Proton Transfer in Cytochrome bo_3 Ubiquinol Oxidase of *Escherichia coli*: Second-Site Mutations in Subunit I That Restore Proton Pumping in the Mutant Asp135→Asn[†]

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ABSTRACT: The ubiquinol oxidase, cytochrome bo_3, of *Escherichia coli* is a member of the respiratory heme−copper oxidase family and conserves energy from the reduction of dioxygen to water by translocation of protons across the bacterial membrane. Mutation of an aspartic acid residue (Asp135) to asparagine in subunit I of this enzyme was previously found to impair proton translocation [Thomas et al. (1993) *Biochemistry 32*, 10923−10928]. This residue is located in an interhelical "loop" between transmembranous helices II and III, which contains six well-conserved residues (Asn124, Pro128, Gly132, Asp135, Pro139, and Asn142). Site-directed mutagenesis was performed to study the function of this entire domain. Nonconservative mutations of Asn124 and Asn142 also resulted in a loss of proton translocation, whereas their conservative substitution to glutamine had no effect. Mutations in eight other positions within this domain did not affect proton translocation. Introduction of an acidic group at positions 139 or 142, but not at eight other tested positions, restored proton pumping in the Asp135→Asn mutated protein. These results suggest that the C-terminal part of the domain may be α-helical and that the entire "loop" plays an important structural and functional role as part of an input channel of the proton translocation machinery.

Cytochrome bo_3 is the primary membrane-bound terminal oxidase of *Escherichia coli* grown under high-aeration conditions. It catalyzes the oxidation of ubiquinol and the reduction of oxygen to water in a process coupled to translocation of protons across the cell membrane (Puustinen et al., 1989, 1991). This enzyme belongs to the family of heme−copper oxidases which includes aa_3-type cytochrome *c* oxidases from bacteria and mitochondria (Garcia-Horsman et al., 1994; Saraste, 1990; Calhoun et al., 1994; Hosler et al., 1993). The members of this family are structurally and functionally related. They are characterized by a binuclear heme iron−copper center where the oxygen chemistry takes place and by a low-spin heme that transfers electrons from the substrate (quinol or cytochrome *c*) to the binuclear center [for reviews, see Babcock and Wikström (1992), Garcia-Horsman et al. (1994), Calhoun et al. (1994), Trumpower and Gennis (1994)]. In cytochrome bo_3 the low-spin heme is a protoheme but the oxygen-reactive heme is of type O (Puustinen & Wikström, 1991; Puustinen et al., 1992; Wu et al., 1992). Subunits I of this enzyme family possess a high degree of sequence homology. Mainly on the basis of extensive mutagenesis and analysis of the bacterial oxidases cytochrome aa_3 from *Rhodobacter sphaeroides* and cytochrome bo_3 from *E. coli*, it has been shown that both the low-spin heme and the binuclear center are ligated by specific histidine residues in transmembrane helices of this subunit (Chepuri et al., 1990a; Hill et al., 1992; Calhoun et al., 1993a−c; Lemieux et al., 1992; Minagawa et al., 1992; Shapleigh et al., 1992a,b; Thomas et al., 1993a, 1994).

Although this work has provided a picture of the structural environment around the metal centers, there is still little information available on the structures involved in proton translocation. It has been shown that subunits I and II comprise the minimal unit of the enzyme sufficient for both electron transfer and proton pumping (Solioz et al., 1982; Finel & Wikström, 1986; Hendler et al., 1991). Due to the high degree of conservation, subunit I is therefore a logical target for analysis. Acidic amino acid residues have been shown to be essential for proton movements both in bacteriorhodopsin (Butt et al., 1989; Henderson et al., 1990; Krebs & Khorana, 1993) and in the bacterial photosynthetic reaction center (Rongey et al., 1993; Shinkarev et al., 1993; Takahashi & Wraight, 1991). Consequently, the five most conserved acidic amino acids in subunit I of cytochrome bo_3 were mutated to the corresponding amide, but only one mutant, Asp135→Asn, was found to exhibit dramatically decreased proton translocation with little change in redox activity (Thomas et al., 1993b; Wikström et al., 1994). This aspartate is located in a "loop" predicted to reside on the cytoplasmic (i.e., proton input) side of the membrane between transmembrane helices II and III (Chepuri & Gennis, 1990; Chepuri et al., 1990b). From sequence alignment of subunits I from over 80 different species, it has been shown that this loop, which comprises approximately 20 residues, contains six very well conserved residues: two asparagines, two

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prolines, a glycine, and an aspartate [see Garcia-Horsman et al. (1994)].

