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Isolation and Characterization of a New Class of Cytochrome *d* Terminal Oxidase Mutants of *Escherichia coli*

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Cytochrome *d* terminal oxidase mutants were isolated by using hydroxylamine mutagenesis of pNG2, a pBR322-derived plasmid containing the wild-type *cyd* operon. The mutagenized plasmid was transformed into a *cyo cyd recA* strain, and the transformants were screened for the inability to confer aerobic growth on nonfermentable carbon sources. Western blot analysis and visible-light spectroscopy were performed to characterize three independent mutants grown both aerobically and anaerobically. The mutational variants of the cytochrome *d* complex were stabilized under anaerobic growth conditions. All three mutations perturb the b_{595} and *d* heme components of the complex. These mutations were mapped and sequenced and are shown to be located in the N-terminal third of subunit II of the cytochrome *d* complex. It is proposed that the N terminus of subunit II may interact with subunit I to form an interface that binds the b_{595} and *d* heme centers.

The cytochrome *d* terminal oxidase functions as one of two terminal oxidases in the inner membrane of *Escherichia coli* (for reviews, see references 12 and 22). This oxidase, along with the cytochrome *o* terminal oxidase complex, catalyzes the oxidation of ubiquinol-8 and the reduction of oxygen to water with the concomitant release of protons into the periplasmic space (12). This release of protons is a major contribution to the development of the proton motive force that drives oxidative phosphorylation. These two oxidases are differentially regulated by oxygen. The cytochrome *o* complex is present in the early log phase of growth (i.e., high aeration), and the cytochrome *d* complex becomes the predominant oxidase in stationary-phase cells (i.e., low aeration) (9, 23, 40).

The cytochrome *d* complex is an integral membrane protein consisting of two subunits, I (58,000 Da) and II (43,000 Da), in a 1:1 ratio (24, 36, 37). There are three heme prosthetic groups associated with this complex comprising cytochromes b_{558}, b_{595}, and *d*. The two cytochromes *b* contain protoporphyrin IX (32), and the heme *d* moiety is a chlorin (44–46). The genes encoding the subunits of the cytochrome *d* complex have been cloned and sequenced (16, 17). Subunit I is encoded by *cydA*, and subunit II is encoded by *cydB*, and these genes are transcribed together in an operon (16).

From the sequence of the two subunits of the cytochrome *d* complex, a topological model has been developed (16) (see Fig. 3) and tested with monoclonal antibody studies (10), proteolysis studies (9a, 30), LacZ fusions (13), and PhoA fusions (37b). The antibody binding and proteolysis experiments also suggest that the site of ubiquinol oxidation is near the periplasmic side of the cytoplasmic membrane, consistent with a scalar mechanism of proton translocation (10, 30). In addition, genetic studies have shown that the cytochrome b_{558} moiety is in subunit I (15). One of the axial ligands to cytochrome b_{558} has been identified by site-directed mutagenesis to be His-186 in subunit I (11). In this paper we describe the isolation of a new class of cytochrome *d* mutants obtained by random chemical mutagenesis of the cloned *cyd* operon.

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MATERIALS AND METHODS

Bacterial strains and plasmids. The strains of *E. coli* K-12 and plasmids that were used in this work are listed in Table 1. RG127 was made *cyd*$^+$ *nadA*$^+$ by conjugation (35) with KL208 and called GO100. GO102 was derived from GO100 by the method of Oden et al. (38). Briefly, pKO12 (38) was transformed into JC7623, and the chromosomal wild-type gene was replaced with the *cydAB'* deletion carried on the plasmid by selecting for Km^r Tc^s transformants. The *cyd* deletion was then transduced with P1 (43) into GO100 by selecting for Km^r and the inability to grow aerobically on lactate and succinate. This deletion removes all of *cydA* and the N-terminal half of *cydB* (38).

Chemicals. All chemicals were reagent grade or better.

