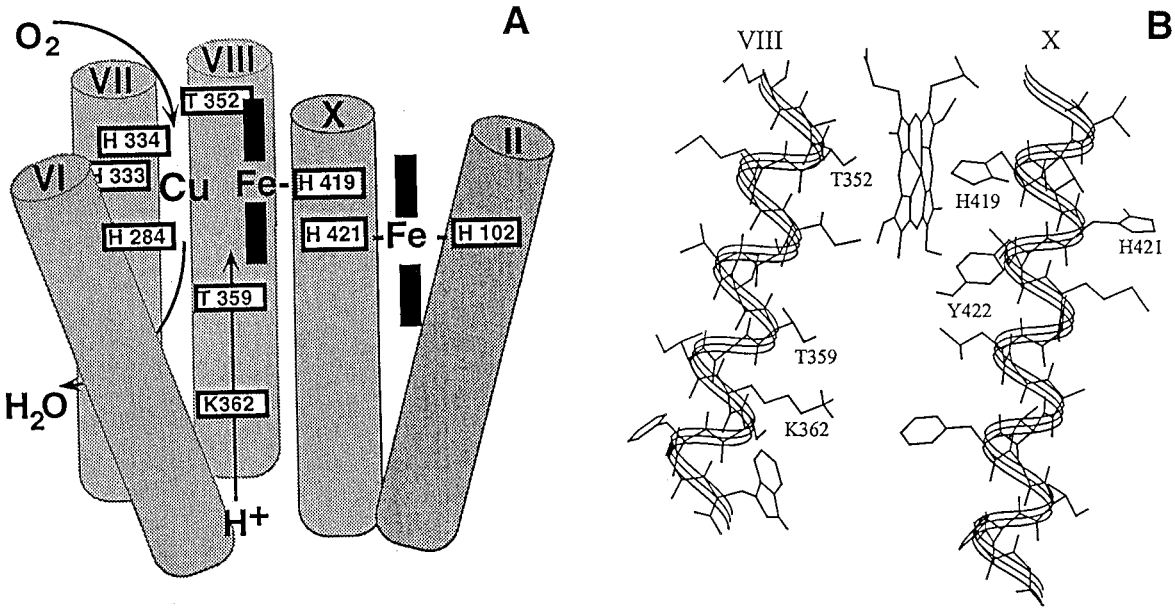

FIGURE 1: (A) Model of five of the transmembrane helices of subunit I of *R. sphaeroides* cytochrome *c* oxidase. The model illustrates the proposed role for helix VIII as a conduit for proton movement to the active site. The assignment of the ligands of heme *a* and the heme a_3-Cu_B center has been previously reported [see Shapleigh et al. (1992b) and Hosler et al. (1993)] and recently confirmed [Iwata et al., 1995; Tsukihara et al., 1995). Readers should refer to the latter references for the actual structures. The "inside" or cytoplasmic face of the bacterial membrane is toward the bottom of the figure, while the "outside" or periplasmic face is toward the top. (B) Computer-generated view of transmembrane helices VIII and X and heme a_3. The hydroxyfarnesyl tail is not shown. The model was prepared on a Silicon Graphics Indigo^2 computer using Quanta 2.0. In this representation helix VIII includes M_{350}-**A**-$\mathbf{T_{352}}$-M-V-**I**-**A**-V-**P**-$\mathbf{T_{359}}$-**G**-I-$\mathbf{K_{362}}$-I-**F**-**S**-$\mathbf{W_{366}}$, while helix X includes V_{417}-A-$\mathbf{H_{419}}$-**F**-$\mathbf{H_{421}}$-$\mathbf{Y_{422}}$-**V**-M-S-L-**G**-**A**-V-**F**-G-I-F-A-G_{435}. (Highly conserved residues are in boldface type.) Although it is not apparent in this two-dimensional drawing, helix VIII is placed behind the heme a_3-Cu_B center as shown in panel A.

denitrificans (Iwata et al., 1995) and bovine mitochondria (Tsukihara et al., 1995, 1996). A schematic of five of the 12 transmembrane helices of subunit I is presented in Figure 1A. Since the protons required for the formation of water at the binuclear center must originate from the inner (negative) side of the membrane, a proton-conducting channel from the inner side to the binuclear center is required. In principle, the channel required for those protons destined to form water could also convey the protons which are pumped across the membrane, at least up to the level of the binuclear center. However, theoretical considerations (Morgan et al., 1994; Iwata et al., 1995) as well as the existence of mutant oxidases that retain electron transfer while losing proton translocation activity (Thomas et al., 1993b; Fetter et al., 1995; Garcia-Horsman et al., 1995) suggest independent pathways to move "substrate" and "pumped" protons through the enzyme.