Here we report a more systematic mutagenesis study of the II−III loop domain of cytochrome bo_3 to further test its possible involvement in proton translocation. Mutations of the well-conserved residues Asn124 and Asn142 cause decoupling of proton translocation from electron transfer, as previously reported for Asp135→Asn (Thomas et al., 1993b). However, mutations in the three other well-conserved residues, Pro128, Gly132, and Pro139, show normal proton-pumping activity. Second-site mutations along with Asp135→Asn were introduced to place the acidic group in other positions of the domain. Two loci were found (at Pro139 and Asn142) where positioning of an acidic group reverses the loss of proton pumping due to the primary mutation at Asp135. These results suggest that the C-terminal segment of the II−III loop domain may be α-helical and that the entire domain may form part of a proton-uptake pathway of the proton-translocation machinery.

MATERIALS AND METHODS

Site-directed mutagenesis (T7-GEN mutagenesis kit; U.S. Biochemical, Cleveland, OH) and cloning were performed as reported previously (Lemieux et al., 1992). The used M13SEΔH template is an M13 derivative containing the 2.18-kbp *SalI*/*Eco*RI fragment from pMC31 (Lemieux et al., 1992). *SalI*/*Eco*RI fragments containing mutations were subcloned into the expression plasmid pMC39 (Lemieux et al., 1992) or into pJT40 (i.e., pMC39 with the addition of an *Xho*I site at 3.38 kbp and a *Hin*dIII site at 3.65 kbp). The mutant protein was expressed in the oxidase-deficient host strain RG 129 (Δ*cyo*,Δ*cyd*; Au & Gennis, 1987). For double mutations, M13SEΔH with the primary mutation was used as a template for the second mutation. The Asp135→Lys/Lys362→Asp double mutant was made by cloning the *Hin*dIII/*Nsi*I fragment from pJT40/Lys362→Asp to pJT40/Asp135→Lys. Both the M13 single-stranded templates and the double-stranded expression plasmids of the mutants were sequenced (Sequenase 2.0 kit; U.S. Biochemical, Cleveland, OH) to confirm the mutation. Complementation analysis of the mutant plasmid was done as described by Lemieux et al. (1992).

Ubiquinol oxidase activity measurements of the mutant and wild type enzymes in bacterial membranes, as well as determinations of cytochrome bo_3 concentration, were done as noted previously (Thomas et al., 1993b). Proton translocation was assayed as described by Puustinen et al. (1989, 1991) and Thomas et al. (1993b). In some cases cells were treated with 200 mM Tris-HCl, pH 8, and 0.5 mM EDTA, for 10 min instead of preparing spheroplasts with lysozyme treatment.

For electrical charge translocation measurements, cells were grown in succinate minimal medium and harvested in the middle of the exponential phase. Washed cells were treated with 200 mM Tris-HCl, pH 8, and 0.5 mM EDTA for 10 min, after which the cells were loaded with sodium, as described by Nakamura et al. (1982). The cells were washed with 100 mM $NaSO_4$, 1 mM $MgSO_4$, 15 mM tricine, and 15 mM MOPS, pH 7.8, and then resuspended in the same medium and kept on ice. The oxygen pulse method used to measure proton translocation was employed (Puustinen et al., 1989, 1991), but instead of H^+ ejection a K^+-selective electrode (Radiometer) was used to measure potassium uptake. The reaction medium contained 100 mM $NaSO_4$, 1 mM $MgSO_4$, 15 mM MOPS, pH 7.2, and 5 mM sodium succinate. Cells were added to anaerobic medium to a concentration of 1−2 mg of protein/mL and incubated anaerobically for 20−40 min to deplete internal ATP. Then 0.5 mM KCl and 10−15 μM valinomycin were added anaerobically. After a further equilibration period of 3−5 min, the reaction was started by a pulse of 2.58 nmol of O_2 as air-saturated water (25 °C, 1 atm). Correction for potassium dilution was made by additions of argon-saturated water.

