Substitution of Asparagine for Aspartate-135 in Subunit I of the Cytochrome *bo* Ubiquinol Oxidase of *Escherichia coli* Eliminates Proton-Pumping Activity†

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Received May 25, 1993; Revised Manuscript Received July 26, 1993®

ABSTRACT: The terminal quinol oxidase, cytochrome *bo*, of *Escherichia coli* is a member of the large terminal oxidase family, which includes cytochrome aa_3-type terminal oxidases from bacteria, plants, and animals. These enzymes conserve energy by linking electron transfer to vectorial proton translocation across mitochondrial or bacterial cell membranes. Site-directed mutagenesis of the five most highly conserved acidic amino acids in subunit I of cytochrome *bo* was performed to study their role in proton transfer. Mutation of only one of these sites, Asp135, to the corresponding amide, results in a dramatic decrease in proton pumping but with little change in electron-transfer activity. However, the conservative mutation Asp135Glu is active in proton translocation. It is proposed that an acidic residue at position 135 in subunit I may be important to form a functional proton input channel of the proton pump.

Cytochrome *bo*, a terminal quinol oxidase in *Escherichia coli*, catalyzes the reduction of dioxygen to water (Minghetti & Gennis, 1988; Minghetti et al., 1992) and couples this activity to vectorial translocation of protons across the cytoplasmic membrane (Puustinen et al., 1991, 1989). This is analogous to proton pumping by both mitochondrial and bacterial cytochrome *c* oxidases (Krab & Wikström, 1978; Solioz et al., 1982; Sone & Hinkle, 1982; Wikström, 1977; Wikström & Krab, 1979). Cytochrome *bo* is also structurally similar to the cytochrome aa_3-type oxidases of bacterial, plant, and animal species (Hosler et al., 1993). There is an especially high degree of amino acid sequence similarity among the subunits I of these enzymes (Chepuri et al., 1990; Lemieux et al., 1992; Santana et al., 1992; Shapleigh et al., 1992b), and together they form a family of terminal oxidases (Saraste, 1990). They also have a common structural motif with respect to the prosthetic groups, *viz.*, a low-spin six-coordinate heme and a binuclear center composed of a high-spin heme in close proximity to a copper atom (Cu_B) (Hill et al., 1992; Shapleigh et al., 1992a). In cytochrome *bo*, the low-spin heme is usually protoheme (heme b_{562}), and the high-spin heme of the binuclear center (heme *o* or o_3) has the novel heme O structure (Puustinen et al., 1992; Puustinen & Wikström, 1991; Wu et al., 1992).

It has been shown that subunits I and II are sufficient for electron-transfer and proton-pumping ability in the related aa_3-type cytochrome *c* oxidase from *Paracoccus denitrificans* (Hendler et al., 1991; Solioz et al., 1982) and that subunit III is not essential for the function of the oxidase from bovine heart mitochondria (Brunori et al., 1987; Finel & Wikström, 1986). Therefore, it is reasonable to assume that amino acid residues of cytochrome *bo* that are critical for the movement of protons during turnover are located within these subunits.

In bacteriorhodopsin, a light-driven proton pump, it has been shown that two aspartic acid residues are directly involved in the translocation of protons across the membrane (Butt et al., 1989; Henderson et al., 1990; Krebs & Khorana, 1993; Mogi et al., 1988). Acidic residues have also been shown to be critical for proton movements in the bacterial photosynthetic reaction center (Rongey et al., 1993; Shinkarev et al., 1993; Takahashi & Wraight, 1991). The purpose of the current work is to test the possibility that acidic residues within subunit I may be important in proton movements within the oxidase. Amino acid sequences of subunits of the terminal oxidase family are known from 75 different species (Bilofsky & Burks, 1988). A comparison of subunit I sequence alignments (Melissa Calhoun, personal communication) reveals several highly conserved acidic amino acid residues, which could potentially be involved in proton translocation.

