Genetic Fusion of Subunits I, II, and III of the Cytochrome *bo* Ubiquinol Oxidase from *Escherichia coli* Results in a Fully Assembled and Active Enzyme†

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ABSTRACT: The cytochrome *bo* ubiquinol oxidase from *Escherichia coli* is a five-subunit enzyme which is a member of the superfamily of heme–copper respiratory oxidases. Three of the subunits (I, II, and III) are homologous to the three mitochondrial encoded subunits of the eukaryotic aa_3-type cytochrome *c* oxidase. Subunits, I, II, and III of the eukaryotic oxidase contain 12, 2, and 7 putative transmembrane spans, respectively. The hydropathy profiles of the subunits of most other members of this oxidase superfamily are consistent with these structures. However, subunit I from the *E. coli* oxidase contains 15 transmembrane spans, with one additional span at the N-terminus and two additional spans at the C-terminus in comparison to the eukaryotic oxidase. The additional transmembrane helix at the N-terminus predicts that the amino terminal residue should be on the periplasmic side of the membrane. By deleting the intergenic region between the *cyo*A and *cyo*B genes, an in-frame fusion between subunit II (*cyo*A) and subunit I (*cyo*B) was generated. This links the C-terminus of subunit II, known to be on the periplasmic side of the membrane, to the N-terminus of subunit I. The resulting oxidase is fully active, and supports the toplogical folding pattern previously suggested for subunit I with the N-terminus in the periplasm. Whereas subunit I of the *E. coli* oxidase has two additional membrane-spanning helices at the C-terminus, subunit III has two fewer helices than does the corresponding subunit III of the eukaryotic oxidase. Since the genes encoding subunits I (*cyo*B) and III (*cyo*C) are contiguous, it is tempting to speculate that the overall structures of subunits I and III are similar in the two oxidases, but that the breaks between subunits I and III occur at different points. If this is true, it should be possible, in principle, to fuse subunits I and III to form a single subunit (I–III). The C-terminus of subunit I and the N-terminus of subunit III are both predicted to be on the cytoplasmic side of the membrane. By deleting the *cyo*B–*cyo*C intergenic region, the fusion of subunits I and III was accomplished, and resulted in an active oxidase. When in-frame fusions were made between all three subunits (II–I–III), the resulting gene product still assembles as part of a functional oxidase. The fused subunit (II–I–III) contains 22 transmembrane spans. These data support the previously proposed topology of the subunits and provide a starting point for defining how the three subunits interact with each other.

The cytochrome *bo* ubiquinol oxidase from *Escherichia coli* is a member of a large superfamily of heme–copper respiratory oxidases which includes the eukaryotic cytochrome *c* oxidases (Babcock & Wikstrom, 1992; Saraste, 1990). The bovine cytochrome *c* oxidase contains 13 subunits: three encoded in the mitochondrion (subunits I, II, and III) and the rest nuclear encoded (Kadenbach et al., 1991). Bacterial members of this superfamily of oxidases contain homologues of the three mitochondrially encoded subunits (Saraste, 1990), but homologues of the nuclear-encoded subunits have not been observed. Studies on the aa_3-type cytochrome *c* oxidase isolated from *Paracoccus dentrificans* have clearly demonstrated that subunits I and II are sufficient both for cytochrome *c* oxidase activity and for coupling the electron-transfer reactions to proton pumping across the membrane (Hendler et al., 1991).

Subunit I is very highly conserved within this superfamily and contains both a low-spin, six-coordinate heme component and the heme–copper binuclear center, which is the site where oxygen is reduced to water (Babcock & Wikstrom, 1992; Saraste, 1990). In the *E. coli bo*-type oxidase (Minghetti et al., 1992; Puustinen & Wikström, 1991), heme B (protoheme IX) occupies the low-spin site (denoted heme b_{562}), whereas heme O (Wu et al., 1992) is the heme component in the binuclear center (denoted heme *o*). In the aa_3-type oxidases, heme A occupies both heme sites (denoted heme *a* and heme a_3). The identity of the two hemes does not correlate with whether a particular oxidase utilizes cytochrome *c* or a quinol as its substrate (Santana et al., 1992).

For those members of the oxidase superfamily that utilize cytochrome *c* as a substrate, subunit II plays a clear function. Subunit II has been implicated in the binding of cytochrome *c* and, in addition, contains a second copper moiety (Cu_A) that is directly involved in the oxidation of ferrocytochrome *c* (Capaldi, 1990a,b; Hill, 1991; Pan et al., 1991; Saraste, 1990). For the quinol oxidases that are members of this superfamily, however, neither the cytochrome *c* binding site nor Cu_A is present, and the role of this subunit is not known (Chepuri et al., 1990; Minghetti et al., 1992).