We expect proton channels in cytochrome oxidase to principally involve a network of residues with polar side chains along with bound water molecules (Nagle & Morowitz, 1978; Nagle & Tristram-Nagle, 1984). While studies on bacteriorhodopsin have shown that carboxylate residues within the membrane domain are critical for proton transfer (Braiman et al., 1988; Butt et al., 1989; Gerwert et al., 1989, 1990; Holz et al., 1989; Krebs & Khorana, 1993), only one conserved acidic residue is found within the transmembrane region of cytochrome oxidase: Glu-286 of helix VI (Iwata et al., 1995). Preliminary results in studies of the E286Q mutant of the *R. sphaeroides* oxidase (unpublished) indicate that this residue is important for function. However, the equivalent mutant in cytochrome bo_3 from *Escherichia coli*, a member of the heme−copper oxidase superfamily, is active and pumps protons (Thomas et al., 1993b). In addition, the gene sequence for the proton-pumping caa_3-type oxidase from *Thermus thermophilus* predicts a threonine at this position (Mather et al., 1993).

Of particular interest has been a group of conserved polar residues located along one face of transmembrane helix VIII (Figure 1B). The short interhelical connection between helix VII and helix VIII [see Figure 1 of Hosler et al. (1993)] originally suggested that helix VIII was close to helix VII and could be modeled adjacent to helix X and the binuclear center. This has now been shown to be true (Iwata et al., 1995).

Previous analysis of site-directed mutants of the quinol oxidase cytochrome bo_3 of *E. coli* suggested that three residues of helix VIII, Lys-362, Thr-359, and Thr-352, might be important in facilitating proton and/or water movement during catalysis (Thomas et al., 1993a). Here we report a more comprehensive analysis of purified mutants of these residues in the aa_3-type cytochrome *c* oxidase of *R. sphaeroides*, an enzyme closely related to the mitochondrial oxidase (Hosler et al., 1992; Cao et al., 1991, 1992). The mutants were examined for steady state activity, for CO binding, by low-temperature Fourier transform infrared (FTIR) spectroscopy, and by resonance Raman spectroscopy to obtain information about the environment of each of the heme centers. Proton pumping activity has also been described (Fetter et al., 1995). The results, suggesting critical functional roles for these helix VIII residues, are interpreted in light of the new structural information.

MATERIALS AND METHODS

Restriction endonucleases and DNA modifying enzymes were obtained from New England Biolabs or Bethesda Research Laboratories. Synthetic oligonucleotides were synthesized at the Biotechnology Center at the University

of Illinois at Urbana−Champaign on an Applied Biosystems model 380A DNA synthesizer. Sequenase enzyme and kits were purchased from US Biochemical. Carbon monoxide (CO) used for FTIR spectroscopy was purchased from Matheson and was 99.5% pure.

Site-directed mutagenesis was carried out using the method of Vandeyar et al. (1988). The vector pJS3, a pRK415-1 derivative containing *cta*D modified to facilitate cloning of fragments (Shapleigh et al., 1992b), was used for cloning and expression of mutants. To mutate Thr-352 and Thr-359, a 455-bp *kpn*I-S*st*I fragment was inserted into the phagemid pT7T3-18U (Pharmacia). Mutagenesis was carried out on single-stranded DNA produced using the helper phage M13KO7. To reintroduce mutants back into pJS3, a *Kpn*I-*Bgl*II subclone of the fragment was used since the *Sst*I site is not unique. Mutagenesis of Lys-362 required the construction of a new vector since the codon for K362 is part of the *Bgl*II restriction site. This was achieved by cloning a 1.65 Kb *Bam*HI-*Hin*dIII fragment of pJS3 into pT7T3-19U. The *Sst*I restriction site in the polylinker of this construct was deleted by restricting with *Sma*I and *Eco*RI, filling in the overhangs with T4 polymerase, and ligating the blunt ends. This construct is designated pBG. A *Kpn*I-*Hin*dIII fragment of pBG was used to move Lys-362 mutants into pJS3. The mutant alleles of *cta*D (subunit I) were expressed in a *R. sphaeroides* strain (JS100) from which the chromosomal copy of *cta*D has been deleted (Shapleigh & Gennis, 1992). The *R. sphaeroides* strains containing the mutant constructs were grown aerobically as described in Hosler et al. (1992).

Preparation of the CO adducts of the oxidase in inner membrane preparations for FTIR spectroscopy, as well as the collection of the low-temperature FTIR difference spectra, was performed as described elsewhere (Shapleigh et al., 1992). The FTIR spectroscopy was performed with purified enzyme which was reconstituted from a 5% cholate solution into proteoliposomes by extended dialysis in the presence of 40 mg of soybean phosopholipids/mL (Asolectin) in 50 mM Tris, pH 8.0.