Media and growth of cells. Strains were grown on LB plates (33) supplemented with 0.3% glucose for anaerobic growth. For aerobic growth, cells were grown on modified LB plates as described previously (3); i.e., the amount of yeast extract (Difco) was lowered to 1 g/liter, and sodium succinate and DL-lactate (Sigma) were both added to 0.3%. This is to avoid background growth due to fermentation as opposed to respiration on nonfermentative carbon sources under aerobic conditions. For liquid cultures, cells were grown aerobically at 37°C in volumes of 150 ml to 1 liter of 1 × M63 minimal medium (43), 0.3% DL-lactate, 0.1% yeast extract, 0.15% Casamino Acids (Difco), 50 μM thiamine hydrochloride (Sigma), 1 μM nicotinic acid (Sigma), 1 mM $MgSO_4$, and 0.1 mM $CaCl_2$ (2). Cells were grown in baffled flasks shaken at 350 rpm and were harvested well into the stationary phase to optimize synthesis of the cytochrome *d* complex. The low oxygen tension resulting from the high cell density causes transcriptional activation of the *cyd* operon (9, 23). Alternatively, cells were grown at 37°C in still cultures in 1- or 2-liter bottles filled to the top and capped as described previously (14). This is referred to as anaerobic growth, although low levels of oxygen are likely, since rigorous precautions against oxygen leakage were not taken.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype	Reference or source
E. coli		
FUN4	F⁻ *cyd-2 cyo-99 recA srl rpsL gal memA401 nadA* Sup⁺	11
GR70N	F⁻ *thi rpsL*	18
GR84N	F⁻ *cyd-2 nadA thi rpsL recA*	17
JC7623	F⁻ *thr-1 leu-6 proA2 his-4 thi-1 argE3 lacY1 galK2 ara-14 xyl-5 mtl-1 tsx-33 rpsL31 supE37 recB21 recC22 sbcB15 sbcC201*	21, 28
RG127	F⁻ *cyd-2 cyo-123 rpsL nadA*	1
KL208	Hfr *relA1*? PO43 of Broda 7	CGSC 4314
GO100	F⁻ *rpsL cyo-123 thi*? *gal*? Δ*lon100*? *relA1*?	This work
GO102	F⁻ *zbg2200::kan* Δ(*cydAB'*)*445 cyo-123 rpsL*	This work
Plasmids		
pNG2	pBR322, *cyd*⁺, Tc^r	17
pACYC184	p15A, Cm^r Tc^r	7
pKO12	pNG2, *zbg2200::kan* Δ(*cydAB'*)*455*, Km^r Tc^r	38
pKO9	pNG2, *cydB9*, Tc^r	This work
pKO17	pNG2, *cydB17*, Tc^r	This work
pKO24	PNG2, *cydB24*, Tc^r	This work

The medium used for anaerobic growth contained 1× M63 minimal medium, 40 mM glucose, 40 mM potassium fumarate (Sigma), 50 μM thiamine-hydrochloride, 1 μM nicotinic acid, 1 mM $MgSO_4$, and 0.1 mM $CaCl_2$. Antibiotics (all from Sigma) were added as needed in the following concentrations: chloramphenicol, 25 μg/ml; tetracycline, 10 μg/ml; and kanamycin, 200 μg/ml.

Mutant isolation. Plasmid pNG2 (17) was mutagenized with hydroxylamine (Mallinckrodt) by the method of Porter et al. (39), except that the incubation in hydroxylamine was done for 24 to 36 h. After dialysis and DNA precipitation (33), the plasmid was transformed into FUN4 (11) and plated onto LB plus 0.3% glucose (LB-glucose) plates containing 20 μg of tetracycline per ml and grown anaerobically in Oxoid chambers at 37°C. After 24 h, colonies were replica plated onto rich agar plates containing 0.3% sodium succinate, 0.3% sodium-DL-lactate, and 20 μg of tetracycline per ml (3). These plates were grown aerobically at 37°C overnight. Mutants were isolated for their inability to confer aerobic growth to FUN4. Approximately 10,000 colonies were screened, and about 30 putative mutants were isolated and purified. Putative mutant plasmids were isolated by using rapid alkaline lysis (33) from anaerobically grown cells. Briefly, cells were grown in 1.5 ml of LB-glucose plus 10 μg of tetracycline per ml for 12 to 24 h before harvest. After isolation, plasmids were resuspended in 20 μl of 10 mM Tris-HCl (Sigma)–1 mM EDTA (Sigma) (pH 8), and 15 μl was used to transform GR84N or GO102.

Transformation of strains. Cells were transformed by using the standard transformation protocol of Hanahan (19) or the one-step method of Chung et al. (8). With the Chung et al. protocol, cells were concentrated 5- to 10-fold before transformation. GO102 was grown in LB-glucose in capped vials flushed with nitrogen. After transformation, this strain was allowed to recover for 1 to 2 h in LB-glucose before it was plated on selective media under anaerobic conditions.

Construction of vectors used in plasmid-plasmid marker rescue. Restriction enzymes were obtained from New England BioLabs or Bethesda Research Laboratories. Agarose and T4 DNA ligase were obtained from Bethesda Research Laboratories. To construct pKO100, pNG2 was digested with *Sph*I and the fragments were separated on a 0.75% agarose gel. The 1.75-kb band was cut out of the gel, and the DNA was electroeluted (Elutrap electo-separation system; Schleicher and Schuell). The DNA was precipitated and ligated with *Sph*I-digested pACYC184 (7). pKO102 was made by digesting pNG2 (17) with *Sph*I and *Nru*I and isolating the 1.2-kb fragment. This fragment was ligated into *Sph*I-*Nru*I-digested pACYC184. Finally, pKO103 was constructed by digesting pNG2 with *Pvu*II and isolating the 1.2-kb fragment. This fragment was then cloned into *Nru*I-digested pACYC184 (Fig. 1).