Recent studies from this laboratory examined the topology of each of the subunits of the *E. coli* cytochrome *bo* oxidase (Chepuri & Gennis, 1990). This was accomplished by constructing gene fusions of the *cyo* genes with the genes encoding alkaline phosphatase and β-galactosidase. The data

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Table I: List of Plasmids and Strains

plasmids	relevant features	source
plasmids		
pMC31	pBR322 derivative that contains the *cyo* operon	Lemieux et al., 1992
pRCO1	derivative of pMC31 with *cyo*A and *cyo*B fused	this work
pRCO2	derivative of pMC31 with *cyo*B and *cyo*C fused	this work
pRCO3	derivative of pMC31 with *cyo*A, *cyo*B, and *cyo*C fused	this work
M13 derivative		
M13XE	M13 construct containing the *Xma*I–*Eco*RI fragment from pL1	Lemieux et al., 1992
strains		
RG129	*cyo*, *cyd*A2, *rec*A, *srl*-300::Tn10*rps*L *thi*, *gal*, *nad*A, *lon*100(?)	Au & Gennis, 1987
GL101	F^-, Str^R, *rps*L, *thi*(?), *gal*(?), *cyo*, *sdh*C::Kan^R, *rec*A Tet^R, lon(100), *srl*300::Tn10	Lemieux et al., 1992
NM522	F^-, lacII^q ZΔM15, *pro*AB/*sup*E, Δ*lac-pro*AB), *thi*-1, Δ(*hsd*SM–*mcr*B)5	laboratory stock
Jmr$^-$	F′, *mcr*AB	gift from Dr. Vandeyer

suggested that the two-dimensional folding patterns of subunits I, II, and III across the membrane are essentially the same as the patterns suggested for the corresponding subunits of the mitochondrial oxidases (Saraste, 1990), but with some interesting differences. Subunit II of the *E. coli* oxidase is proposed to have both N- and C-termini facing the periplasm and to have two transmembered helical segments (Chepuri & Gennis, 1990). This model is consistent with the model of subunit II of the cytochrome *c* oxidases that is based on biochemical studies (Bisson et al., 1982a,b; Capaldi, 1990a,b; Finel, 1988; Saraste, 1990). Subunit I of the *E. coli* oxidase is proposed to have 15 transmembrane helical spans with the N-terminus facing the periplasm and the C-terminus facing the cytoplasm (Chepuri & Gennis, 1990). This folding pattern is also consistent with models of subunit I of the eukaryotic cytochrome *c* oxidases (Saraste, 1990), except that the *E. coli* oxidase has one additional span on the N-terminal side and two additional spans at the C-terminus which are not present in the sequences of most of the other members of this superfamily. Other exceptions, however, are the sequences of subunit I of the cytochrome *c* oxidase from *Bacillus* PS3 (Ishizuka et al., 1990) and of both the aa_3-type (Santana et al., 1992) and caa_3-type (Saraste et al., 1991) oxidases from *Bacillus subtilis*, all of which have the additional putative transmembrane spans observed in the *E. coli* oxidase.

The presence of the two additional transmembrane helices at the C-terminus of subunit I of both the *E. coli* and the three *Bacillus* oxidases correlates with the observation that subunit III of each of these four oxidases lacks two transmembrane spans that are present at the N-terminus of subunit III of all of the other members of this superfamily (Chepuri & Gennis, 1990; Ishizuka et al., 1990; Santana et al., 1992; Saraste et al., 1991). In *E. coli*, *B. subtilis*, and *Bacillus* PS3, the gene encoding subunit III follows the gene encoding subunit I within an operon. Hence, it is natural to speculate that a genetic rearrangement has essentially resulted in transferring a sequence that is normally found at the beginning of subunit III to the end of subunit I (Mather et al., 1990). The caa_3-type oxidase from *Thermus thermophilus* exhibits yet another variation due to an apparent genetic rearrangement. In this case, subunits I and III have been fused, resulting in a single subunit (Mather et al., 1993). These data suggest the possibility of fusing the corresponding subunits of the *E. coli* oxidase.

In this article, it is shown that subunits I and III of cytochrome *bo* from *E. coli* can be fused to yield an active, fully assembled oxidase. Furthermore, it is demonstrated that subunits II and I can also be genetically fused without compromising the assembly or function of the resulting oxidase. Finally, all three subunits are fused and shown to result in an active oxidase species. These experiments are consistent with the proposed topologies of each of the three subunits and also suggest some constraints in how these three subunits interact with each other.