Purification of the wild type cytochrome *c* oxidase and mutant forms, optical and resonance Raman spectroscopies, and electron transfer measurements were performed as described in Hosler et al. (1992). Purification for FTIR studies was performed as described in Mitchell and Gennis (1995). The amount of CO binding, relative to wild type, was determined as in Hosler et al. (1994b).

RESULTS

The following mutant forms of cytochrome *c* oxidase were prepared by site-directed mutagenesis: T352A, T352N, T352I, T359A, K362M, K362A, and K362R. Visible spectra (not shown) of isolated cytoplasmic membranes from mutant strains showed that in every case, except K362R, the absorbance band at ∼605 nm due to heme *a* is present. Membranes of cells synthesizing K362R contain little or no heme *a*, indicating poor expression or instability of this mutant oxidase.

The activity of the mutant oxidases cannot be readily evaluated in the membrane due to the presence of an additional cytochrome *c* oxidase in *R. sphaeroides*, a cbb_3-type (García-Horsman et al., 1994; Shapleigh et al., 1992a). In addition, RR spectroscopy as well as optical spectroscopy in the Soret region requires that the oxidases be purified. T352A, T359A, K362M, and K362A were selected as representative mutant forms to be purified and further examined.

Table 1: Absorbance Maxima (from Absolute Reduced Spectra), Heme *a* and a_3 Content, CO Binding, and Electron Transfer Activity of the Helix VIII Mutants

oxidase	α_{max} (nm)	$Soret_{max}$ (nm)	Soret/α of the fully reduced oxidase	CO binding (% of wild type)	electron transfer activity (% of wild type)
wild type	605.4	444.4	5.4	(100)	(100)
T352A	603.0	∼441[a]	6.3[a]	35	<10
T359A	605.4	444.4	5.5	100	35
K362M	604.8	443.4	5.5	55	<2
K362A	604.8	443.4	5.3	60	<2

[a] The level of *b* and *c* cytochrome contaminants in this sample shifts the Soret maximum by approximately 1 nm (from 442 nm) and increases the Soret/α ratio from 5.4 to 6.3, as determined by comparison with a spectrum of a wild type sample containing similar amounts of *b* and *c* cytochromes.

The activity, absorbance characteristics, and CO binding behavior of the four purified mutants is summarized in Table 1. All four have low or no activity but contain normal amounts of heme *a* and heme a_3 as revealed by the Soret/α peak absorbance ratio of the fully reduced oxidases. The Soret/α ratio is a sensitive indicator of selective loss of either heme a_3, as evidenced by a marked decrease [see Hosler et al. (1994b)], or heme *a*, as indicated by a sharp increase [see Vanneste (1966)]. Small but significant blue shifts are seen in all of the optical spectra except that of T359A. Further differences among the mutants, as well as the structural and functional significance of these differences, are discussed below.

Oxidase Activity and UV/Visible Spectroscopy of Purified Mutant Oxidases. Purified T352A has less than 10% of the activity of the native enzyme (Table 1). The absolute UV/visible spectrum reveals a blue shift of ∼2.5 nm for both the α and the Soret peaks, indicating changes in the local environments of both hemes *a* and a_3 (Table 1; note that 1 nm of the Soret blue shift is ascribed to a spectral shift due to contamination by the cbb_3-type oxidase). The amount of CO that binds to heme a_3 in this mutant is reduced to 35%−40% of that observed with wild type (Table 1), indicating structural perturbation of the heme a_3-Cu_B center.

In contrast, T359A is essentially wild type in its spectral characteristics and CO binding (Table 1), indicating that the loss of threonine at this position does not significantly affect the structure of the metal centers. The electron transfer activity of T359A, however, is maximally 35% of that of wild type (some preparations have lower specific activity). This suggests that Thr-359 has some rate-limiting effect on turnover of the oxidase.

Neither of the mutant oxidases containing alterations of Lys-362 has measurable activity. The reduction of the heme *a* band at 605 nm by ascorbate/TMPD or dithionite is immediate. Heme a_3, however, is not reduced with excess ascorbate/TMPD, as evidenced by the Soret/α value and the finding that no CO binds under these conditions (data not shown). Hence, reduction of heme a_3 via heme *a* is inhibited in the Lys-362 mutants. Reduction by dithionite similarly results in rapid reduction of heme *a*, but the rate of reduction of heme a_3 varied from a few minutes to several hours,

