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Possible proton relay pathways in cytochrome *c* oxidase

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ABSTRACT **As the final electron acceptor in the respiratory chain of eukaryotic and many prokaryotic organisms, cytochrome *c* oxidase (EC 1.9.3.1) catalyzes the reduction of oxygen to water and generates a proton gradient. To test for proton pathways through the oxidase, site-directed mutagenesis was applied to subunit I of the *Rhodobacter sphaeroides* enzyme. Mutants were characterized in three highly conserved regions of the peptide, comprising possible proton loading, unloading, and transfer sites: an interior loop between helices II and III (Asp132Asn/Ala), an exterior loop between helices IX and X (His411Ala, Asp412Asn, Thr413Asn, Tyr414Phe), and the predicted transmembrane helix VIII (Thr352Ala, Pro358Ala, Thr359Ala, Lys362Met). Most of the mutants had lower activity than wild type, but only mutants at residue 132 lost proton pumping while retaining electron transfer activity. Although electron transfer was substantially inhibited, no major structural alteration appears to have occurred in D132 mutants, since resonance Raman and visible absorbance spectra were normal. However, lower CO binding (70–85% of wild type) suggests some minor change to the binuclear center. In addition, the activity of the reconstituted Asp132 mutants was inhibited rather than stimulated by ionophores or uncoupler. The inhibition was not observed with the purified enzyme and a direct pH effect was ruled out, suggesting an altered response to the electrical or pH gradient. The results support an important role for the conserved II–III loop in the proton pumping process and are consistent with the possibility of involvement of residues in helix VIII and the IX–X loop.**

Cytochrome *c* oxidase (EC 1.9.3.1), a key enzyme in aerobic energy metabolism, reduces oxygen to water, yielding substantial energy that drives the formation of a proton gradient; however, the mechanism of coupling between oxygen reduction and proton translocation remains obscure.

Recognition of the strong homology between mitochondrial and bacterial enzymes (1, 2) has stimulated the application of molecular genetic tools to the analysis of the oxidase mechanism. The genes for cytochrome *c* oxidase from *Rhodobacter sphaeroides* have been cloned, sequenced, deleted, and reintroduced into the bacterium, and sequence comparisons reveal a high degree of homology with the three mitochondrially encoded subunits of mammalian oxidase (3–7). Extensive site-directed mutagenesis of the largest subunit, COX I, has permitted the assignment of the ligands for the three redox active metal centers, heme *a*, heme a_3, and Cu_B (7–10), suggesting that all three metal centers are located in COX I toward the outer side of the membrane, while substrate and pumped protons come from the inside (11). Thus some kind of proton channel or relay system is required to convey protons to the site of oxygen reduction, the heme a_3–Cu_B center, and beyond. It is reasonable to look for residues involved in proton pumping near this center, although indirect coupling and involvement of other subunits are also possible.

This paper reports the results of analysis of mutant forms of *Rb. sphaeroides* cytochrome *c* oxidase with respect to their proton and electron transfer activity. Three regions in COX I were targeted, each of which contains highly conserved residues capable of participating in a proton relay system (see Fig. 1). The results strongly suggest a critical role in proton pumping for an aspartate in the loop between helices II and III as well as possible involvement of helix VIII and the IX–X loop region.

MATERIALS AND METHODS

Site-directed mutagenesis was done by using various restriction fragments of Cox I as described (10). The D132N/A mutants were made by using a 450-bp fragment coding for residues 68–226, and both were sequenced to check for any secondary mutations. The mutants in helix VIII were made by using a 450-bp fragment coding for residues 225–378.

Determination of CO spectra, the pyridine hemochrome assay, resonance Raman spectroscopy, proton pumping, and electron transfer were measured as described (5). The method for reconstitution of oxidase into soybean phospholipid vesicles was modified to include preincubation of the purified oxidase with 6.7 mg of cholate detergent per nmol of enzyme for 2 hr before addition to the cholate/phospholipid mixture (Robert Kirken and Larry Prochaska, personal communication). Other methods and experimental conditions are described in the figure legends.

RESULTS

Fig. 1 indicates the predicted locations of the residues subject to mutagenesis in this study: H411A, D412N, T413N, and Y414F in the IX–X loop; T352A, P358A, T359A, and K362M in helix VIII; and D132N/A in the II–III loop. All were purified as in ref. 5 and characterized as described below. K362M was inactive and T352A had very low activity and could not be purified sufficiently to permit proton pumping measurements.