MATERIALS AND METHODS

Materials. The restriction enzymes *Eco*RI, *Nsi*I, *Hin*dIII, and *Msp*I were purchased from Bethesda Research Laboratories. The restriction enzymes *Hha*I and *Xma*I were obtained from New England Biolabs, as were T4 DNA ligase and T4 DNA kinase. T4 DNA polymerase, exonuclease III, and sequenase kits were obtained from U. S. Biochemical Corp. The nucleotides dATP, dGTP, dTTP, and 5Me-dCTP were purchased from Pharmacia LKB Biotechnology, Inc. ATP and phenylmethanesulfonyl fluoride were purchased from Sigma. Oligonucleotides used to generate mutations, or as sequencing primers, were obtained from the Biotechnology Center at the University of Illinois (Urbana, IL). Goat anti-rabbit IgG(H+L) alkaline phosphate conjugate and prestained SDS–PAGE standards were purchased from Bio-Rad Laboratories.

Fusion of cyoA and cyoB. *cyoA*, encoding subunit II of the cytochrome *bo* complex and *cyo*B encoding subunit I are separated by 24 base pairs in the *cyo* operon (Chepuri et al., 1990). The intergenic region plus the stop codon of *cyo*A and the first codon of *cyo*B were deleted using the oligonucleotide, 5′-GCGGAATCCGCCCATTTCGGAAAATTATCA-3′, which spans the deleted region with 15 base pairs on each side. The deletion mutagenesis was performed with minor variations by following the method of Vandeyar (Vandeyar et al., 1988) for site-directed mutagenesis. The single-stranded DNA template was prepared from an M13 derivative M13XE (see Table I), which was constructed by cloning the *Xma*I–*Eco*RI fragment pMC31 into M13mp18 (Lemieux et al., 1992). The deletion mutation was verified on the M13 phage by DNA sequencing using the USB Sequenase kit protocol. The smaller *Xma*I–*Hin*dIII fragment, which contains the desired deletion, was subcloned from the M13 phage into plasmid pMC31. The resulting plasmid is designated as pRC01 (see Figure 1). The mutation was confirmed after subcloning by double-stranded DNA sequencing. The double-stranded DNA was prepared by using a modified method of DNA minipreparation (Sambrook et al., 1989).

Fusion of cyoB and cyoC. *cyo*B encoding subunit I and *cyo*C encoding subunit III of the cytochrome *bo* complex overlap by 11 base pairs (Chepuri et al., 1990). An oligonucleotide, 5′-AATGGCAACGATACTTTGAC-3′, was designed to delete the T in the stop codon TGA of *cyo*B, resulting in an in-frame fusion of *cyo*B and *cyo*C. The first three amino acids of the subunit III, methionine, alanine, and threonine, are subsequently removed. The deletion mutagenesis protocol was essentially that of Vandeyer (Vandeyar et al., 1988), using M13XE as the single-stranded DNA template.