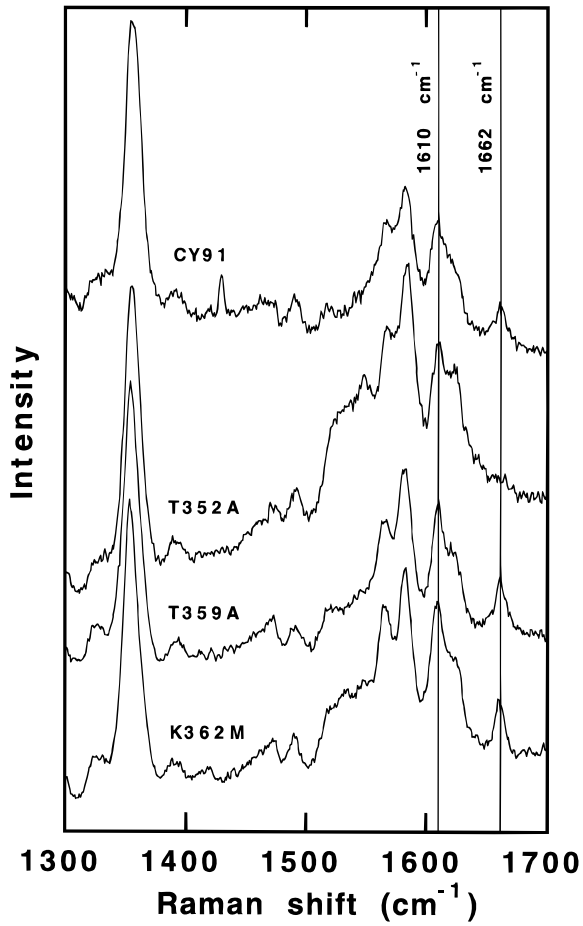

FIGURE 2: High-frequency region of the resonance Raman spectrum of dithionite-reduced wild type cytochrome *c* oxidase (CY91), T352A, T359A, and K362M. The concentration of the samples was approximately 40 mM. Special care was taken to ensure the complete or near-complete reduction of K362M (see text) prior to the acquisition of the RR spectrum. Full reduction of K362M is suggested by presence of n_4 at 1355 cm^{-1} and by the amplitude and position of the formyl stretch of heme a_3 at 1662 cm^{-1}. The formyl stretching frequencies of heme *a* (1610 cm^{-1}) and heme a_3 (1662 cm^{-1}) are marked. The excitation wavelength was 441.6 nm.

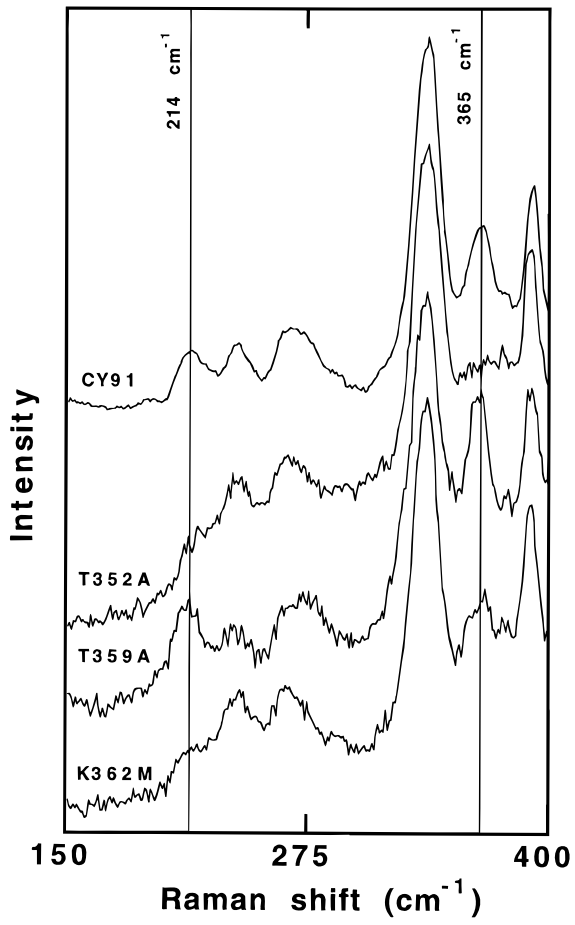

FIGURE 3: Low-frequency region of the resonance Raman spectrum of the reduced wild type (CY91) and mutant cytochrome oxidases. The Fe−N_{his} stretch of heme a_3 (214 cm^{-1}) and the porphyrin ring bending mode specific for heme a_3 (365 cm^{-1}) are marked. Conditions are as in Figure 2.

depending on the history of the preparation. After brief storage at 4 °C biphasic reduction of heme a_3 was observed, indicating at least two different conformations of the preparation. The Soret/α value of 5.6 indicated that both hemes were present in normal amounts.