Electron Transfer, Spectral, and Structural Properties. As shown in Table 1, all of the purified mutants have lower activity than the 1700 sec^{-1} turnover (molecular activity) of the wt. Excluding D132N/A, activities range from 300 sec^{-1} to 1300 sec^{-1}. The D132N mutant has a lower turnover, only 70 sec^{-1}, raising the possibility that this activity could be from a con-

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Abbreviations: CCCP, carbonylcyanide *m*-chlorophenylhydrazone; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; RCR, respiratory control ratio; Val, valinomycin; wt, wild type.

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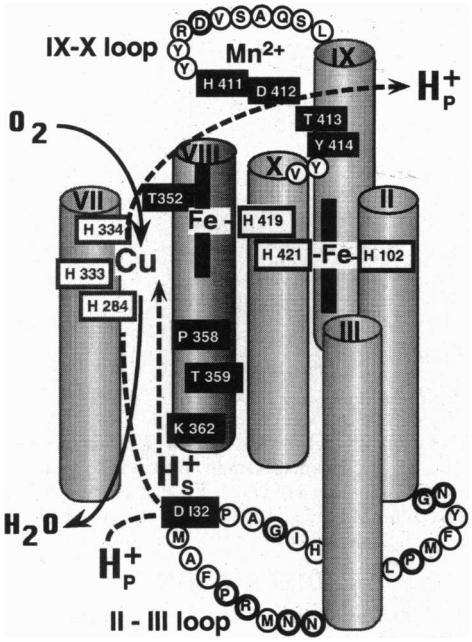

FIG. 1. Model of the active site region of cytochrome *c* oxidase indicating residues analyzed by mutagenesis (filled boxes, white letters). This model shows 6 of the 12 predicted transmembrane helices of subunit I. These contain five of the proposed histidine ligands of heme *a*, heme a_3, and Cu_B. Helix VI, not shown, contains a third Cu_B ligand, H284, which is indicated. The dashed arrows indicate the hypothetical, tested pathways for protons through the protein. Two possible pathways are indicated for substrate (H_s^+) and pumped (H_p^+) protons as suggested in ref. 12. Other highly conserved residues are indicated with bold circles.

taminating oxidase, such as cytochrome cbb_3 (14, 15). However, visible spectra (Fig. 2) show the enzyme to be highly pure of the cbb_3 oxidase and analysis by SDS gel shows the same purity and subunit composition as wt. This mutant is also oxidized to the same extent as wt in the native membrane, as is the case for other active mutants, whereas inactive mutants usually show almost complete reduction. In addition, the D132A mutant shows essentially identical properties to D132N.

Most of the mutants studied here have spectral characteristics indicative of a native binuclear center, and insofar as any alteration in the visible and Raman spectra is observed, it does not correlate with the loss of the ability to pump protons. Specifically, Y414F shows a 5-nm α band red shift and H411A shows a 0.8-nm α band blue shift (16), but neither is altered in proton pumping efficiency. In contrast, the severe loss of proton pumping activity in the D132 mutants is accompanied by no alteration in the visible spectra or in the Raman spectra, as shown for D132N (Fig. 2; for wt spectra see ref. 5). However, after bubbling the reduced enzyme with 1 ml of CO, which gives 100% conversion of wt cytochrome oxidase to a CO-bound form (5), the D132 mutants were only 70–85% saturated. A similar slightly lower CO binding (84%) is observed when H411 is converted to alanine (16), a mutant with high activity and normal proton pumping. Pyridine hemochromagen analysis of D132N and H411A shows no loss of heme A, suggesting only minor disturbance of the heme pocket. Whether this represents an altered affinity for CO or a heterogeneous population is not clear.

Reconstitution and Respiratory Control. The activities of the mutants assayed before and after reconstitution are listed in Table 1. There is apparent loss of activity in most cases because the assay conditions used to measure turnover after reconstitution do not give maximal activities (see legend of Table 1). In contrast, the mutants D132N/A and D412N show no loss of activity, suggesting that they are stable to reconstitution and less sensitive to the assay conditions.