Mapping plasmid-borne *cyd* mutations. Plasmids unable to confer aerobic growth were transformed into GO102 by anaerobic selection on LB-glucose plus tetracycline. Transformants were purified and then transformed again with each of the pACYC184-derived vectors (pKO100, pKO102, and pKO103). Selection for strains harboring both plasmids was done on LB-glucose medium plus tetracycline and chloramphenicol under anaerobic conditions. Strains were purified, streaked onto rich agar plates containing succinate and lactate, and grown aerobically at 37°C for 24 h.

DNA sequencing. Mutant plasmids were isolated by alkaline lysis and CsCl (Beckman) equilibrium density centrifugation (33) and then denatured by the method of Hattori and Sakaki (20). The dideoxy-chain terminating method (42), Sequenase (U.S. Biochemical Corp.), α-^{35}S-dATP (New England Nuclear), and primers to the known *cyd* sequence (University of Illinois Biotechnology Center, Genetic Engineering Facility) were used to sequence the denatured templates as recommended by the manufacturer. The reaction mixtures were analyzed on 6% polyacrylamide (Bio-Rad) gradient gels (4).

Preparation of *E. coli* cell membranes. Cells were grown aerobically or anaerobically in liquid culture as described above. Cells were harvested, washed with 0.1 M potassium phosphate buffer (pH 7), and either used immediately or stored at 70°C. Cells were resuspended in 0.1 M potassium phosphate (pH 7)–2 mM phenylmethylsulfonyl fluoride (Sigma)–0.5 μg of leupeptin (Boehringer-Mannheim) per ml at 2 g (wet weight) of cells in 30 ml of buffer. Cells were broken by one or two passages through a French press (SLM-Aminco) and then centrifuged at 12,000 × *g* for 20 min at 4°C to pellet the membrane fraction. The membranes were then resuspended in 2 to 5 ml of fresh buffer by using a glass homogenizer, and the homogenate was centrifuged at 12,000 × *g* for 10 min at 4°C to remove unbroken cells. The supernatant was then centrifuged at 200,000 × *g* for 1 h at 4°C to pellet the membrane fraction. The protein concentration was determined by the bicinchonic acid (BCA) method (Pierce Chemical).

Visible light spectroscopy. Membranes were prepared as described above and used fresh. Spectra were taken on a computer-driven DW-2 spectrophotometer (SLM-Aminco) modified by On-Line Systems, Inc. The cytochrome *d* complex is oxygenated as isolated (31), and no further oxidant was used. Samples were diluted with buffer as needed and reduced with dithionite. Reduced-minus-oxygenated difference spectra were collected and normalized to identical protein concentrations. The amounts of heme per milligram of protein were calculated by using a molar extinction coefficient of 17.5 mM^{-1} cm^{-1} and $A_{560-580}$ for *b* heme (25)