RESULTS

Figure 1 shows a two-dimensional model of subunit I of cytochrome bo_3 of *E. coli*. The first set of mutants included changes in the six most conserved residues within the II−III "loop" domain (Table 1). The redox activity was high enough to support aerobic growth of the oxidase-deficient strain RG129 (*cyo*, *cyd*), except for Pro128→Asp that could not be further analyzed. Relative to the wild type, the redox activity ranges from 20% to 120% depending on the position of the mutation and the residue that it was changed to, but in all cases this activity was high enough to allow measurements of proton translocation (see Discussion). These measurements were carried out in spheroplasts, and the results are presented as H^+/e^- ratios in Table 1. For the wild type this ratio is near 2.0 (at pH 6−7), as has been reported previously (Puustinen et al., 1989, 1991; Verkhovskaya et al., 1992): 1 proton/electron is released extracellularly due to the oxidation of ubiquinol, and the second is vectorially pumped across the membrane from the cytoplasm. This yields an overall translocation of two electrical charge equivalents across the membrane per electron transferred from ubiquinol to dioxygen, as has been verified independently (Wikström et al., 1994). In some of the mutants described here, the q/e^- ratio of charge translocation was determined independently to verify the proton ejection measurements (see Materials and Methods).

As shown in Table 1, the Asp135→Asn, Asp135→Lys, Asn124→His, Asn124→Asp, and Asn142→Val mutants are deficient in proton pumping. The observed decrease in the H^+/e^- ratio is not due to an increased proton permeability of the membrane in these mutants, since all of them show a slow protonic decay after proton ejection following an oxygen pulse [cf. Thomas et al. (1993b)]. On the other hand, the mutants Pro128→Ala, Gly132→Ala, Gly132→Arg, Pro139→Ala, and Pro139→Glu show wild type proton-translocation activity; Pro128→Asp does not complement growth and could, hence, not be tested. It is interesting to note that for all residues critical for vectorial proton translocation, i.e., Asn124, Asp135, and Asn142, a conservative change to a chemically analogous residue such as glutamine and glutamate, respectively, supports proton-translocating activity (Table 1). Moreover, Asn142→Asp retains some proton-translocation activity whereas Asn142→Val does not, indicating the necessity of a polar residue at this site.

A second set of mutations (Table 2) is classified here as changes in less conserved residues. It is necessary to mention that the *E. coli* cytochrome bo_3 can be considered