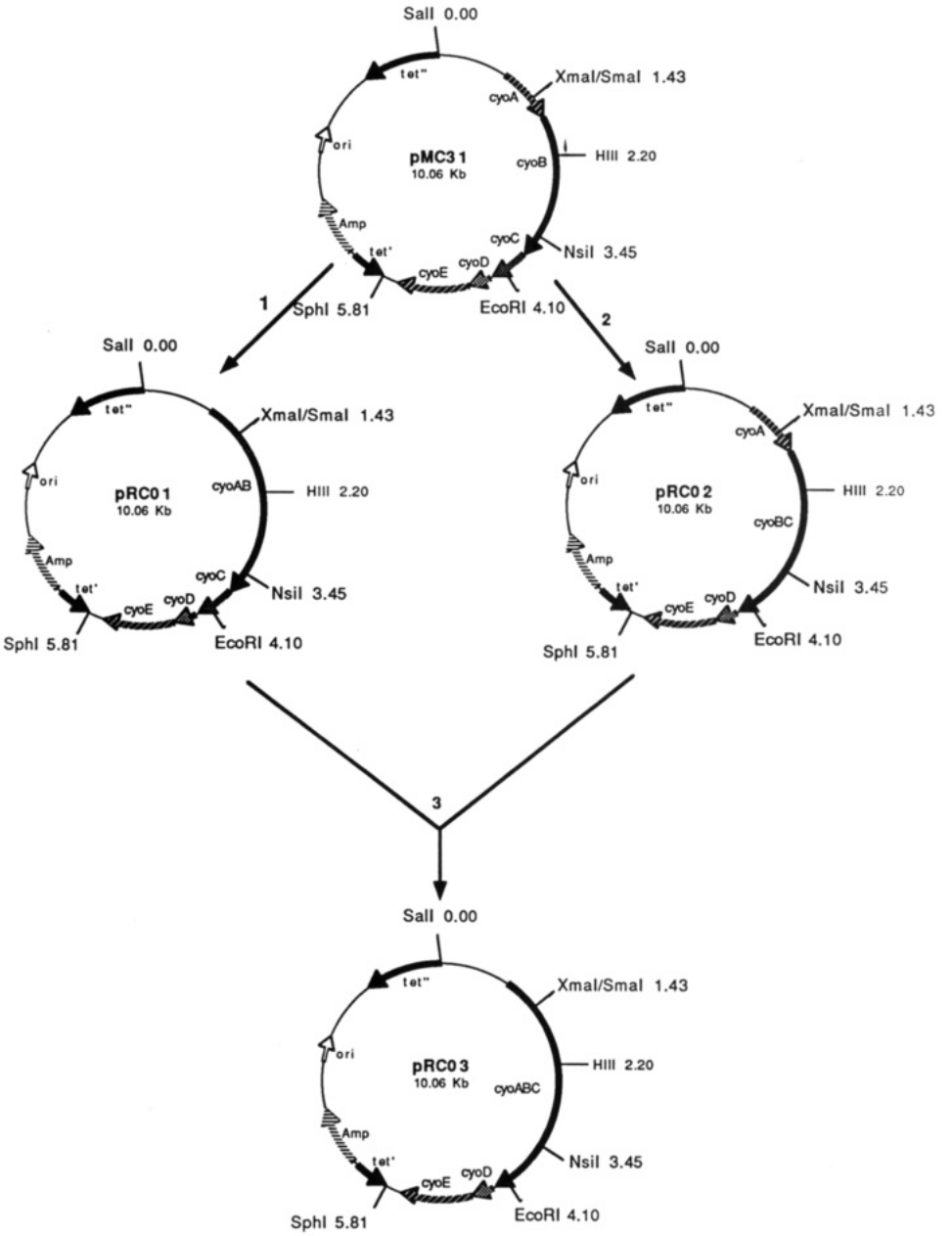

FIGURE 1: Construction of the three plasmids that contain fused genes. The genes *cyo*A, *cyo*B, and *cyo*C, correspond to subunits II, I, and III of the cytochrome *bo* complex, respectively. Arrow 1: The plasmid pRC01 was generated from pMC31 by fusing *cyo*A and *cyo*B in-frame, as described in the text. Arrow 2: One base pair in the stop codon of *cyo*B was deleted to generate pRC02. Arrow 3: The smaller *Nsi*I–*Eco*RI fragment of pRC02 replaced the corresponding region in pRC01 to yield pRC03.

After the deletion mutation was identified on the M13 phage, the mutated *Nsi*I–*Eco*RI fragment was subcloned into plasmid pMC31. The resulting plasmid is designated pRC02 (see Figure 1). The mutation was confirmed with double-stranded DNA sequencing.

Construction of Plasmid pRC03. Plasmids pRC01 and pRC02 were digested with restriction enzymes *Eco*RI and *Nsi*I. After electrophoresis on a low-melting agarose gel, the smaller *Eco*RI–*Nsi*I fragment from pRC02, which contains the fusion mutation of *cyo*B and *cyo*C, was ligated to the larger fragment from pRC01, which has the fusion mutation of *cyo*A and *cyo*B using T4 DNA ligase. The resulting plasmid is designated pRC03 (see Figure 1). The presence of both fusion mutations in pRC03 was confirmed with double-stranded DNA sequencing.

Complementation Analysis. In order to determine whether the subunit-fused oxidases are physiologically functional, genetic complementation was conducted. This was accomplished by following a previously published procedure (Lemieux et al., 1992), using a host strain, RG129, which cannot grow aerobically on nonfermentable substrates due to mutations in both the *cyo* and *cyd* operons which encode the two respiratory oxidases in *E. coli*.