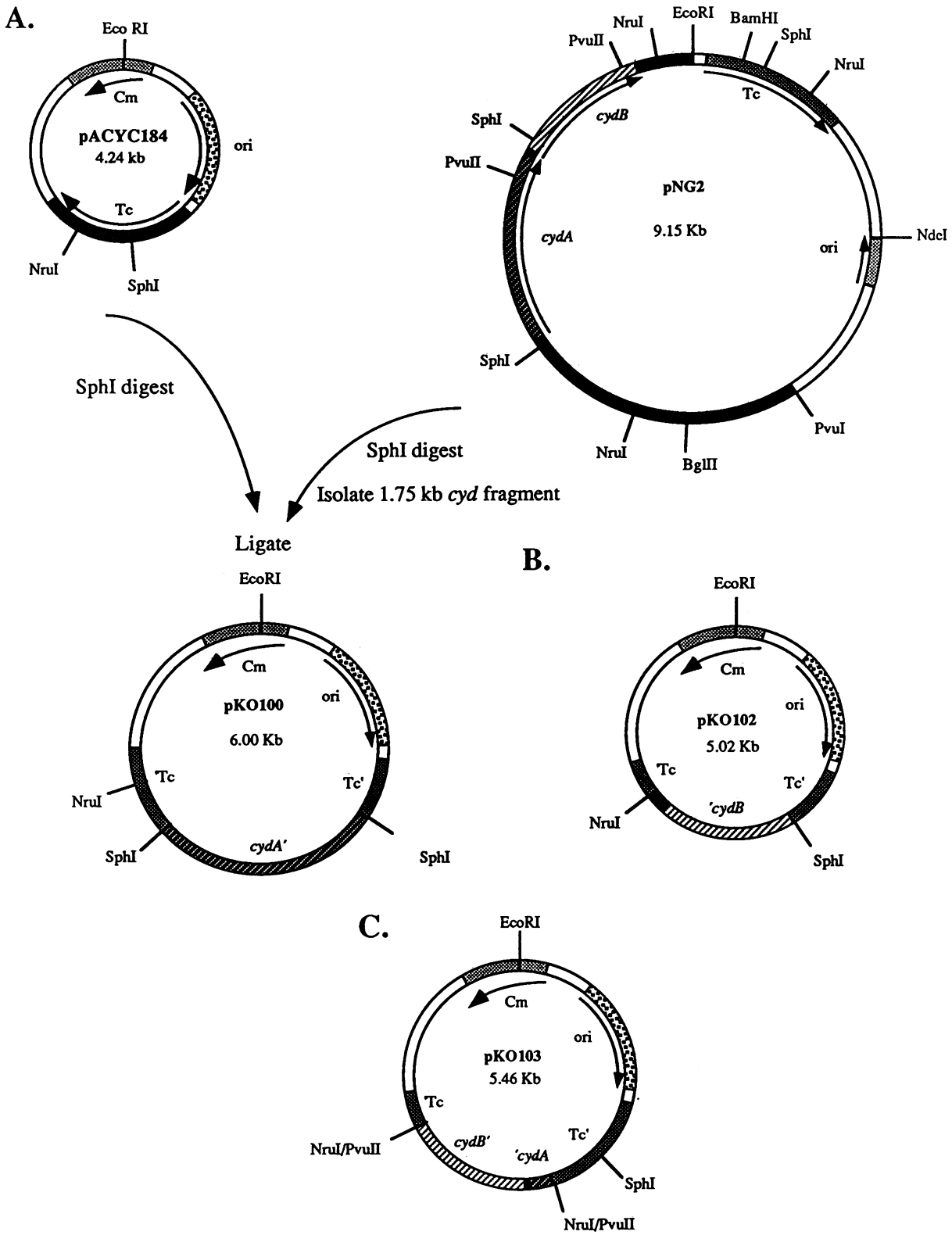


FIG. 1. Construction of vectors for plasmid-plasmid marker rescue. A set of pACYC184-derived plasmids was constructed that contain different portions of the *cyd* structural genes. See Materials and Methods for experimental details. The figure includes the following: chromosomal DNA (■), vector DNA (□), *ori* (▩), antibiotic resistance genes (▩), *cydA* (▨), *cydB* (▨). (A) pKO100; (B) pKO102; (C) pKO103.

and a molar extinction coefficient of 7.4 mM^{-1} cm^{-1} and $A_{628-607}$ for *d* heme (32).

Immunoblotting of *E. coli* membranes. Samples (5 to 40 μg of membrane protein) were separated electrophoretically by a 12.5% sodium dodecyl sulfate–polyacrylamide gel with 1-mm spacers with the Laemmli Tris-glycine discontinuous buffer system (29) and then transferred to nitrocellulose (Bio-Rad) by electrophoresis (Bio-Rad Mini Protean and Transblot systems). Prestained molecular weight markers (low range; Bio-Rad) were used as standards. Western immunoblotting was performed by the method of Burnette (6) with the following modifications. The nitrocellulose sheets were washed with Tris-buffered saline (TBS; 10 mM Tris-HCl, 500 mM NaCl [pH 8.0]) and blocked with 3% gelatin (Bio-Rad) in TBS. Washes were done with TBS–0.05% Tween-20 (Bio-Rad) after every incubation. Primary antibodies were added in TBS–1% gelatin and incubated for 1 h. After the protein bands were washed, they were visualized by incubation with goat anti-rabbit immunoglobulin G conjugated with alkaline phosphatase (Bio-Rad) and assayed for alkaline phosphatase activity as recommended by the manufacturer. The primary antibodies used were strip-purified polyclonal antibodies to subunits I and II and monoclonal antibody A16-1 to subunit I (26). The method used to strip purify the polyclonal antisera to subunits I and II of the cytochrome *d* complex is as follows (5, 41). Approximately 0.5 to 1 mg of pure cytochrome *d* complex was electrophoresed on a 12.5% sodium dodecyl sulfate–polyacrylamide gel with a continuous trough as a well. The gel was blotted to nitrocellulose, and after transfer the protein bands were visualized with 0.2% Ponceau S (Sigma) in 3% (wt/vol) trichloroacetic acid (Mallinckrodt). Bands were marked with a pencil, and the blot was destained with distilled water. The strips containing the protein bands were cut out of the blot and blocked with 3% gelatin in TBS for 1 h, washed twice with TBS, and then placed in 2 to 5 ml of the appropriate diluted antiserum. Strips were incubated for 5 to 60 min, depending on the antibody affinity, and then washed three times with TBS. Antibodies were eluted into 1.5 ml of 0.1 M glycine (pH 2.5), and after 2 to 5 min the solution was neutralized with 1 M Tris base. The strips were washed with TBS and stored in TBS with 0.1% sodium azide at 4°C. Antibody solutions were concentrated to 100 to 200 μl with using Centricon concentrators (Amicon) and stored at −20°C. Samples of 50 to 100 μl of strip-purified antibodies to subunit I and 200 μl of the subunit II antibodies were used per 15-ml volume of TBS–1% gelatin.