Resonance Raman Analysis of Purified Mutants. Resonance Raman spectroscopy of aa_3-type cytochrome *c* oxidases is particularly valuable in that it yields information concerning the local protein environment of each of the two hemes (Babcock, 1988; Ching et al., 1985). The RR spectra of three of the purified mutants are shown in Figures 2 and 3. In all three mutants, two RR bands specifically associated with heme *a*, the 1610 cm^{-1} formyl stretch and the 1624 cm^{-1} vinyl stretch, are identical to those of wild type. Consistent with its native spectral characteristics, T359A also shows normal heme a_3 modes, including the formyl stretch at 1662 cm^{-1}, the ring bending mode at 365 cm^{-1}, and the Fe−N_{his} stretch at 214 cm^{-1}.

The other two mutants shown in Figures 2 and 3, T352A and K362M, show significant, but quite different, alterations in their heme a_3 signals. For T352A, the 365 and 1662 cm^{-1} modes are absent, while the Fe−N_{his} stretch at 214 cm^{-1} is detectable, but at greatly diminished amplitude. The loss or attenuation of the RR modes reflecting heme a_3−protein interactions must be reconciled with the fact that the optical absorbance data show that heme a_3 is present in normal amounts (Table 1). Structural changes that favor the existence of multiple conformations of the heme a_3−Cu_B center can result in broadening of the heme a_3-specific RR bands to the point where they cannot be resolved, as previously noted for mutations of the ligands of Cu_B and heme a_3 (Calhoun et al., 1993; Hosler et al., 1993; Shapleigh et al., 1992b). Thus, the RR spectrum of T352A suggests a disordered heme a_3-Cu_B pocket in which the proximal ligation of heme a_3 by His-419 of helix X is maintained but has several energy states. Since the heme *a*-specific RR modes indicate a normal environment for the low-spin heme center, the conformational changes induced by the substitution of alanine for Thr-352 must be relatively localized and not be the result of a general perturbation of the entire protein structure, consistent with proximity of Thr-352 to the heme a_3−Cu_B active site (Iwata et al., 1995; Tsukihara et al., 1996), as shown in Figure 1B.

In the RR spectrum of K362M, both the Fe−N_{his} stretch at 214 cm^{-1} and the porphyrin bending mode at 365 cm^{-1} are present but attenuated (Figure 2). The nature of the base line in the low-frequency region makes quantitation difficult, but the amplitude of the 214 and 365 cm^{-1} modes are