Cell Growth for Analysis of Mutant Oxidases. Cells were grown as previously described (Lemieux et al., 1992). The concentrations of both mutant and wild-type cytochrome *bo* complexes in membranes were determined from the CO–dithionite-reduced minus dithionite-reduced difference spectrum (Lemieux et al., 1992). The extinction coefficient of the peak-to-trough absorbance for the Soret band in the CO difference spectrum used was 135 mM^{-1} cm^{-1} (Kita et al., 1984), although another study suggests a value that is larger: 287 mM^{-1} cm^{-1} (Puustinen et al., 1991).

Western Immunoblotting Analysis. The procedure followed for Western immunoblotting analysis was as described previously (Harlow & Lane, 1988). The separating gel for

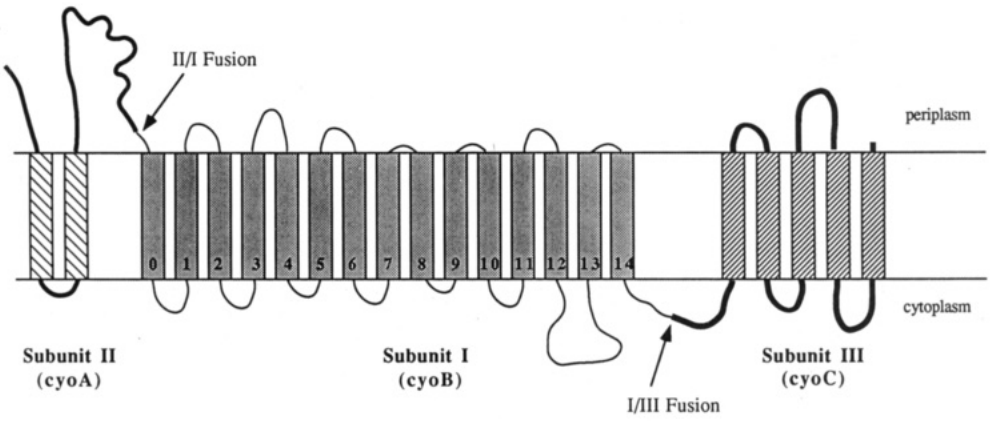

FIGURE 2: Schematic drawing of the membrane topology of subunits I, II, and III of the cytochrome *bo* complex. This topological model is based on the hydropathy profiles and gene fusion studies (Chepuri & Gennis, 1990). Arrows indicate fusion sites. "II/I fusion" denotes the fusion of subunit II and subunit I. "I/III fusion" denotes the fusion of subunit I and subunit III. The transmembrane helices of subunit I are numbered so as to maintain the traditional scheme for cytochrome *c* oxidase.

sodium dodecyl sulfate–polyacrylamide gel electrophoresis was 10% polyacrylamide. The cytochrome *bo* complex used as a control in the analysis was purified as previously described (Minghetti et al., 1992).

Assay for Oxidase Activity. Rates of oxygen consumption at 37 °C were measured with a YSI Model oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, OH) and a temperature-controlled 1.8-mL electrode chamber (Gibson). The buffer contained 50 mM potassium phosphate (pH 7.5) plus 2 mM dithioerythritol and 555 μM ubiquinol-1. The concentration of O_2 in the air-saturated buffer at this temperature was assumed to be 250 μM. The reaction was initiated by injecting 5 μL of appropriately diluted membrane preparation. The background activity prior to the addition of membranes was subtracted.

RESULTS

Three plasmids were generated as described in the previous section which contain variants of the *cyo* operon. Plasmid pRC01 has a fusion in which the *cyo*A and *cyo*B genes have been used in-frame, which should yield a single combined subunit II–I in place of subunits II and I. Similarly, pRC02 contains an operon in which the *cyo*B and *cyo*C genes have been fused in-frame, thus yielding a single subunit I–III in place of subunits I and III. The operon contained in pRC03 has these two fusions combined and should yield a single subunit II–I–III in place of the three separate subunits. Figure 2 shows a schematic diagram of subunits I, II, and III with the folding pattern suggested from previous studies and with the fusion junctions indicated. Each of the three plasmids encoding the subunit fusions is able to restore the ability of an oxidase-deficient strain (RG129) to grow aerobically on nonfermentable substrates. Hence, it is concluded that each of these constructs encodes a viable ubiquinol oxidase. Membranes were prepared from each of the strains encoding the subunit-fused oxidases and were used for further analysis.