RESULTS

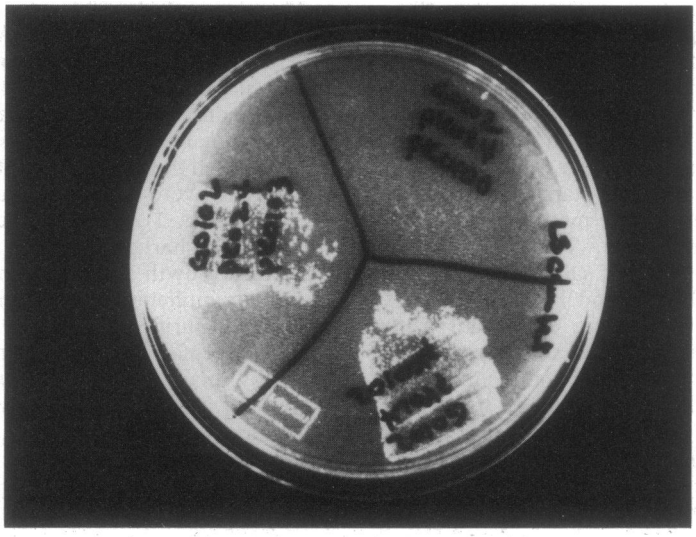
Initial characterization of cytochrome *d* complex mutants. As described in Materials and Methods, approximately 30 plasmid mutants were obtained. Since these mutants were isolated on a multicopy plasmid, overexpression of the defective cytochrome occurred, allowing the mutants to be initially characterized by the color of the cell pellets. Cells containing pNG2, the pBR322-derived plasmid with the cloned *cyd* operon (17), are bright green because of the heme *d* component in the overproduced oxidase. Cells containing pNG10 are red since they only produce subunit I, which is the cytochrome b_{558} component of the oxidase (18). About 40% of the plasmids did not overexpress cytochrome and were not characterized further. Of the remaining plasmids, 7 resulted in a brown cell pellet and 10 resulted in red cells. Two mutants plasmids, pKO17 and pKO24, which confer a brown color to the cell pellet, were selected and characterized. Another plasmid, pKO9, which results in a red cell pellet, was also characterized (Table 1).

Mapping and identifying the mutations in pKO9, pKO17, and pKO24 by marker rescue. All plasmids were transformed into GO102, a *cyo* Δ*cyd* mutant, and were selected under anaerobic growth conditions by using tetracycline. The transformants could not grow aerobically because there was no functional oxidase expressed either from the chromosome or from the plasmid. A set of mapping vectors based on pACYC184 were constructed as described in Materials and Methods (Fig. 1). Each of these vectors was transformed into GO102 harboring pKO9, pKO17, or pKO24 under anaerobic growth conditions in the presence of tetracycline and chloramphenicol. Transformants containing both plasmids were purified and tested on modified LB plates containing succinate and lactate for the ability to grow aerobically by marker rescue between plasmids. The results obtained with pKO24 are shown in Fig. 2. Figure 2A shows that marker rescue occurred with two plasmids, pKO103 and pKO102, indicating that the piece of DNA rescuing the mutation in pKO24 is in *cydB* between the *Sph*I and *Pvu*II sites (Fig. 2B). Any aerobic growth resulting from reversion is insignificant when compared with that obtained from the plasmid-plasmid marker rescue. pKO9 and pKO17 were mapped with the same set of pACYC184-derived vectors. The mutation in pKO17 is also in *cydB* between the *Sph*I and *Pvu*II sites, whereas pKO9 maps between the *Pvu*II site in *cydA* and the *Sph*I site in *cydB* (Fig. 2B).

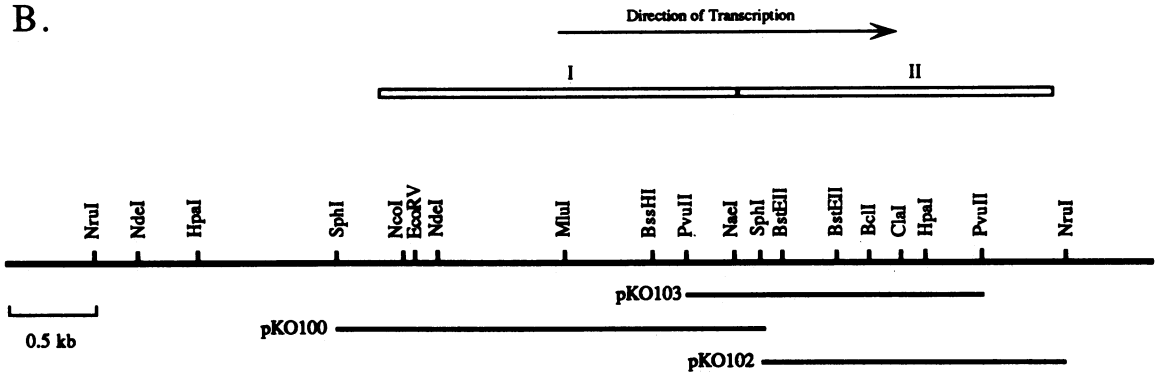
Double-stranded sequencing of CsCl-purified plasmids identified the mutations lying within the regions defined by the marker rescue experiment. The locations of these mutations in a topological model of cytochrome *d* complex subunits are shown in Fig. 3. pKO9 contains a C-to-T transition in the noncoding strand at bp 1682 in *cyd* (16). This results in a change from GGG (glycine) to GAG (glutamic acid) at amino acid 31 in subunit II. The mutation in pKO17 is another C-to-T transition at bp 1796 in the noncoding strand. This changes GGC (glycine) to GAC (aspartic acid) at amino acid 69 in subunit II of the cytochrome *d* complex. The mutation in pKO24 is also a glycine-to-aspartic acid change, whereby GGT is changed to GAT by another C-to-T transition at bp 1967 in the noncoding strand. These mutations are now identified as *cydB9*, *cydB17*, and *cydB24*.