Table 1. Comparison of mutant and wild-type (wt) oxidases: Activity before and after reconstitution, respiratory control ratios (RCRs), and proton pumping efficiency

Enzyme	Activity purified, sec^{-1}	Vesicle activity, sec^{-1}				
		Initial	+ Val	+ Val + CCCP	RCR	H^+/e^-
bh	600	30	100	200	6	0.7–0.8
wt	1700	200	400	900	5	0.6–0.8
D132N	70	80	50	70	0.9	0
D132A	50	50	20	20	0.4	0
P358A	600	100	200	400	4	0.5–0.7
T359A	500	50	100	200	4	0.7–0.8
H411A	800	100	300	400	4	0.5–0.6
D412N	300	100	300	400	4	0.5–0.6
T413N	1300	200	400	700	3	0.5–0.6
Y414F	700	100	200	500	4	0.5–0.7

Proton pumping values were calculated by using data similar to that in Fig. 5. Activity of the purified enzyme was measured polarographically in 50 mM potassium phosphate, pH 6.5/0.056% lauryl maltoside/2 mg of cholate-solubilized soybean phospholipid, at 25°C, with 30 μM cytochrome *c* kept fully reduced with 2.8 mM ascorbate and 1.1 mM *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD). Activity in the vesicles was measured by using the reaction conditions as in Fig. 3, assuming no loss of the oxidase during the reconstitution procedure. In most cases activity measured for the reconstituted vesicles is significantly lower than that of the purified enzyme due to different assay conditions, including lower TMPD levels, higher pH, and some degree of inverted orientation of the enzyme [for beef heart (bh), wt, and D132 mutants, orientation was ≥70% outward-facing, assayed as in ref. 13]. RCRs are calculated by dividing the activity after valinomycin (Val) plus carbonylcyanide *m*-chlorophenylhydrazone (CCCP) addition by the initial activity. H^+/e^- ratios are calculated as described in the legend to Fig. 5. Activities and H^+/e^- are the average from two or more independent reconstitutions.

Addition of cytochrome *c* to the reconstituted oxidase does not cause much increase in oxygen consumption if the vesicles are intact and the oxidase is inserted properly, because a proton gradient is immediately established that inhibits electron transfer activity. Addition of a protonophore (CCCP) and an ionophore (Val) releases the proton and electrical gradient and stimulates the O_2 consumption rate of the native reconstituted enzyme. Significant stimulation shows intactness of the vesicles and good incorporation of the oxidase (Table 1). All of the vesicles except those of the D132 mutants show expected increased activity. In contrast, the D132 mutants show decreased activity in response to either Val or CCCP (Fig. 3) or nigericin (data not shown). Nevertheless, the fact that the reconstituted D132 mutants do respond to a change in the proton or electrical gradient is evidence that the oxidase inserts properly and that an intact membrane is maintained. Indeed, disruption of the vesicles with detergent overcomes the inhibition, presumably by completely eliminating membrane gradients. Furthermore no inhibition is observed with the purified enzyme at similar concentrations of lipid, Val, CCCP, or nigericin.

A direct effect of altered internal or external pH on the activity of the mutant was also considered. However, no difference is observed in the pH response of purified wt and D132N (Fig. 4); both activities increase with decreasing pH, as previously reported for the beef heart enzyme (17).

Proton Pumping. Fig. 5 *Upper* shows recordings of the change in oxidation state of cytochrome *c* after addition to the oxidase-containing vesicles; Fig. 5 *Lower* shows the concomitant changes in pH in the extravesicular solution. Shown are wt, D132N, which does not pump protons, and T413N, an example of a mutant that has normal proton pumping efficiency. Reduced cytochrome *c* is rapidly and completely oxi-