Site-directed mutagenesis of the five most highly conserved acidic residues of subunit I was utilized to investigate their role, if any, in proton translocation. Amino acid substitutions for each of these acidic residues cause a reduction in the oxidase activity, and in one mutant, Asp135Asn, proton pumping appears to be decoupled from the electron-transfer activity. Aspartate-135, which is located in a hydrophilic interhelical domain on the cytoplasmic side of the membrane, appears to be important for proton-pumping activity.

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 addition of an *Xho*I site at 3.38 kbp), and the mutant protein was expressed in the host strain GL101 (Lemieux et al., 1992).

The base pair change(s) resulting in each mutant was (were) confirmed by sequencing after subcloning into the plasmid

† This work was supported by grants from the Sigrid Juselius Foundation (to M.W.) and the Acadamy of Finland (MRC), the National Science Foundation (DMB 89-04614, to J.O.A.), and the U.S. Department of Energy (DE-FG-02-87ER13716, to R.B.G.).

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

0006-2960/93/0432-10923$04.00/0 © 1993 American Chemical Society

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 sequenced using the Sequenase kit from United States Biochemical (Cleveland, OH).

Cells were grown for membrane isolation to a density of 100 Klett units in LB–lactate medium according to the previously described protocol (Lemieux et al., 1992). 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 difference spectra were recorded at 77 K using samples of 8 mg/mL membrane protein. All 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 genetic complementation, mutant enzyme activity, and proton pumping, plasmids containing the mutations were transformed into the strain RG129 (Lemieux et al., 1992). This strain lacks both respiratory oxidases (*cyo*, *cyd*) and cannot grow aerobically on nonfermentable substrate in the absence of a plasmid-derived functional oxidase. The nature of the *cyo* mutation in RG129 is not known. The strain is *rec*A to prevent marker rescue by recombination. Complementation of aerobic growth was determined as noted (Chepuri et al., 1990). The strain GK100 (Δ*cyo* Δ*cyd*) (Zuberi, unpublished results) was also used to test genetic complementation in some mutants.

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 Clark-type oxygen electrode, a Johnson Foundation (University of Pennsylvania, Philadelphia, PA) amplifier, and a closed, stirred glass reaction chamber. The solution contained 50 mM Tris-HCl (pH 7.4), 0.5 mM EDTA, and 165 μM ubiquinone-1, and 4.5 mM dithiothreitol (DTT) was present to keep the ubiquinone in its reduced state. Ubiquinone-1 was a gift from Hoffmann–LaRoche A.G. (Basel, Switzerland).

The concentration of cytochrome *bo* was determined spectrophotometrically using detergent-solubilized membranes. The peak-to-trough absorbance difference between 415 and 430 nm was determined in a CO-reduced *minus* reduced visible difference spectrum, using an extinction coefficient of 287 mM^{-1} cm^{-1} for the two-heme enzyme (Puustinen et al., 1991). It is noted that other values for this extinction coefficient have been published, 89 mM^{-1} cm^{-1} (Cheesman et al., 1993) and 135 mM^{-1} cm^{-1} (Kita et al., 1984). Until this discrepancy is resolved, the choice of values is somewhat arbitrary, but does not comprise the validity of any of the work discussed in this paper.

FTIR sample preparation and instrument conditions were similar to those noted previously (Shapleigh et al., 1992a). Crude membranes containing cytochrome *bo* in the host strain GL101 (Lemieux et al., 1992) were used to prepare the reduced CO adduct for FTIR. One milliliter of isolated membranes from approximately 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; 1 mL 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 septum was replaced with the appropriate centrifuge cap, and then the sample was pelleted at 160000*g* for 2 h. Again under a flow of CO, the supernatant was decanted, and CO-saturated glycerol was 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 27-μm 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 presented as a "light" *minus* "dark" 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. There was no further averaging, smoothing, or other correction to the spectra.