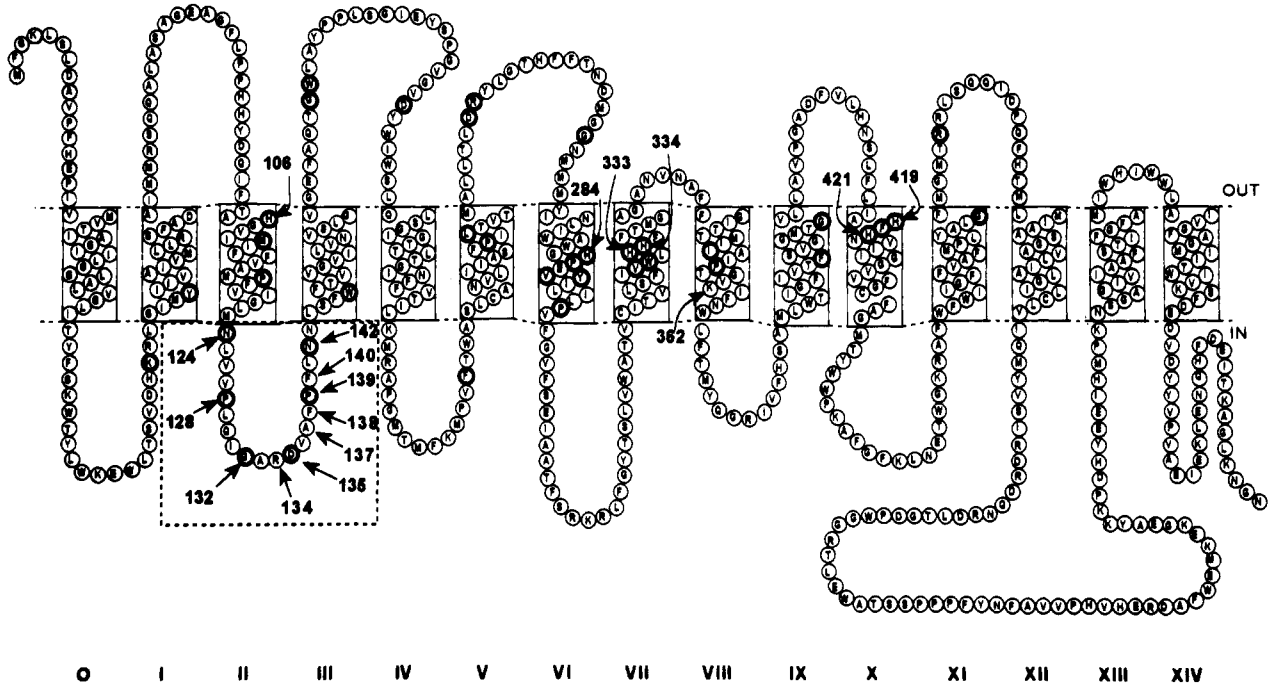

FIGURE 1: Amino acid sequence of subunit I of *E. coli* cytochrome bo_3, showing 15 predicted transmembranous helices (0−XIV). Conserved residues are in bold open circles. The "loop" domain between transmembranous helices II and III is highlighted. For performed mutations, see Tables 1−3 and the text. The six invariant histidines that are proposed to be metal ligands are indicated by residue numbers.

Table 1: Electron Transfer and Proton Pumping for Cytochrome bo_3 Mutants in Conserved Residues

strain	complementation[b]	activity[c]	H^+/e^- (and q/e^-)[d,f]
wild type[a]	+	100	1.8−2.0 (1.8−2.0 q/e^-)
N124H	+	16	1.0−1.2
N124D	+	56	1.1−1.4
N124Q	+	110	1.8−2.1
P128A	+	115	1.8−2.5 (1.7 q/e^-)
P128D	−	−	−
G132A	+	113	1.7−2.0
G132R	+	nd	2.1−2.2
D135N	+[e]	45[e]	1.0−1.2[e] (1.0−1.1 q/e^-)
D135K	+	23	1.2−1.4
D135E[e]	+	45	1.7−1.8
P139A	+	67	1.6−2.0
P139E	+	46	1.6−2.0
N142V	+	22	0.9−1.2
N142D	+	48	1.4−1.6
N142Q	+	109	1.7−2.0

[a] Wild type used for proton pumping, oxidase activity, and complementation data were obtained from plasmid-encoded expression in the RG129 strain. [b] Complementation in a mutant is defined as the ability to support aerobic growth as the sole oxidase on lactate minimal media in the RG129 host strain. [c] Activity is expressed as percent of the wild type oxidase activity (600 e^-/s). [d] A range of H^+/e^- ratios is shown; data were collected (at pH 6−7) from several oxygen pulses made on at least two independent growth batches prepared by two different protocols (see Materials and Methods). [e] Taken from Thomas et al., 1993b. [f] Ratio of translocated electrical charge equivalents/electron (see Materials and Methods).

Table 2: Electron Transfer and Proton Pumping for Cytochrome bo_3 Mutants in Less Conserved Residues[a]

strain	complementation[b]	activity[c]	H^+/e^- (and q/e^-)[d,f]
A133D	+	nd	2.0−2.3
R134P	+	112	1.7−1.9
A137D	+	nd	1.6−2.0
F138R	+	55	1.3−1.6 (0.9 q/e^-)
F138G	+	63	1.6−2.2
F140R	+	nd	1.6−2.0
R134P/F140R	+	nd	2.0−2.2

[a] For footnotes b−d and f, see the corresponding footnotes to Table 1.

an exception regarding the residues Arg134 and Phe140. In approximately 90% of over aligned 80 subunit I sequences, the residue at position 134 is a proline and the residue aligned with position 140 is an arginine. There are only three other sequences reported where residue 140 is a phenylalanine (or a tyrosine); residue 134 is then also an arginine in these cases, as in cytochrome bo_3. These are the sequences of cytochromes aa_3-600 and caa_3 from *Bacillus subtilis* (Santana et al., 1992; Saraste et al., 1991) and cytochrome caa_3 from the thermophilic bacterium PS3 (Sone et al., 1988). To mimic the structure of the majority of the oxidases in this region, we constructed the individual mutants Arg134→Pro and Phe140→Arg, as well as the double mutant Arg134→Pro/Phe140→Arg. All three are active in proton translocation (Table 2), showing that the "majority structure" in this domain can be accommodated by the *E. coli* enzyme.