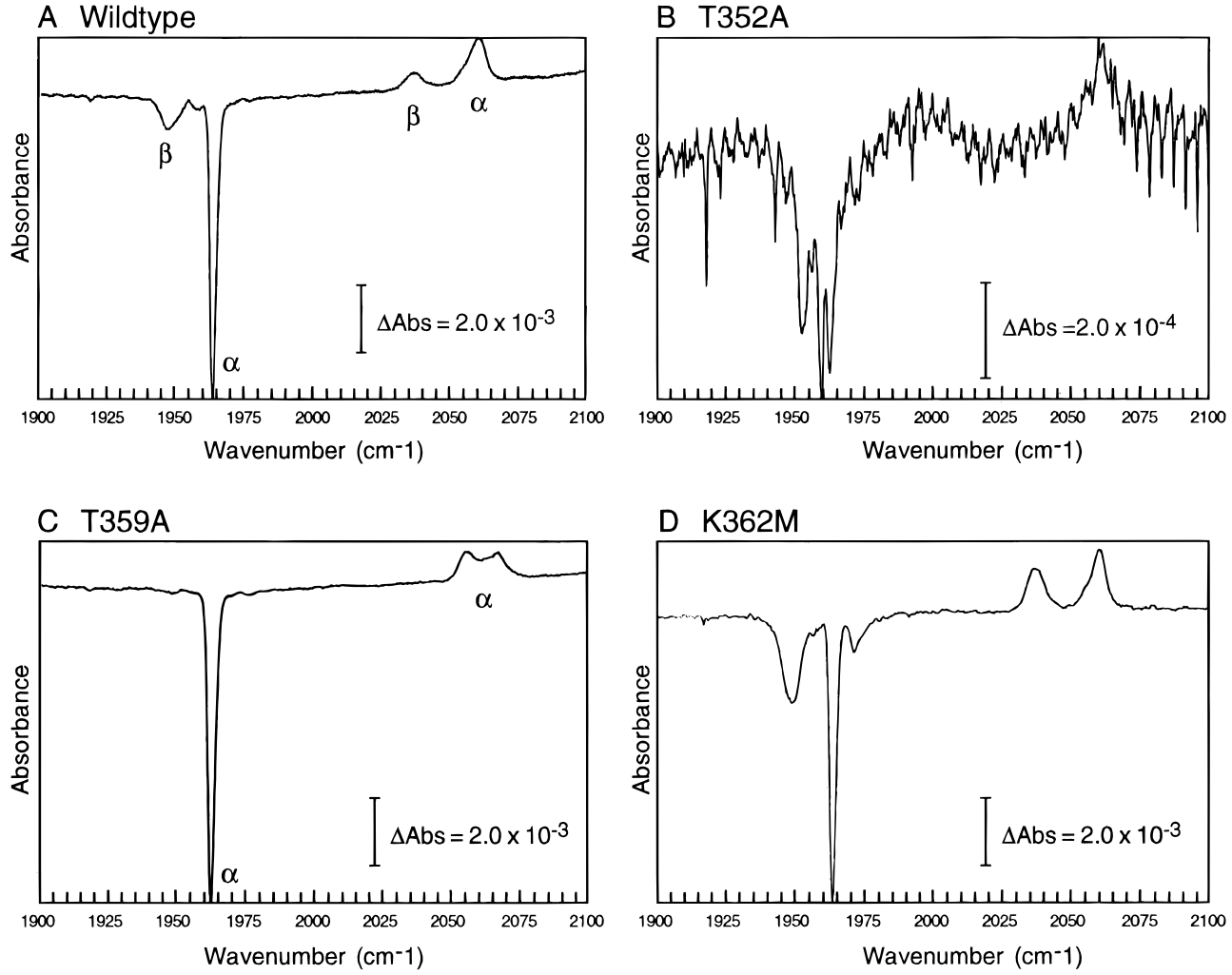

FIGURE 4: Low-temperature Fourier transform infrared (FTIR) difference spectra (light-minus-dark) of wild type and mutant cytochrome oxidases. All the spectra were obtained using purified enzyme reconstituted into phospholipid vesicles at pH 8.0. The spectrum of T359A was recorded at 10 K, while the others were recorded at 20−35 K.

approximately 50% of that of the wild type enzyme. The heme a_3 formyl stretch at 1662 cm^{-1}, however, is wild type in both its position and amplitude (Figure 2), indicating that heme a_3 of K362M remains high-spin and that the oxidase is fully reduced. The RR spectrum of purified K362A (not shown) is identical to that of K362M. Since the protein environment of the heme a_3 formyl group in K362M and K362A appears undisturbed, it is not likely that the changes seen in the low-frequency region of these spectra result from significant disruption of the heme a_3-Cu_B center, such as is seen for T352A. Rather, the RR spectra indicate that the structural change induced in the Lys-362 mutants is localized to the region of the axial ligand of heme a_3.

FTIR Analysis of CO Adducts. Low-temperature FTIR absorbance difference spectroscopy is another useful tool for probing the local molecular environment of the heme a_3-Cu_B center, since at cryogenic temperatures CO can be photolyzed from the heme iron and trapped on the copper (Alben et al., 1981). The stretching frequency of CO, bound to either heme a_3 or Cu_B, depends upon the immediate environment of the metal center. The FTIR absorption light-*minus*-dark difference spectrum of the CO adduct of the aa_3-type cytochrome *c* oxidase of *R. sphaeroides* is almost identical to that of the oxidase from bovine mitochondria (Shapleigh et al., 1992a). Oxidases from each of these organisms exhibit two molecular conformations, designated α and *β* (Fiamingo et al., 1986; Shapleigh et al., 1992a). In the aa_3-type oxidase of *R. sphaeroides*, the Fe—C≡O α form has a maximum at 1964 cm^{-1}, while the Cu—C≡O α form is seen at 2060 cm^{-1} (Shapleigh et al., 1992a). The *β* forms have maxima at 1950 cm^{-1} for the Fe—C≡O and 2039 cm^{-1} for the Cu—C≡O adducts (Shapleigh et al., 1992a). The cbb_3-type cytochrome *c* oxidase of *R. sphaeroides* is expressed to high levels in strains that synthesize nonfunctional forms of the aa_3-type oxidase. The FTIR absorption difference spectrum of the CO adduct of the cbb_3-type oxidase does not exhibit multiple molecular forms (Fe—C≡O, 1952 cm^{-1}; Cu—C≡O, 2065 cm^{-1}; García-Horsman et al., 1994; Shapleigh et al., 1992a). FTIR analysis can be performed with either isolated inner membrane preparations or purified enzyme reconstituted into proteoliposomes (Shapleigh et al., 1992a). Use of inner membranes in strains containing nonfunctional cytochrome aa_3, however, can result in significant spectroscopic interference due to the CO adduct of the cbb_3-type cytochrome *c* oxidase.

The FTIR absorption difference spectra of CO adducts of T352A (Figure 4) and T352I (not shown), performed on purified enzyme, have broad peaks in the 1960−1970 cm^{-1} region, probably arising from Fe—C≡O species in the perturbed binuclear centers. This is consistent with the RR spectrum of T352A, which also indicates disruption of the heme a_3 environment. The Cu—CO band is also shifted

compared to the wild-type, closer to 2070 than 2060 cm^{-1}.