For proton-pumping assays, wild-type *E. coli* and mutants were grown aerobically on LB or minimal medium, containing 1% (w/v) lactate or 1% (w/v) succinate (Lemieux et al., 1992). Lactate was the preferred carbon source, because then the isolated spheroplasts exhibited only minimal amounts of endogenous NADH-linked respiration that could not be inhibited by capsaicin (8-methyl-*N*-vanillyl-6-nonanamide, Sigma) (Yagi, 1990). Proton-pumping experiments were performed as noted by Puustinen et al. (1989, 1991). The medium contained 100 mM KCl, 100 mM KSCN, 100 mM sucrose, 3 mM $MgCl_2$, 0.5 mM HEPES, and 2.5 mM potassium succinate. In some cases, KCl and KSCN were replaced by 150 mM KCl and 0.25 μg/mL valinomycin. Correction for background pH drift was done by computer after digitizing the raw data (Verkhovskaya et al., 1992).

Computer-aided structure predictions were performed using the pSAAM program for Windows provided by the Biotechnology Center, Urbana, IL (Crofts, 1987, 1992).

RESULTS

A set of highly conserved acidic amino acid residues in subunit I of *E. coli* cytochrome *bo* (Figure 1) was chosen for site-directed mutagenesis. The mutations, which are listed in Table I, were made at each of these residues with the hope of creating strains that exhibit oxidase activity but lack proton-pumping activity. As shown in Table I, all mutants but Glu286Ala have substantial, albeit reduced, oxidase activity and have the ability to support aerobic growth when expressed in the oxidase-deficient strain RG129 (*cyo cyd*). The reported turnover numbers reflect varying degrees of quinol oxidase activity among the active mutants, relative to wild type.

It should be noted, however, that when the Glu286Gln mutant is expressed in a different oxidase-deficient strain, GK100 (Δ*cyo* Δ*cyd*), rather than in RG129 (Table I), it does not support aerobic growth on nonfermentable substrates. This indicates that genetic complementation can depend, in some cases, upon the host strain. Of the many mutations so far examined in subunit I of cytochrome *bo*, including Glu286Ala (Table I), the only one which shows such a dramatic difference in phenotype when expressed in RG129 *versus* GK100 is Glu286Gln. The reason is not known.

Proton-pumping assays were performed with each of the mutants displaying oxidase activity (Table I). The H^+/e^-