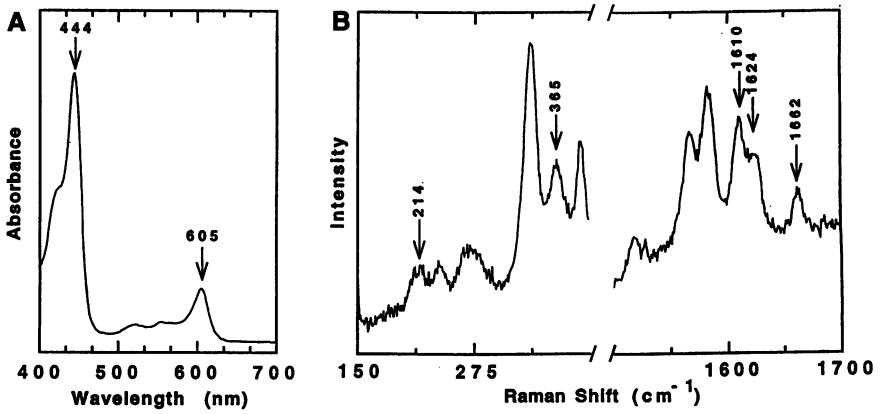

FIG. 2. Visible and resonance Raman spectra indicating a native active site of purified D132N-cytochrome *c* oxidase. (*A*) Visible spectrum: 0.8 μM in 100 mM potassium phosphate/0.2% lauryl maltoside, pH 7.0, reduced by dithionite. (*B*) Resonance Raman spectra: 35 μM oxidase in 100 mM KH_2PO_4, reduced with dithionite, excitation at 441.6 mn (10). The following modes are indicated: 214 cm^{-1}, Fe-N_{his} stretch of heme a_3; 365 cm^{-1}, ring bending of heme a_3; 1611 cm^{-1}, formyl stretch of heme *a*; 1624 cm^{-1}, vinyl stretch of heme *a*; 1662 cm^{-1}, formyl stretch of heme a_3.

dized in all cases, though with a half-time about 3-fold greater for D132N than for wt or T413A. This 3-fold difference in rate is observed at 0.8 μM cytochrome *c* and is in marked contrast to the >20-fold difference in maximal velocity observed at 30 μM cytochrome *c* with the purified enzyme. These results suggest that the mutation has a profound effect on internal rates of electron transfer that might only become limiting at saturating cytochrome *c*.

The proton concentration changes shown in Fig. 5*A Lower* are typical for wt enzyme. Acidification is followed by alkalinization due to proton leakage back into the vesicles in response to internal proton consumption. A similar result is obtained with T413A (Fig. 5*C*), but D132N (Fig. 5*B*) shows no acidification, only alkalinization. D132A showed results similar to D132N. In all three cases shown, addition of the uncoupler CCCP allows proton equilibration across the membrane and only net rapid alkalinization is seen. Stimulation of the rate of alkalinization after addition of CCCP is evidence that the vesicles were maintaining a proton gradient. Even D132N showed stimulation under these assay conditions where Val had already been added, consistent with the oxygen consumption assay (Fig. 3). The other mutants (Table 1) showed proton pumping behavior similar to wt and T413N, giving normal H^+/e^- ratios.

DISCUSSION

General Considerations. Bacteriorhodopsin provides the only protein model for a proton pumping mechanism that is backed by extensive structural and mechanistic data. Several aspartic acids and an arginine have been identified as part of a proton pumping pathway for which the driving force is a light-induced conformational change of retinal (18, 19). It is suggested that the path for protons also involves water molecules within the protein to complete a proton relay system (18) similar to that proposed by Nagle and Morowitz (20).

To test whether the proton pumping model provided by bacteriorhodopsin is applicable to cytochrome oxidase, it is important to identify mutants in which proton pumping is inhibited. A complication in interpreting any effects on proton pumping is the dual role of protons in the oxidase reaction: four protons are consumed from the inside of the membrane for each pair of electrons transferred, two protons in the reduction of oxygen to water and two in the pumping reaction. Different proton paths may exist for substrate and pumped protons, since substrate protons must directly access the oxygen intermediates at the binuclear center while pumped protons may not.

If there is only one path for protons to the binuclear center, then blocking this path would be expected to inhibit electron