Among the three other positions tested in this category (Table 2), proton translocation is impaired in the mutant Phe138→Arg, as confirmed by the measurement of the q/e^- ratio (Table 2). However, this appears to be due to a secondary structural perturbation because the Phe138→Gly mutant shows normal proton translocation.

It has been proposed that the residue Lys362, located in helix VIII, may be involved in the protonation of the binuclear site (Hosler et al., 1993; Thomas et al., 1993a). A possible salt bridge between this residue and an acidic group, perhaps Asp135, has also been discussed (Fetter et al., 1995). Substitutions of Lys362 specifically perturb the binuclear site and are associated with loss of catalytic activity (Hosler et al., 1993). To test the idea of proximity of these residues, we constructed the Lys362→Asp and Asp135→Lys mutants.

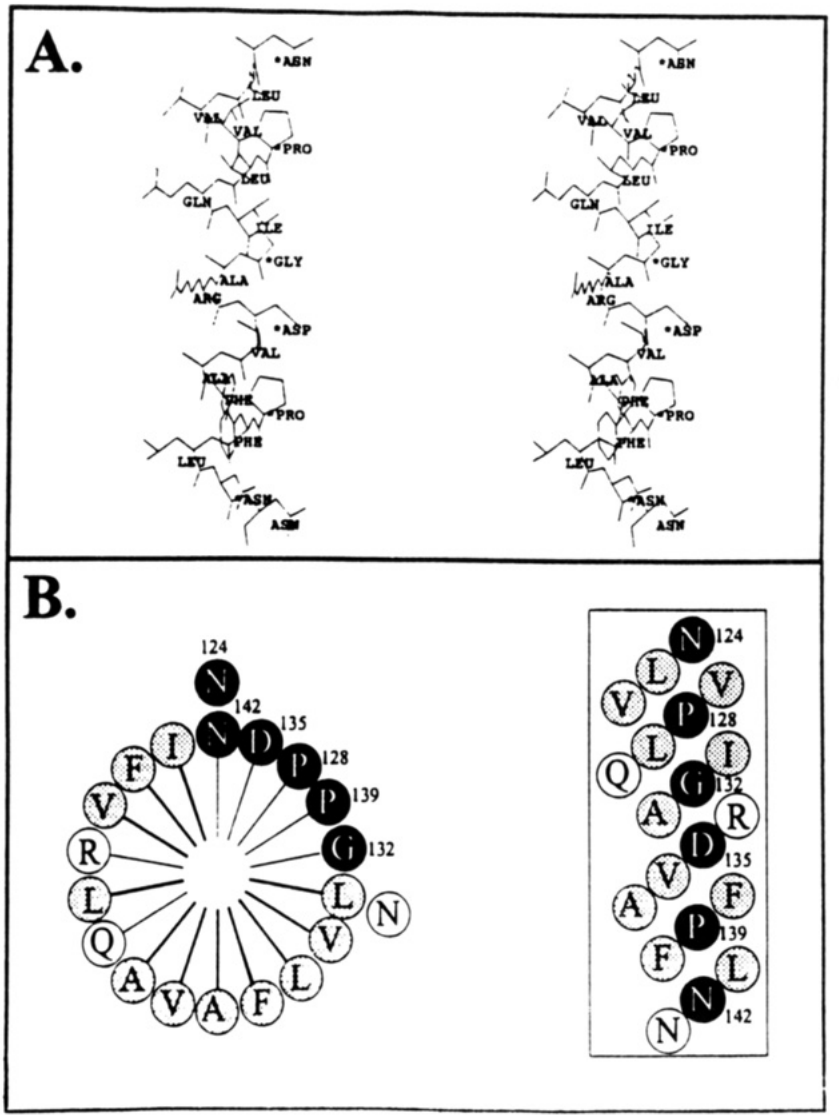

FIGURE 2: Helical model of the domain between transmembranous helices II and III. A: Stereo representation of the domain from Asn124 and Asn142 generated with the computer program HyperChem 2 for Windows (Autodesk Inc., 1993). The polypeptide is configured as an α-helix, which places the most highly conserved residues (indicated by asterisks) along one face. B: Helical wheel and split cylinder models of the same domain as in panel A. Filled solid circles represent the most highly conserved residues. Shaded residues (with bold stems) are hydrophobic. Also indicated by open circles are Arg134, which is a proline in most other subunit I sequences, and Gln130 and Asn 143, which are polar residues that are less conserved than the set of six residues indicated by filled circles.

As expected, substitution of Lys362 by aspartate does not yield functional oxidase, and Asp135→Lys abolishes proton translocation, as does Asp135→Asn (Table 1). The double mutant Lys362→Asp/Asp135→Lys is devoid of redox activity (not shown). These results do not support the idea of a salt bridge between the two residues.

Figure 2 shows that all six well-conserved residues of the "II−III loop" domain would cluster along a one-quarter surface of an α-helix. Although this might be a remarkable coincidence, such a distribution is suggestive of a helical structure. To test this, and the possibility that the acid function apparently required for proton translocation may not be restricted to position 135, we moved the acidic group to several other positions in the domain, using the double mutants listed