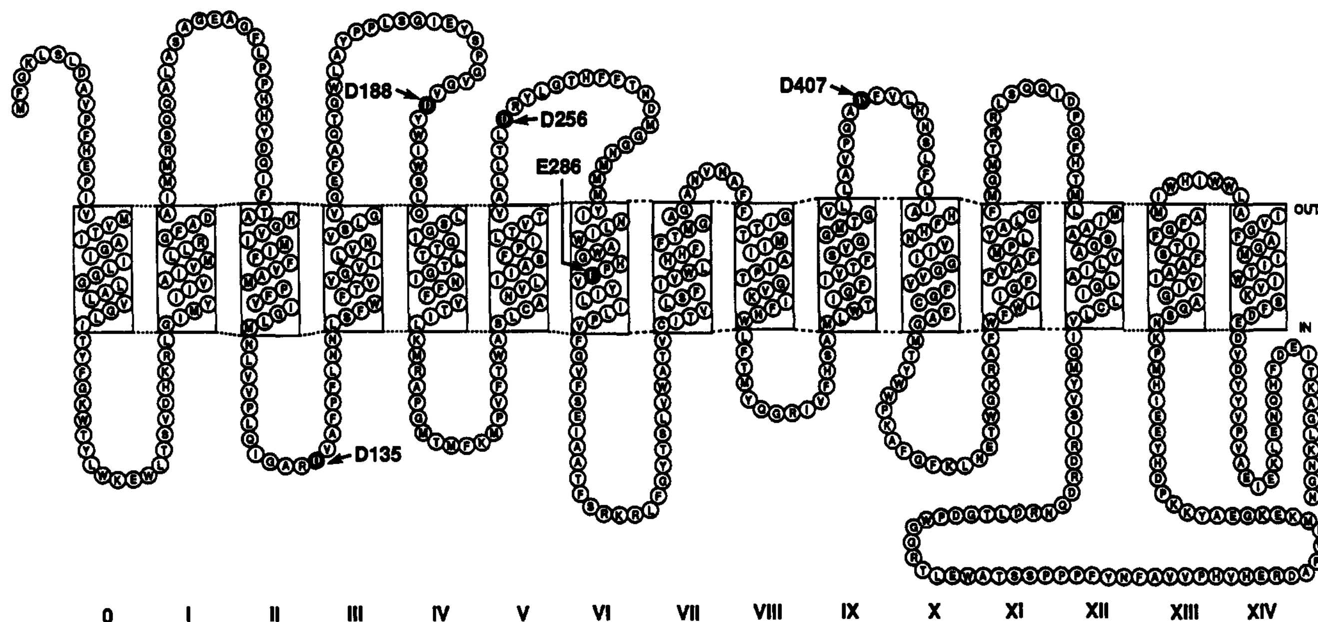

FIGURE 1: Two-dimensional topological model of subunit I of the *bo*-type oxidase from *Escherichia coli*. The most highly conserved acidic residues are highlighted in black. Of the 75 sequences aligned from GenBank (Bilofsky & Bruks, 1988), Glu286 is missing in 3 sequences (*Sulfolobus acidocaldarius*, *Thermus thermophilus* ba_3, and *Thermus thermophilus* caa_3), Asp135 is missing from only 2 sequences (*Sulfolobus acidocaldarius* and *Thermus thermophilus* ba_3), Asp188 is absent in 3 prokaryotic oxidase sequence (*S. acidocaladarius*, *Bacillus subtilis* aa_3-600, and *Bradyrhizobium japonicum 1*), Asp256 is missing in 2 prokaryotic oxidases (that from *S. acidocaldarius*, and the *Thermus thermophilus* ba_3), and Asp407 is missing in 4 oxidase sequences (*S. acidocaldarius*, *Paramecium aurelia*, *Paramecium tetraurelia*, and sea urchin).

Table I: Oxidase and Proton-Pumping Activity for the Cytochrome *bo* Mutants

			H^+/e^- ratio[d]		
strain	complementation[b]	activity[c]	pH 6	pH 7	pH 8
wild type[a]	+	100	1.8–2.0	1.5–1.8	0.7–0.8
D135E	+	45	1.7–1.8	1.5	1.1–1.3
D135N	+	45	1.0–1.2	1.0–1.1	0.8–1.0
D188N	+	53	1.8–1.9	1.7	1.1
E286Q	+	69	1.8–1.9	1.5–1.6	0.8
E286A	–	NA[e]	NA	NA	NA
D256N	+	25	1.8–2.0	1.8	1.2
D407N	+	31	1.8–1.9	1.5–1.6	1.1

[a] Wild type used for proton-pumping and oxidase activity assays was from GO103 at chromosomal levels. Complementation data for wild type were obtained from plasmid-encoded expression in RG129. [b] 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. [c] Specific activity is expressed as percent of wild-type activity (1140 e^-/s). [d] The range of observed H^+/e^- ratios is shown; data were collected from several oxygen pulses with minimally two independent growth batches of *E. coli*; for wild type and the D135N, D135E, and E286Q mutants, the data are more extensive and were collected using several different growth batches. The reported ratios were obtained by linear back-extrapolation of protonic decay to $t = 0$ (see Materials and Methods). [e] NA, not applicable.

ratio for the wild type is near 2 at pH 6–7, as previously reported (Puustinen et al., 1989), but at pH >8, the ratio declines to a value near unity (Table I), which has been ascribed to decoupling of proton translocation from electron transfer (Verkhovskaya et al., 1992). One proton per electron is presumed to be released to the periplasmic side of the membrane as a direct result of separation of the electronic and protonic charges upon ubiquinol oxidation. This event also occurs in cytochrome *bd*, the second quinol oxidase of *E. coli* (Anraku & Gennis, 1987). The second proton is thought to be due to proton pumping vectorially across the membrane, as in the structurally related cytochrome *c* oxidases. This proton-pumping function is lacking from the structurally unrelated cytochrome *bd* (Puustinen et al., 1991).