Characterization of overexpressed *cydB9*, *cydB17*, and *cydB24* under different growth conditions. During the course of the experiments described above, it was noted that the color of the cell pellet changed depending on the growth conditions used. To rule out host differences, the mutant plasmids were grown aerobically and anaerobically in the same host, GR84N. This strain can be grown aerobically by utilizing the cytochrome *o* complex encoded by the *cyo* gene in the chromosome. Figure 4 shows the reduced-minus-oxygenated spectra of membranes isolated from strains grown aerobically on lactate and normalized to 1 mg of protein per ml. It is clear that the spectroscopic features are dominated by the plasmid-encoded cytochromes. Table 2 shows the amounts of cytochromes *b* and *d* associated with strains carrying plasmids with mutant *cyd* genes. From the data in Table 2, it is apparent that GR84N/pKO24 membranes contain *d* heme, but only ~40% of the amount contained in GR84N/pNG2 wild-type control membranes. The amount of *b* heme in the membranes of this strain is about the same as that in the strain that overproduces the wild-type oxidase. GR84N/pKO17 contains about 70% of the cytochrome *b* and 10% of the cytochrome *d* when compared with the amounts in the wild-type control, and GR84N/pKO9

A.



B.



Plasmid to test	Rescue by: pKO100	pKO102	pKO103
pKO9	+	--	+
pKO17	--	+	+
pKO24	--	+	+

FIG. 2. Marker rescue of plasmid-borne mutants of *cyd*. (A) GO102/pKO24 transformed with pKO100, pKO102, and pKO103, shown clockwise in the photograph. Double transformants were streaked on rich agar plates with 0.3% DL-lactate and 0.3% sodium succinate plus tetracycline and chloramphenicol. (B) Summary of mapping results on pKO9, pKO17, and pKO24 with the vectors shown in Fig. 1. The heavy line represents the portion of chromosomal DNA containing the *cyd* operon. Boxes representing *cydA* (subunit I) and *cydB* (subunit II) are shown above the restriction map.

has only about 30% of the cytochrome *b* normally associated with the overexpressed wild-type cytochrome *d* complex. This mutant also has no cytochrome b_{595} or *d*. Western blots show that both subunits are present in the membranes of the mutants, except for pKO9, in which subunit II is missing (Fig. 5). The cytoplasm was not examined spectroscopically or immunologically for the presence of any components of the cytochrome *d* complex that might not be incorporated in the membrane.