in Table 3. An aspartate residue in place of Asn124, Pro128, Ile131, Gly132, Val136, Ala137, Phe138, or Leu141 does not restore proton translocation in the primary Asp135→Asn mutant. However, when an acidic group is placed at position 139 (instead of proline) or 142 (instead of asparagine), the proton-pumping ability is recovered. This is also the case for the double mutant where Asp135 was changed to alanine and the proline at position 139 was changed to glutamate (Table 3).

Table 3: Electron Transfer and Proton Pumping for the Cytochrome bo_3 Double Mutants[a]

strain	complementation[b]	activity[c]	H^+/e^- (and q/e^-)[d,f]
N124D/D135N	+	21	1.0−1.2 (0.8−0.9 q/e^-)
P128D/D135N	−	−	−
I131D/D135N	+	nd	1.1−1.2
G132D/D135N	+	66	0.9−1.4 (0.9−1.1 q/e^-)
V136D/D135N	+	nd	0.9−1.0
A137D/D135N	+	nd	1.0−1.3
F138D/D135N	+	nd	0.9−1.2
P139E/D135N	+	95	1.6−2.2 (1.5−1.6 q/e^-)
P139E/D135A	+	nd	1.6−2.1
L141D/D135N	+	nd	1.0−1.2
N142D/D135N	+	33	1.6−2.2

[a] For footnotes b−d and f, see the corresponding footnotes to Table 1.

DISCUSSION

The conclusion that a mutation has specifically affected proton translocation requires proton pumping to be affected more significantly than the linked redox reaction. This may be determined as a lowered H^+/e^- or q/e^- ratio. However, the measured H^+/e^- ratio may also be lowered artefactually if the redox reaction is slowed down to the extent that proton ejection can no longer compete kinetically with the natural proton permeability of the membrane. In the mutants studied here, in which proton translocation is impaired, respiration is generally also inhibited. But in all cases, electron transfer still occurs with a turnover time of <20 ms, which is almost 3 orders of magnitude faster than the characteristic proton conductance of the spheroplast membranes ($\tau_{1/2} > 10$ s). We hence conclude that the reported mutations have primarily affected a proton transfer pathway. On the other hand, inhibition of proton transfer is, *a priori*, expected to cause inhibition of respiration as well, due to the linkage of the two functions. Therefore, the observed lowering of the H^+/e^- ratio means that this linkage has been weakened or lost by perturbation of the structure. It is interesting that in many cases where a mutation inhibits both proton translocation and electron transfer (see Asn124 and Asn142 mutants, Table 1) *both* activities are restored by a mutation to an amino acid side chain chemically analogous to that in wild type enzyme.