Figure 3 shows the spectra of the membranes. In Figure 3A the CO-reduced minus reduced spectra are shown, and in Figure 3B are the dithionite-reduced minus air-oxidized spectra taken at 77 K. The data presented in Figure 3A show the diagnostic peak and trough in the Soret band resulting from CO binding to the heme *o* component of the dithionite-reduced oxidase. The optical spectra of the subunit-fused oxidases are essentially identical to that of the wild-type control. The data in Figure 3B show the split α band (555 and 563.5 nm) previously shown to be due primarily to the low-spin heme b_{562} component of the oxidase (Minghetti et al., 1992). The

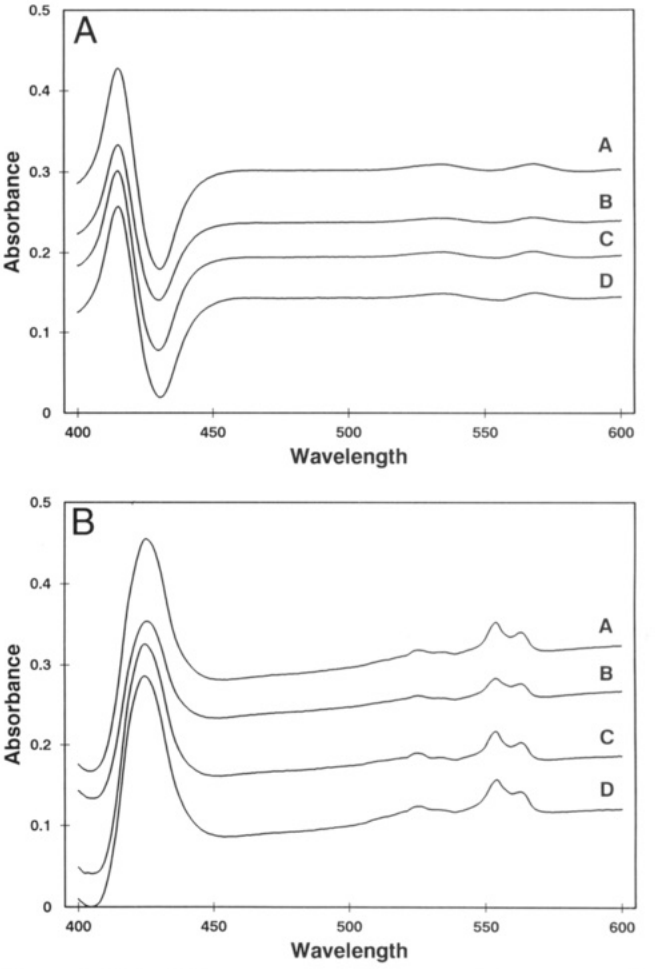

FIGURE 3: UV–visible spectra of membranes containing mutant and wild-type cytochrome *bo* complexes. Panel A: CO–dithionite-reduced minus dithionite-reduced difference spectra. The membrane protein concentration in all cases was 2 mg/mL. Panel B: Low-temperature (77 K) dithionite-reduced minus air-oxidized spectra. All spectra were recorded at a protein concentration of 8 mg/mL. Plasmids pRC01, pRC02, pRC03, and pMC31 were expressed in the RG129 strain background, in which the chromosomal *cyo* operon is not expressed. Spectrum A, wild-type control (pMC31). Spectrum B, II–I fusion (pRC01). Spectrum C, I–III fusion (pRC02). Spectrum D, II–I–III fusion (pRC03).

spectra of the subunit-fused oxidases appear identical to that of the wild-type control. Together, these data suggest no perturbation to either heme component due to the subunit fusions. This is further substantiated by the data summarized