The FTIR analysis of T359A, performed using purified enzyme, is particularly interesting with regard to the α/β conformers (Figure 4) since only the α conformers are observed in this mutant. Splitting of the Cu−CO peak is also more pronounced in this mutant than in the wild type samples.

The FTIR spectrum of the K362M mutant is nearly identical to wild type, suggesting that this amino acid substitution has only relatively subtle effects on the structure of the heme−copper binuclear center (Figure 4) in agreement with the resonance Raman findings. The data support the conclusion that the K362M mutation does not substantially perturb the distal side of heme a_3 or the environment of Cu_B.

DISCUSSION

For each molecule of dioxygen that is reduced to water by cytochrome *c* oxidase, four "substrate" protons are obtained from the cytoplasm, while another four 'pumped' protons are translocated through the protein and released on the opposite side of the membrane. Hence, the enzyme must contain specific residues to facilitate the movement of protons during turnover. Recent studies of bacteriorhodopsin (Krebs & Khorana, 1993), the bacterial photosynthetic reaction center (Okamura & Feher, 1992), and cytochrome P450s (Raag et al., 1991; Ravichandran et al., 1993) have demonstrated that polar amino acids play critical functions in facilitating proton movements during enzyme turnover.

Previous modeling efforts have identified helix VIII of subunit I as a likely proton conduit in cytochrome *c* oxidase, particularly polar residues Thr-352, Thr-359, and Lys-362 (Thomas et al., 1993a; Hosler et al., 1993; Calhoun et al., 1994). The predicted importance of these residues has been conformed by the high-resolution structure of the cytochrome *c* oxidase of *P. denitrificans* (Iwata et al., 1995) and of beef heart (Tsukihara et al., 1996). (Note that Thr-352, Thr-359, and Lys-362 in *R. sphaeroides* correspond to Thr-344, Thr-351, and Lys-354 in *Paracoccus* and Thr-309, Thr-316, and Lys-319 in bovine, respectively.) Thr-352 is adjacent to the Cu_B ligand His-333 (His-325 in *Paracoccus*, His-290 in bovine) and is proposed to stabilize this conformation of the enzyme active site by a hydrogen bond to His-333 (Iwata et al., 1995). Both Thr-359 and Lys-362 are components of an apparent channel leading from the cytoplasmic surface of the enzyme to the heme−copper center (Iwata et al., 1995; Tsukihara et al., 1996). This channel has been proposed to convey protons needed to form H_2O (Iwata et al., 1995; Thomas et al., 1993a; Hosler et al., 1993; Calhoun et al., 1994).

The three polar residues of helix VIII selected for examination in this work are highly conserved in 75 subunit I sequences (Calhoun, 1993). The data presented here show that Thr-352, Thr-359, and Lys-362 are important for the function of the aa_3-type oxidase of *R. sphaeroides*, as they are in the heme−copper cytochrome bo_3 quinol oxidase of *E. coli* (Thomas et al., 1993a).

Mutation of Thr-352 Perturbs the Heme a_3-Cu_B Center. Both RR and FTIR analysis show that mutation of Thr-352 results in significant disruption of the heme−copper binuclear center, even though a small level of electron transfer activity is retained. The EPR spectrum (not shown) of T352A indicates retention of a spin-coupled heme a_3-Cu_B pair and the likely presence of normal levels of Cu_B. Quantitation of the Cu_A signal showed that the EPR spectrum represented the entire population of the T352A sample (data not shown). The low CO binding by this mutant suggests that either the binuclear site does not saturate under these conditions where the wild type does or the mutant can adopt two different conformations that bind (35%), or do not bind (65%), CO. In either case, the results are indicative of an altered heme α_3−Cu_B center, as also evidenced by RR and FTIR. The data are consistent with the structure of the *Paracoccus* oxidase, which shows Thr-352 to be distal to Cu_B, possibly supplying a hydrogen bond to the Cu_B ligand, His-333, when the heme−copper center is oxidized (Iwata et al., 1995). Since the replacement of Thr-352 by serine in cytochrome bo_3 yields an enzyme with 60% activity and a wild type FTIR spectrum (Thomas et al., 1993a) it does appear that a hydrogen bonding residue at position 352 may be important, though not absolutely essential. The purified T352A mutant of the *E. coli* oxidase retains 18% of the wild type steady state activity, and the single-turnover kinetics of the fully reduced mutant oxidase with O_2 are very similar to the wild type (Svensson et al., 1995). The exact role of Thr-352 in the mechanism of the oxidase remains to be resolved experimentally.