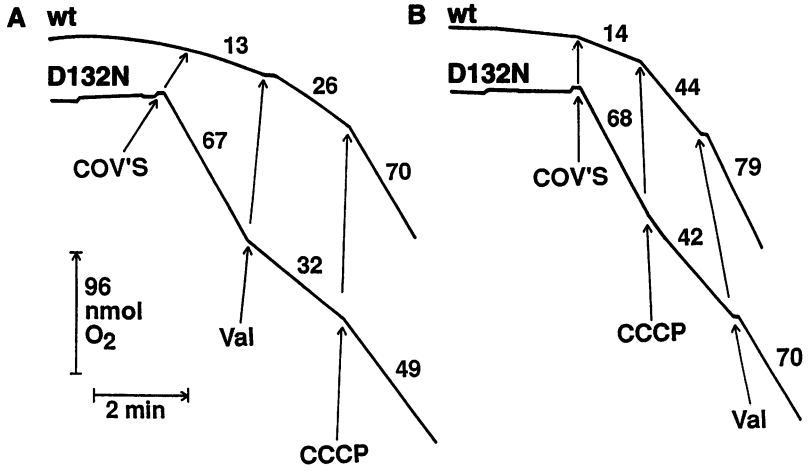

FIG. 3. Effects of Val and CCCP on the oxygen consumption of reconstituted wt and mutant D132N cytochrome oxidase. Rates of oxygen consumption were measured polarographically at 25°C in 10 mM Hepes/41 mM KCl/38 mM sucrose, pH 7.4, with 5.6 mM ascorbate, 0.28 mM TMPD, and 30 μM cytochrome *c*. After adding 5 μl of wt vesicles or 100 μl of D132N vesicles to 1.8 ml of medium in a stirred cell, controlled activity was measured. The effect is shown for addition of Val (4.4 μM) followed by CCCP (5.6 μM) (*A*) or CCCP followed by Val (*B*).

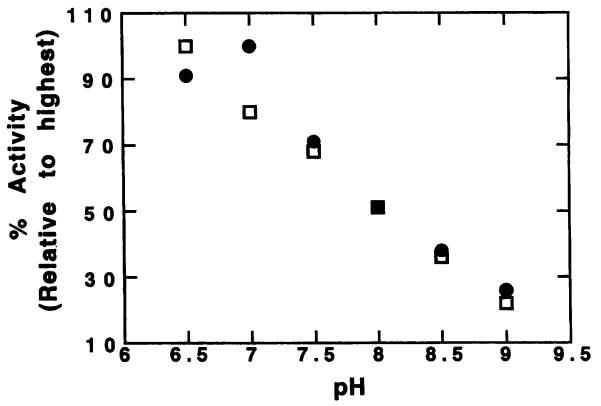

FIG. 4. Comparison of the effect of pH on the activity of purified D132N and wt cytochrome *c* oxidase. Activities were assayed polarographically using 0.003 μM wt or 0.06 μM D132N in 50 mM potassium phosphate for pH 6.5–7.5 and in 50 mM Tris for pH 7.5–9, including 0.056% lauryl maltoside, no phospholipids, but using the concentrations of cytochrome *c*, ascorbate, and TMPD as indicated in Table 1. Relative activities were calculated as a percent of the highest turnover measured: for wt (1000 sec^{-1}) (●); for D132N (70 sec^{-1}) (□).

transfer and proton transport equally, unless protons were used preferentially for reduction rather than for pumping. Even if two separate paths exist, inhibition of the proton pumping pathway could slow electron transfer insofar as there is tight coupling between the two processes.

Although theoretical and mechanistic arguments can be made for obligatory coupling (21), there is evidence for decoupling or slipping in cytochrome *c* oxidase (22). Reaction of oxidase with *N,N'*-dicyclohexylcarbodiimide, or removal of subunit III, reduces the number of protons pumped per electron transferred (23–25). Other evidence suggests that proton pumping by the fully reduced enzyme is only 50% efficient (26, 27). The proton-to-electron ratio may also vary depending on the rate of electron input to cytochrome oxidase in whole mitochondria and reconstituted vesicles (28, 29).

Rationale for Mutant Selection. Site-directed mutagenesis in cytochrome aa_3 was focused on residues that could participate in a proton transfer network and were highly conserved. Likely candidates are amino acids with side chains having available protons. In the case of proline, it is relatively common in transmembrane helices and has been proposed to play a role in ion transport by increasing the flexibility of the helix (30).

According to the bacteriorhodopsin model, acidic residues should be prime candidates for a proton relay system. Only one conserved acidic residue, E286, is predicted to exist in the membrane domain of cytochrome oxidases. While the E286N mutant of cytochrome aa_3 is inactive, the same mutation in the homologous cytochrome *bo* is active and pumps protons (31); therefore a critical role in proton pumping is unlikely.