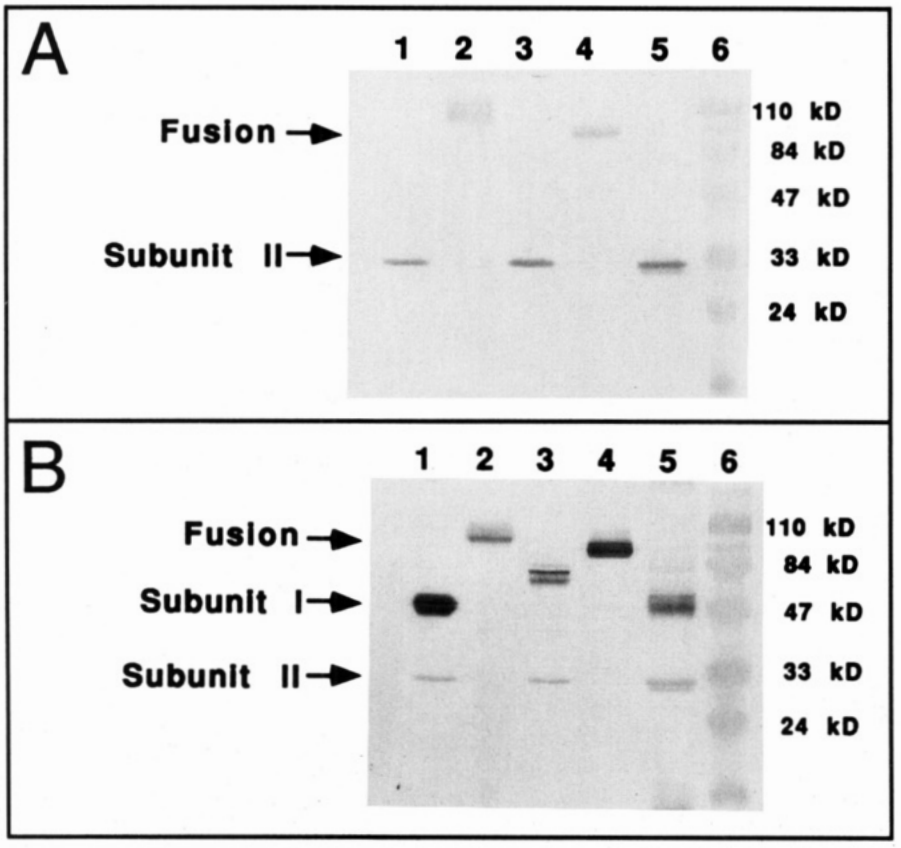

FIGURE 4: Western immunoblot analysis of cytoplasmic membranes from strains expressing the mutant *cyo* operons. The host strain for expressing plasmids was RG129. Panel A: Western immunoblot with anti-subunit II antiserum. Panel B: Western immunoblot with anti-subunit I antiserum. All antisera were strip-purified. All lanes, except lanes 5 and 6, were loaded with 10 μg of membrane proteins: lane 1, membrane containing wild-type cytochrome *bo* complex (pMC31); lane 2, mutant cytochrome *bo* complex with subunits I, II, and III fused together (pRC03); lane 3, mutant cytochrome *bo* complex with subunits I and III fused (pRC02); lane 4, mutant cytochrome *bo* complex with subunits II and I fused (pRC01); lane 5, 2 μg of the pure cytochrome *bo* complex; lane 6, protein molecular weight standards. The antibodies used in B cross-react to some extent with subunit II, resulting in a faint band corresponding to subunit II in lanes 1, 3, and 5.

Table II: Cytochrome *bo* Content and Ubiquinol-1 Turnover in Membranes of RG129 (*cyo cyd*) Containing Plasmids Expressing either Wild-Type or Subunit-Fused Oxidase

plasmid	mutant oxidase	ubquinol-1 oxidase turnover[a]	concentration of *bo*-type oxidase in membrane[b]
pMC31	wild-type	7.44×10^3	921
pRCO1	II–I linked	7.13×10^3	823
pRCO2	I–III linked	7.88×10^3	829
pRCO3	II–I–III linked	7.65×10^3	882

[a] Moles of O_2 utilized per mole of cytochrome *bo* complex. [b] Picomoles of cytochrome *bo* per milligram of membrane protein.

in Table II. The spectra shown in Figure 3A were used to quantify the amount of cytochrome *bo* oxidase present in each membrane sample. The data are virtually the same for each sample, indicating that the subunit fusions do not alter the extent to which each oxidase variant is overproduced and do not result in instability either *in vivo* or during isolation of the membranes. The turnover of each of the fused-subunit oxidases was measured using ubiquinol-1 as the substrate. The data, summarized in Table II, once again show no substantial difference from the wild-type control.

Figure 4 shows the results of Western immunoblotting of each of the membrane samples using antibodies directed against either subunit II (Figure 4A) or subunit I (Figure 4B). The results confirm the presence of fused subunits. The highest molecular weight species is seen in lane 2, containing the sample in which subunits I, II, and III are fused. The same band is visualized using the antibodies to either subunit I or subunit II. No antibodies are available against subunit III. In lane 3 (Figure 4B) it is clear that subunit I is now fused with subunit III and has a larger size than in the wild-type control. However, subunit II has the same size as in the wild-type control (lane 3, Figure 4). In lane 4, both antibodies visualize the same band, which is the fusion of subunits I and II, and this fused product has a smaller size than that observed in lane 2, which contains all three subunits linked together. Note that the antibodies against subunit I (Figure 4B) cross-react to some extent with subunit II. In no case are additional bands apparent that would suggest substantial proteolytic degradation of the fused subunits. The fact that several bands appear as doublets suggests, however, that some degradation may be occurring.