It is difficult to obtain structural information about specific protein domains, or residues, involved in proton translocation by the heme−copper oxidases. Some insight was obtained recently from the Asp135→Asn mutant, where proton translocation is decoupled from electron transfer (Thomas et al., 1993b; Wikström et al., 1994). However, this finding is beset with the well-known difficulty associated with site-directed mutations in enzymes of unknown structure: a secondary structural effect could explain such an isolated observation, without direct functioning of the aspartate residue in proton transfer. To try to overcome this difficulty, we studied in some detail the entire "loop" domain between transmembrane helices II and III where Asp135 is located (Figure 1). This domain of about 20 amino acids contains six residues (Asn124, Pro128, Gly132, Asp135, Pro139, and Asn142) that are conserved in virtually all respiratory oxidases (except those of the cytochrome *cbb*$_3$-type; cf. below). Their distribution is suggestive of α-helical secondary structure (Figure 2), even though these residues are not typical for helices, and computer-aided structure predictions (Crofts, 1992) yield a reasonably high score for a buried helix. Such a helix would also be amphiphatic: with few exceptions, the less conserved residues are nonpolar, making up the remaining three-quarter helical surface. It is also of interest to note that if this domain is truly a cytoplasmic "loop" (Figure 1), it would lie far from the metal centers, which are near the opposite side of the membrane (Hosler et al., 1993).

In addition to the Asp135→Asn reported earlier (Thomas et al., 1993b), mutations of Asn124 and Asn142 also impaired proton pumping, except when substituted conservatively with glutamine. Mutations in less conserved residues lead to loss of proton translocation in one case, but this appeared to be due to a less specific structural perturbation (see Results and Table 2). The finding that three mutations in the "II−III loop" domain specifically affect proton translocation supports the notion (Thomas et al., 1993b) that this domain may indeed be of key importance for the proton-translocating function of the enzyme. However, in the family of heme−copper oxidases, the polar II−III loop residues are not conserved in the cytochromes of *cbb*$_3$-type (van der Oost et al., 1994), yet at least the *cbb*$_3$-type enzymes in *Paracoccus denitrificans* (Raitio & Wikström, 1994) and *Rh. sphaeroides* (unpublished results) have been shown to pump protons. On the other hand, there are acidic residues in the cytoplasmic loops between transmembrane helices IV and V and between VIII and IX in subunit I of these enzymes. Hence, some variation in the structures responsible for proton channeling into the enzyme may occur, as has been shown for the bacterial photosynthetic reaction centers (Takahashi & Wraight, 1991).

Of the six well-conserved residues, the three with polar character (Asn124, Asp135, and Asn142) are apparently crucial. Yet, in each case the side chain can be lengthened by one methylene group without loss of proton-translocating activity. But most significantly, the carboxylic group, originally at position 135, can be moved to two other unique positions (139 and 142) without loss of function. Assuming a helical secondary structure, these positions would be on the same face as the 135 locus but displaced from it toward the C-terminus by one and two helical turns, respectively (Figure 2). In contrast, placing the acidic group at position 136, 137, 138, or 141, which would be on the opposite helical face, does not restore activity, nor did any of the tested relocations toward the N-terminus, even to position 132 which (in a helix) would lie on the same face as position 135 (Table 3, Figure 2). These findings strongly suggest that the C-terminal region of the "II−III loop", between residues 135 and 142, may indeed be α-helical. Interestingly, a recent structure prediction suggests that the transmembrane helix III might, in fact, start already at residue 136 in cytochrome *bo*$_3$ from *E. coli* (Jones et al., 1994). This would place Asp135 on the membrane interface and include residues 136−142 as part of helix III, which would be consistent with our results. On the other hand, the fact that a phenylalanine at position 140 is almost unique for the *E. coli* enzyme (see above), and is usually an arginine, may well change this prediction.

The II−III "loop" domain clearly requires both acidic and potentially hydrogen-bonding amide residues in order for proton translocation to occur normally. The C-terminal residues (in positions 135−142) may provide a hydrogen-bonded network for proton transfer along one polar side of a helix, which is likely to be in contact with some as yet unidentified proteinaceous structure of the enzyme. This network might well include bound water molecules as found in the bacterial photosynthetic reaction center (Ermler et al., 1994). The way in which the "loop II−III" domain interacts with the rest of the subunit is obviously of greatest significance to understanding the function. For example, it might be folded into the membranous part of the enzyme, thus interacting with transmembrane helices. Work is in progress to create second-site revertants of mutations in this domain to gain answers to these intriguing questions.

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