Mutation of Thr-359 Modifies Activity and the Ratio of α to β Conformers. The replacement of Thr-359 by alanine in the *R. sphaeroides* cytochrome *c* oxidase reduces enzymatic activity to 20%−35% of the wild type, while the optical and RR analyses show that the structure of the heme a_3−Cu_B center is undisturbed. Proton-pumping analysis (Fetter et al., 1995) indicates a normal efficiency (H^+/e^- ratios the same as wild type). This is consistent with the location of Thr-359 in the *Paracoccus* oxidase (Iwata et al., 1995) and suggests that the loss of steady state activity is not due to disruption of the heme−copper center. The data would support a role of Thr-359 in facilitating transfer of protons to the heme−copper center required for oxygen reduction, or for pumping, assuming that proton uptake will limit electron transfer in either case. The equivalent mutant of the *E. coli* cytochrome bo_3 quinol oxidase has been reported previously (Thomas et al., 1993a; Svensson et al 1995). The purified T359A mutant of the *E. coli* oxidase has 15% of the wild type activity (Svensson et al., 1995) and also has an unperturbed heme−copper center when the FTIR difference spectrum of the CO adduct is used as the criterion (Thomas et al., 1993a). Hence, Thr-359 may be involved in proton translocation, but can be bypassed to some extent when an alanine is in this position. The T359S mutant of the *E. coli* oxidase also shows no structural perturbations and retains ~60% of wild type activity (Thomas et al., 1993a). Svensson et al. (1995) showed that the single-turnover reaction kinetics of the fully reduced T359A mutant of the *E. coli* oxidase with O_2 are near wild type. Hence, the proposed role of this residue in the substrate or "chemical" channel (Iwata et al., 1995) to facilitate the movement of protons specifically destined for forming H_2O may be questioned.

The FTIR absorbance difference spectrum of the CO adduct of T359A in the *Rhodobacter* oxidase is not identical to that of the wild type enzyme, in that the α forms of the metal carbonyls predominate while the β forms are absent. It is possible that two alternative hydrogen bonding interactions of the Thr-359 hydroxyl in the fully reduced, CO-

ligated form of the enzyme could produce the α and β conformers of the binuclear center that are visualized by FTIR at low temperature.

Influence of Lys-362 Modification on the Heme a_3−Cu_B Center. The structure of the *Paracoccus* oxidase suggests that Lys-362 is a central residue in the substrate channel (Iwata et al., 1995). This lysine is located 15 Å away from the heme a_3 Fe. Previous studies showed that replacement of Lys-362 in the *E. coli* oxidase by either leucine, methionine, or asparagine results in elimination of the steady state activity to a value less than 1% of the wild type (Thomas et al., 1993a; Svensson et al 1995). Similarly, the replacement of Lys-362 by methionine or alanine in *R. sphaeroides* cytochrome *c* oxidase inactivates the enzyme (Table 1). The spectroscopic properties of the K362M mutant of the *E. coli* oxidase have not been analyzed in detail but indicate no structural alteration in the vicinity of the metal centers compared to the wild type (Thomas et al., 1993a). In contrast, the more in depth resonance Raman analysis of the purified K362M mutants of the *Rhodobacter* oxidase reveal a distinct perturbation of the Fe−histidine bond of heme a_3, suggestive of more than one conformational state. This may be related to the altered CO binding exhibited by this mutant and the slight shifts in the absorbance spectra (Table 1). The origin of this perturbation is not known, nor is it clear that these changes are related to the drastic effect on catalytic function exhibited by the K362 mutants.

One interesting feature of the K362M and K362A mutants is the difficulty of reducing heme a_3 by dithionite. It has been demonstrated that reduction of the heme−copper center is accompanied by the uptake of protons by the oxidase (Mitchell & Rich, 1994). If the K362M and K362A mutants have blocked the proton uptake pathway, this could prevent reduction of these metal centers. However, Iwata et al. (1995) suggest that the protons taken up during the initial reduction of the heme−copper center are destined to be "pumped" protons rather than substrate protons, while the model assigns K362 to the substrate channel. This assignment, or the model, may thus need to be reconsidered, a conclusion also supported by the single-turnover studies of the K362M mutant of the *E. coli* oxidase, showing nearly wild type behavior upon mixing the reduced enzyme with O_2 (Svensson et al., 1995). Clearly, these questions will require additional experimentation to be resolved. One experimental difficulty is that these mutants exhibit hysteretic effects and conformational heterogeneity, as monitored by the kinetics of heme a_3 reduction.

Further complexity for the role of Lys-362 arises from the recent report that this residue is myristolated in *Neurospora* cytochrome *c* oxidase (Vassilev et al., 1995). However, it is not yet known if the modification is stoichiometric, or if the modified enzyme is active (R. Brambl, personal communication) and no myristyl is seen on this residue in the bovine enzyme crystal structure (Tsukihara et al., 1996). On the basis of the studies of the *Rhodobacter* oxidase, it would be surprising if the myristoylation would yield an active enzyme species.

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