IX–X Loop. Among the mutants analyzed in this extramembrane region, T413N is most like wt with only slightly lower electron transfer activity and normal pumping, arguing against an important mechanistic role. Modification of D412 to N, on the other hand, causes strong inhibition of electron transfer but minimal alteration of spectral properties (16) and no change in protons per electron pumped. Y414F and H411A have shifted α bands (16) and inhibited electron transfer but again unaltered pumping efficiency. Thus there is no positive evidence to implicate residues 411, 412, and 414 in proton pumping, but a role in this process cannot be ruled out since their overall activity is diminished. It should be noted that previous studies indicate that residues 411 and 412 participate in a Mn/Mg binding site; loss of the metal after mutation of these residues could account for the loss of function (16).

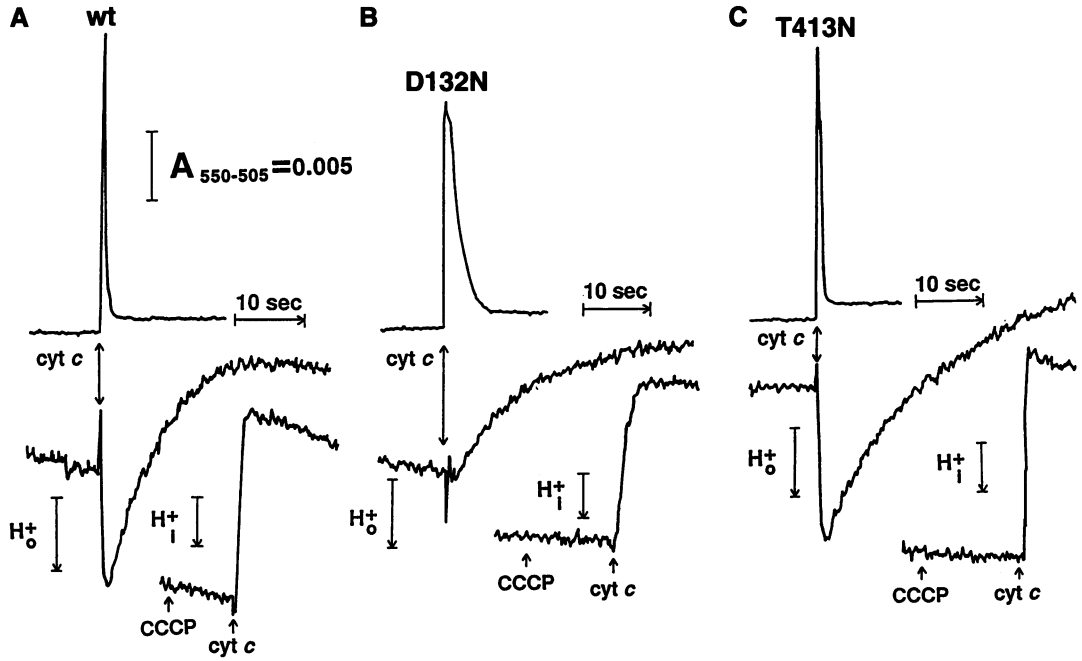

FIG. 5. Spectral analysis of proton pumping and cytochrome *c* oxidation by reconstituted wt and mutant cytochrome *c* oxidases. Both activities were assayed at 22°C in 2.5 ml of 50 μM $NaHCO_3$/45 mM KCl/44 mM sucrose, with or without 50 μM phenol red, pH 7.4, with 0.08 nmol of cytochrome *c* oxidase reconstituted into soybean phospholipid vesicles. Following addition of the vesicles to a stirred cell in an Aminco DW2a spectrophotometer, Val (final concentration, 3.2 μM) and dilute CCCP (final concentration, 0.4 nM) were used to equilibrate the vesicles. (*Upper*) Oxidation of reduced cytochrome *c* at 550 minus 505 nm in the absence of phenol red. (*Lower*) Extravesicular pH changes, as measured at 556.8 minus 504.7 nm by phenol red. Rapid acidification occurred upon addition of 2.1 nmol of cytochrome *c* (final concentration, 0.8 μM) to wt (*A*) or Thr413Asn (*C*) but not in the case of Asp132Asn (*B*). In the presence of concentrated CCCP (5 μM), addition of cytochrome *c* produced the expected alkalization. Addition of 0.5 nmol of HCl standard before (H^+_o) and after (H^+_i) CCCP addition caused the changes in absorbance indicated and was used to calculate the protons extruded and the protons consumed, respectively. The H^+/e^- ratio can be calculated from the protons extruded (H^+), knowing the amount of cytochrome *c* added (e^-).