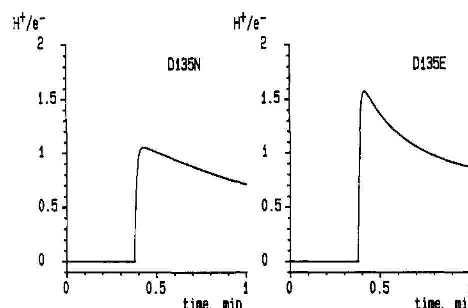

FIGURE 2: Proton translocation in *E. coli* mutant sphaeroplasts in KCl/KSCN medium at pH 6.2. Left panel, Asp135Asn mutant; right panel, Asp135Glu mutant. 5.16 nmol of O_2 was injected into the anaerobic sphaeroplast suspensions to initiate proton translocation.

Of the tested mutants, only Asp135Asn is deficient in the ability to pump protons (Table I). All other mutants having respiratory activity, including Asp135Glu and Glu286Gln, exhibit wild-type proton-pumping ability. Figure 2 compares two proton ejection traces from experiments with the Asp135Asn and Asp135Glu mutants. It is clear from the slow protonic decay that the proton permeability of the Asp135Asn mutant membrane has not been increased. Therefore, the lowered H^+/e^- ratio cannot be a trivial result of accelerated protonic backflow across the membrane in this mutant.

Figure 3A compares the visible CO difference spectra of the mutants with that of wild type. All mutants studied bind CO at levels at or near wild type, indicating that the heme *o* environment is unperturbed. Analogous dithionite-reduced *minus* air-oxidized visible spectra at 77 K suggest that the low-spin b_{562} heme site is also unperturbed in the mutants (Figure 3B). FTIR spectroscopy was further used to characterize the structural integrity of the binuclear center in the Asp135Asn mutant, which was found not to pump protons. A "light" minus "dark" CO FTIR difference spectrum recorded at 20 K is shown in Figure 4A and compared to that of the

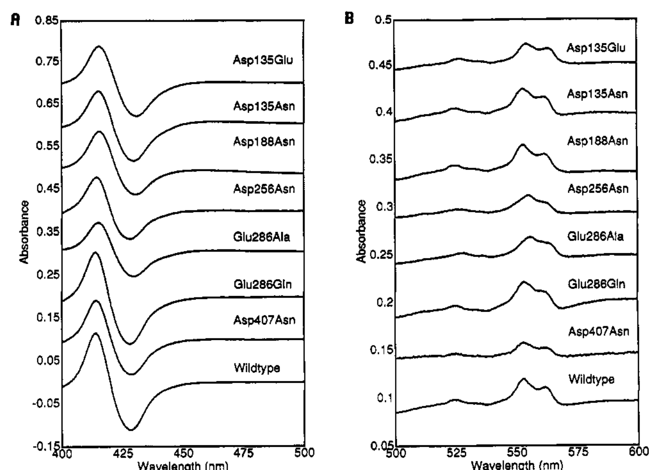

FIGURE 3: (A) Dithionite-reduced plus CO *minus* dithionite-reduced visible spectra of the Soret region at room temperature of membrane suspensions containing the indicated cytochrome *bo* variants. (B) Dithionite-reduced *minus* air-oxidized visible spectra at 77 K of the α-region of the indicated cytochrome *bo* variants.