DISCUSSION

The data presented in this article clearly demonstrate that subunits I, II, and III of the cytochrome *bo* ubiquinol oxidase from *E. coli* can be genetically linked without any deleterious consequences. The level of overproduction, the spectroscopic characteristics, and the enzymatic properties appear unaltered compared to those of the wild-type control. The fusion of all three subunits should have a molecular weight of 133 000 and have 22 transmembrane spans, as indicated in the schematic diagram in Figure 2. Previous work suggested that the C-terminus of subunit II and the N-terminus of subunit I were both on the periplasmic side of the bacterial membrane. The fact that an in-frame fusion can be made without ill effects indicates not only that this topology is correct but that the two ends of the subunits which are fused must normally be close

to each other, or at least can be brought together without any structural changes of consequence.

The topologies of the three subunits shown in Figure 2 are partially based on an examination of in-frame gene fusions of these subunits to the mature portion of alkaline phosphatase (*pho*A). One of the fusions reported (Chepuri & Gennis, 1990) had a junction near the cytoplasmic side of helix 0 (Figure 2). Surprisingly, this *cyo*B–*pho*A gene fusion had relatively high alkaline phosphatase activity, suggesting that the N-terminal portion of subunit I could function as a signal sequence to export the mature portion of alkaline phosphatase to the periplasm. This result, however, would normally be interpreted as indicating that helix 0 of subunit I has the opposite orientation as that shown in Figure 2. However, since this interpretation is inconsistent with virtually all of the other topological data (Chepuri & Gennis, 1990), it was previously concluded that the ability of helix 0 to function as a signal sequence (i.e., N-terminus inside) must be context-dependent, and it does not do so as part of subunit I. The data presented in the current work confirm this judgement. The fact that the N-terminus of subunit I can be linked to the C-terminus of subunit II definitively shows that the N-terminus of subunit I is on the periplasmic side of the membrane, as shown in Figure 2. This is also consistent with the "positive in" rule (Andersson et al., 1992), since the interhelical connection between helices 0 and 1 has a net charge of +2, and the N-terminal extension prior to helix 0 has a net charge of −1.

The ability to fuse the C-terminus of subunit I with the N-terminus of subunit III is also consistent with the topologies proposed previously (Chepuri & Gennis, 1990). Although there is no convincing sequence homology, it is reasonable to conclude that helices 13 and 14 subunit I of the *E. coli* oxidase correspond to the first two putative transmembrane helices of subunit III of most of the other members of the heme–copper oxidase superfamily (Mather et al., 1990, 1993). Whereas subunit III of the *E. coli* oxidase has a predicted five transmembrane spans, most other subunit III sequences have seven transmembrane spans. The *Bacillus* PS3 caa_3-type oxidase (Ishizuka et al., 1990) and both the caa_3-type (Saraste et al., 1991) and aa_3-type (Santana et al., 1992) oxidases from *B. subtilis* also have only five transmembrane spans in subunit III along with two "extra" transmembrane spans at the C-terminus of subunit I. Since in *E. coli*, PS3, and *B. subtilis* the gene encoding subunit III immediately follows that encoding subunit I (Ishizuka et al., 1990; Santana et al., 1992; Saraste et al., 1991), it is reasonable to speculate that a genetic rearrangement could readily produce this result. The fact that subunits I and III of the oxidase from *T. thermophilus* are naturally fused (Mather et al., 1993) demonstrates that the overall structure of the oxidase is tolerant of such variations. The current data with the *E. coli* oxidase dramatically emphasize this point.

Nothing is currently known about how the transmembrane domains of subunits I, II, and III interact with each other. The fact that subunits I and II can be fused as shown in the current work does not limit the possibilities, since the hydrophilic domain of subunit II to which the fusion is made with helix 0 of subunit I is so large. However, the conclusion that helices 13 and 14 of the *E. coli* and the three *Bacillus* oxidases correspond to the first two transmembrane spans which are "normally" part of subunit III is significant. This conclusion is consistent with the current work and is driven by the patterns observed in the various prokaryotic oxidases, as already discussed. If correct, it seem likely that these two helices form at least a portion of the interface between subunits I and III, regardless of whether they are a part of either subunit I or subunit III.

Finally, it is noted that the artificial fusion generated between subunits I and III in the *E. coli* oxidase has its natural counterpart in the oxidase from *T. thermophilus* (Mather et al., 1993). This suggests the possibility of some oxidases, yet to be characterized in which subunits I and II, or even subunits I, II, and III, may be fused in Nature.

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