Under anaerobic growth conditions, more cytochrome *d* is

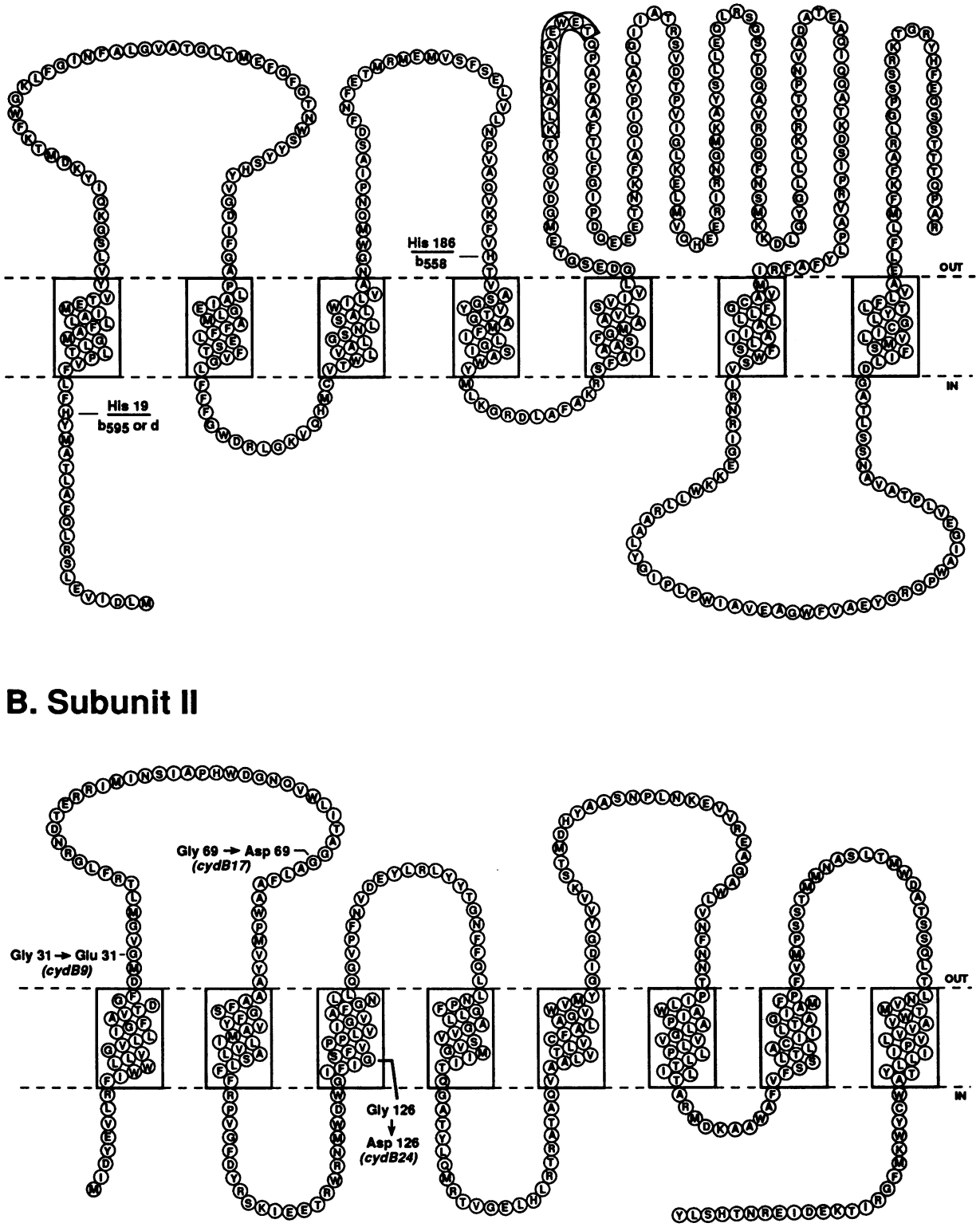


FIG. 3. Topological models of the two subunits of the cytochrome *d* complex, modified from the data of Green et al. (16). The three mutations identified in this work are shown in subunit II. Previous work has implicated His-19 in subunit I as a potential axial ligand of cytochrome b_{595} or *d*; the same components are perturbed by the three mutations in subunit II (11). Also shown are His-186 in subunit I, an axial ligand of cytochrome b_{558}, and the binding site (boxed residues) for monoclonal antibodies that specifically inhibit ubiquinol oxidase activity (10).