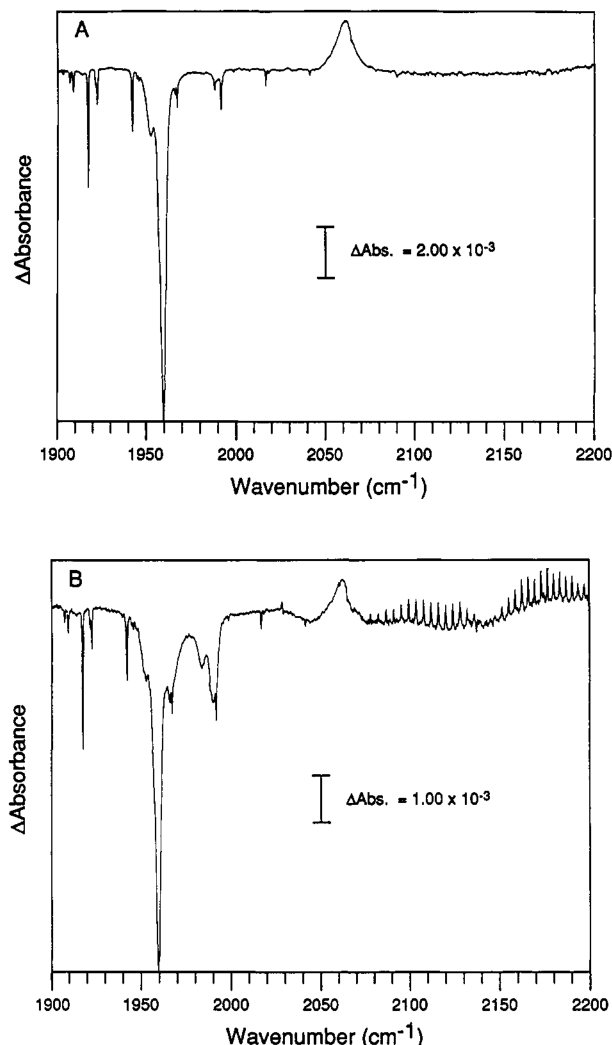

FIGURE 4: "Light" *minus* "dark" FTIR absorbance difference spectra recorded at 20 K and a path length of 27 μm. The absorption band at 1959 cm^{-1} corresponds to the Fe—C≡O vibrational mode, and the absorption band at 2063 cm^{-1} corresponds to the Cu—C≡O vibrational mode. The 1984-cm^{-1} band is the Fe—C≡O vibrational mode from the alternate oxidase, cytochrome *bd*. The band at 1990 cm^{-1} is probably from improperly assembled cytochrome *bo*. Panel A, wild type; panel B, Asp135Asn. The background noise in the spectrum in panel B is due to atmospheric CO.

wild type (Figure 4B). The center frequencies of the characteristic Fe—C≡O (1959 cm^{-1}) and Cu—C≡O (2063 cm^{-1}) bands are identical to those of the wild-type control (Hill et al., 1992). These data support the conclusion that the decoupling effect seen with Asp135Asn is not the result of a structural perturbation to the binuclear center.

DISCUSSION

The family of terminal heme–copper oxidases, including cytochrome *bo* from *E. coli* as well as mitochondrial cytochrome *c* oxidase, functions as redox-driven proton pumps. On the average, one proton is pumped across the membrane for each electron transferred to reduce dioxygen. In addition, one further ("chemical") proton per electron is required to form water at the binuclear center. Both of these protons are taken up from the inside of the membrane (Wikström, 1988), i.e., the cytoplasmic side in *E. coli*. The binuclear heme iron–copper center is located within the membranous domain of the oxidase, and recent site-directed mutagenesis work (Hosler et al., 1993; Lemieux et al., 1992; Minagawa et al., 1992; Shapleigh et al., 1992b) indicates that it may be located relatively close to the outer membrane surface, i.e., the periplasmic side of the *E. coli* membrane. Therefore, at least one proton-conducting structure, or "channel", is likely to be required to facilitate uptake of both "chemical" as well as "pumped" protons from the bacterial cytoplasm.