Indeed, a role for this non-redox active metal in proton pumping can be envisaged.

Helix VIII. Because K362M was inactive and T352A was difficult to purify and had low activity, neither could be tested for proton pumping. However, these activity characteristics and altered spectral properties support the idea of helix VIII being proximal to the binuclear center but do not establish its role. Mutants T359A and P358A show reduced activity, about 30% of wt, but no significant change in spectral properties or proton pumping efficiency, as measured by H^+/e^-. Since the native spectral characteristics of these mutants indicate no general disruption of protein structure, the observed loss of activity could be due to a limitation of proton access to the active site. Indeed, if two pathways for substrate and pumped protons exist, as indicated in Fig. 1, inhibition of a substrate proton pathway might be expected to give these results. Similarly, inhibition of a tightly coupled pathway for pumped protons would equally limit electron transfer. Thus these results are consistent with a central role for helix VIII in proton transfer.

II–III Loop. Conversion of D132 in the II–III loop to N or A caused a dramatic inhibition of electron transfer and these were the only mutations that eliminated proton pumping while retaining the ability to reduce oxygen at a significant rate. Visible absorbance spectra show that neither mutation has a significant effect on heme *a* or heme a_3. The absence of changes in the vibrational modes of either heme *a* or heme a_3 in resonance Raman analysis of D132N is further strong evidence of native heme environments. Only the CO binding appears to be slightly altered. Heterogeneity of the sample or a minor structural change in the binuclear center (possibly in the vicinity of spectrally silent Cu_B) may have occurred such that the CO binding equilibrium is altered. Although CO binding would suggest a quantitatively similar structural perturbation in H411A (16), it remains competent in proton pumping.

Lack of evidence of a significant structural change at the binuclear center favors the idea that electron transfer activity in the D132 mutants is limited either by availability of substrate protons to the active site (single pathway model) or by the obligatory coupling of proton pumping to electron transfer (two-pathway model). Either scenario, along with the predicted location of the II–III loop, would implicate D132 as a critical residue in a proton relay system from the intracellular side of the membrane. Similar conclusions have been reached from studies of mutants in the homologous residue in cytochrome *bo* of *Escherichia coli* (31). However, in that study, proton pumping was carried out in spheroplasts, where the detailed response to pH and electrical gradients could not be easily examined.

When incorporated into phospholipid vesicles, mutants of D132 show an unusual response to addition of ionophores or protonophores: their activity is inhibited rather than stimulated by release of either the proton or the electrical gradient. A possible explanation for this response is that the mutation has changed the pH sensitivity of the mutant. Although the pH inside and outside of the vesicles should be the same after reconstitution, when Val is added before beginning the proton pumping assay, external alkalinization is observed. This implies a potassium gradient (higher internal $[K^+]$) but is also consistent with the observation of Madden and Redelmeier (32) that there is a higher pH inside than outside of the vesicles (>8.6, versus 7.4). If Asp132Asn were less active than wt at low pH, then addition of Val (or CCCP or nigericin) might inhibit the enzyme activity. However, the pH dependence of the activity of the purified D132N is essentially identical to wt, arguing against a simple pH effect. Thus it seems more likely that release of a gradient of pH or charge is involved in the inhibition. If D132 were normally involved in a salt bridge holding the II–III loop in a stable conformation, loss of its negative charge would make this region more conformationally flexible and could alter its sensitivity to changes in the electrical/pH gradient. Alternatively, the loss of D132 as an entry point for protons could bring into play a new, less efficient pathway that might respond differently to an electrochemical potential.

Conclusions. None of the residues examined by mutagenesis in the IX–X extracellular loop or helix VIII preferentially inhibits proton pumping but, since a number retain native spectral properties and have lower or no activity, their involvement in proton transfer remains a real possibility. More definitively, loss of proton pumping with retention of low but significant electron transfer activity in the case of mutants at D132 is strongly suggestive of a critical role in proton pumping. It remains to be determined whether D132 is a direct participant in a proton relay system or an important determinant of a structural element that is essential for this process.

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