By analogy with bacteriorhodopsin (Henderson et al., 1990; Krebs & Khorana, 1993), one might expect acidic amino acid residues to be directly involved in facilitating proton uptake and release by such "channels". Since it is reasonable to assume that the mechanism of proton transfer is analogous among the structurally related members of the terminal oxidase family, the present study was focused upon the five most highly conserved acidic amino acid residues within subunit I. It is noted that no acidic residue is totally conserved in all of the 75 sequences of subunit I which were aligned (Calhoun, unpublished results), so it is not surprising that 4 of the acidic residues were found not to be essential in the current work. However, studies with bacterial photosynthetic reaction centers have clearly shown that functionally critical acidic residues need not be phylogenetically conserved among all species (Takahashi & Wraight, 1991).

Of the five most highly conserved acidic residues within subunit I (Figure 1), only Glu286 can reasonably be placed within the lipid bilayer. Glu286 is present in 72 of the 75 available sequences of subunit I, but is absent in the sequences of 3 prokaryotic oxidases: *Sulfolobus acidocaldarius* (Lübben et al., 1992), and both the ba_3-type (Genebank, LO9121) and caa_3-type (Mather et al., 1993) oxidases from *Thermus thermophilus*. Of most significance is the fact that the caa_3-type oxidase from *Thermus thermophilus*, which has a threonine in place of Glue286, has been shown to pump protons (Yoshida & Fee, 1984).

The data presented in this work demonstrate that Glu286 is not essential for proton pumping in the *E. coli* cytochrome *bo*. We have reported previously that glutamine can be placed in this position without substantial loss of oxidase activity. However, for reasons not yet understood, this mutation leads to the incorporation of heme O in the low-spin heme site, instead of protoheme, in more than half of the total enzyme population (Puustinen et al., 1992). An alanine substituted at this position does not yield a functional oxidase, so an important structural and/or functional role for Glu286 in the *E. coli* cytochrome *bo* cannot be ruled out. Nevertheless, the current data do indicate that Glu286 does not have an essential

role in proton translocation, in contrast to that of the two aspartic acid residues within the proton channel of bacteriorhodopsin.

Substitutions for the aspartic acid residues at positions 188, 256, and 407 cause some reduction in the specific electron-transfer activity of the enzyme, but the proton-pumping stoichiometry (the H^+/e^- ratio) is wild type. Hence, a specific role for these three acidic residues in proton pumping is not likely. However, dramatically lower oxidase activity is observed in several cases (e.g., Asp256Asn). Important functions for some of these residues, including the facilitation of proton movements, cannot be excluded. This will require further work to clarify.

Substitution of asparagine at position 135 of cytochrome *bo* causes a dramatic drop in the H^+/e^- ratio from 2.0 to a value near 1.0, accompanied by only a modest drop in quinol oxidase specific activity. Asp135 is present in all but 2 of the 75 sequences of subunit I, that for *S. acidocaldarius* (Lübben et al., 1992) and the ba_3-type oxidase from *Thermus thermophilus*. Neither of these two oxidases has been shown to pump protons.

The measured H^+/e^- ratio can be lowered in several ways, many of which are uninteresting. The proton permeability of the membrane may be increased to an extent that the backflux of protons overwhelms proton ejection. If this were to occur, then the H^+/e^- ratio would appear to be decreased, although no change in the proton-pumping stoichiometry will have actually occurred. The measured H^+/e^- ratio might also decline due to a substantial decrease in the rate of electron transfer, and hence in the rate of coupled proton translocation. In this case, a normal rate of proton backflux could overhwelm the reduced rate of proton ejection, again leading to a lowered measured H^+/e^- ratio despite the fact that proton pumping *per se* is unaffected. Both these possible pitfalls were carefully excluded in the present work. Note, for example, that several mutants (e.g., Asp256Asn) have quinol oxidase activities lower than that of the Asp135Asn mutant, and yet exhibit wild-type proton-pumping stoichiometry (Table I).

We conclude, therefore, that the proton-pumping activity itself is deficient in the Asp135Asn mutant. This constitutes the first example of a site-directed, specific structural perturbation that abolishes proton pumping in any one of the terminal oxidases. Previous covalent modification by DCCD of a conserved glutamic acid residue in subunit III also similarly causes a loss of proton pumping (Casey et al., 1980; Prochaska et al., 1981), but this must be due to an unspecific or secondary effect, because site-directed mutagenesis of this amino acid results in wild-type proton translocation (Haltia et al., 1991).

It is of particular interest to note that the H^+/e^- ratio of Asp135Asn is similar (unity) to the value of wild type at pH 8, when proton pumping in the wild-type enzyme is abolished, or largely depressed (Table I) (Verkhovskaya et al., 1992). This is consistent with the conclusion that the proton release from ubiquinol upon its oxidation (yielding $H^+/e^- = 1$) still occurs toward the outer aqueous (periplasmic) phase in the Asp135Asn mutant and that the depression of the H^+/e^- ratio at lower pH is indeed due to a specific loss of proton pumping.

It is premature to conclude that these results show a direct role of Asp135 in proton transfer, since its substitution by asparagine might cause a structural change that affects proton transfer indirectly. On the other hand, no spectroscopic evidence was found for structural perturbations near the low-spin heme or near the binuclear site. A major structural perturbation caused by this mutation can be ruled out by the wild-type FTIR spectrum shown in Figure 4. More subtle local changes in conformation, either nonspecific or due to the disruption of an allosteric mechanism, cannot be excluded.

The normal proton pumping found by substituting a glutamic acid residue at position 135 indicates, in any case, that if this position is occupied by an acidic residue, normal proton pumping is sustained. The extra methylene group of glutamic acid can apparently be accommodated. However, it cannot be excluded that proton transfer might have been slowed down with glutamic acid at site 135, relative to the rate with aspartic acid at this site. The present data only show that if this has occurred, it does not limit overall proton transfer sufficiently to cause a significant decline in the H^+/e^- ratio.

Despite the fact that an indirect effect on proton pumping cannot be excluded at this time, it may nevertheless be of interest to consider some of the implications of a direct involvement of Asp135 in proton transfer. The Asp135 site is located on the cytoplasmic side (inside) of the membrane (Figure 1) where it is part of a loop that contains several well-conserved residues (Saraste, 1990). Therefore, if Asp135 is indeed directly involved in proton transfer, it may form part of a proton input channel from the cytoplasmic aqueous space. Computer-aided structure predictions using the pSAAM program (Crofts, 1987, 1992) suggest that this domain might form a buried structure. It has been shown (Verkhovskaya et al., 1992) that the proton-pumping activity of wild-type cytochrome *bo* is lost at high pH, and this was confirmed here (Table I). It is possible, therefore, that the Asp135Asn mutation leads to the same decoupling effect. The local pH at the input site of the molecular proton-pumping machinery may be raised during turnover due to a blockage of proton transfer into this site. At high pH, the probability of protonation of essential proton-carrying residues of the pump is lowered. If the pump can switch these residues into their output configuration at a sufficient velocity in their unprotonated state, then the pump will perform unproductive, futile, catalytic cycles (decoupling), and the H^+/e^- ratio will fall.

Although such speculation is interesting and suggests the direction of future research to test these possibilities, it must be stressed that at this point all that can be said with certainty is that the substitution of Asp135 by Asn results in a drastic reduction in the proton-pumping activity of the *E. coli* cytochrome *bo*. The molecular mechanism remains to be determined.

ACKNOWLEDGMENT

We thank Tamma Zuberi for providing the GK100 strain, Melissa Calhoun and John Hill for discussions and assistance with the FTIR, and Marina Verkhovskaya for help with the figures.

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