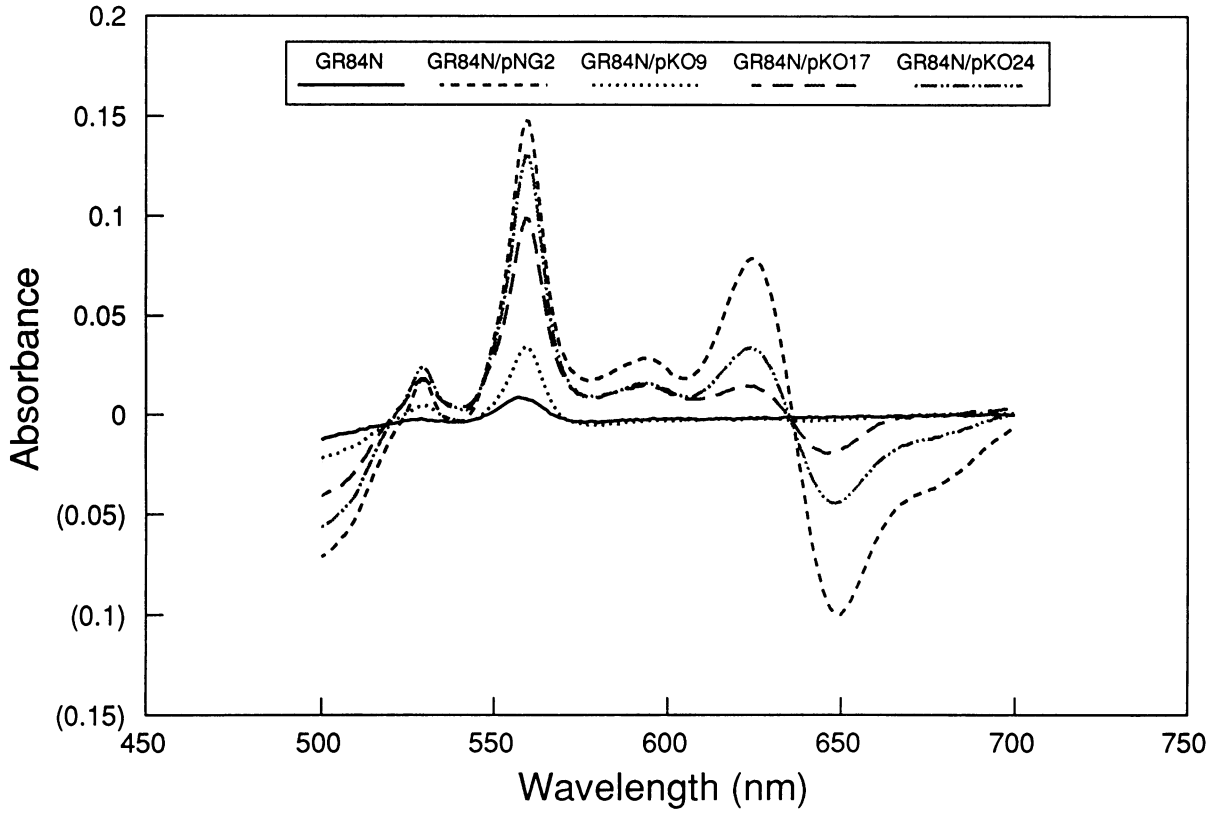


FIG. 4. Reduced-minus-oxygenated spectra of membranes containing wild-type and mutant cytochrome *d* terminal oxidases. Membranes were isolated as described in Materials and Methods from cultures grown aerobically with lactate as a carbon source. All spectra were normalized to a protein concentration of 1 mg/ml.

present relative to cytochrome *b* in all of the mutants (Fig. 6 and Table 2). Especially noteworthy is GR84N/pKO9, in which there are detectable amounts of cytochromes b_{595} and *d* when the cells are grown anaerobically. As can be seen in Fig. 7, this correlates with the presence of subunit II, which is not observed in this strain when it is grown aerobically (Fig. 5). In addition, membranes from GR84N/pKO17 cells grown anaerobically have an aberrant chlorin peak that is blue shifted 2 to 3 nm from the wild-type peak (627 versus 630 nm). From the Western blots (Fig. 5 and 7), it appears that little proteolysis occurred during the sample preparation. Therefore, in vitro proteolysis does not appear to explain the increased content of cytochrome *d* in the strains expressing pKO9 or pKO17 (Table 2). Note that in all cases, the mutant oxidases do not support aerobic growth when expressed in a *cyo* Δ*cyd* strain (GO102). Membranes of

TABLE 2. Cytochrome content of membranes from strains expressing wild-type and mutant *cyd* alleles

Growth and strain	Cytochrome *b*		Cytochrome *d*		Cytochrome *b*/ cytochrome *d* ratio
	nmol/mg	%	nmol/g	%	
Aerobic growth					
GR84N	0.7	9		0	
GR84N/pNG2	7.5	100	8.2	100	0.9
GR84N/pKO9	2.3	31		0	
GR84N/pKO17	5.2	69	0.9	11	5.7
GR84N/pKO24	6.9	92	3.4	41	2.1
Anaerobic growth					
GR84N	0.4	10	0	0	0.8
GR84N/pNG2	3.9	100	4.8	100	0.8
GR84N/pKO9	2.7	69	0.9	19	3.2
GR84N/pKO17	4.4	113	3.8	79	1.2
GR84N/pKO24	3.8	97	3.4	71	1.1

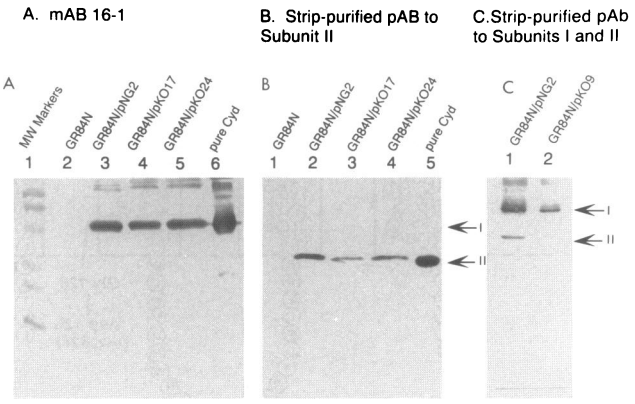


FIG. 5. Western blots of membranes from strains grown aerobically. Protein amounts are as follows: pure cytochrome *d* oxidase, 2.5 μg; GR84N membranes, 20 μg; all others, 5 μg. Blots were probed with the following antibodies: (A) Monoclonal antibody 16-1 to subunit I (27); (B) strip-purified polyclonal antibodies to subunit II; (C) mixture of strip-purified polyclonal antibodies to subunits I and II. The arrows indicate subunits I and II.

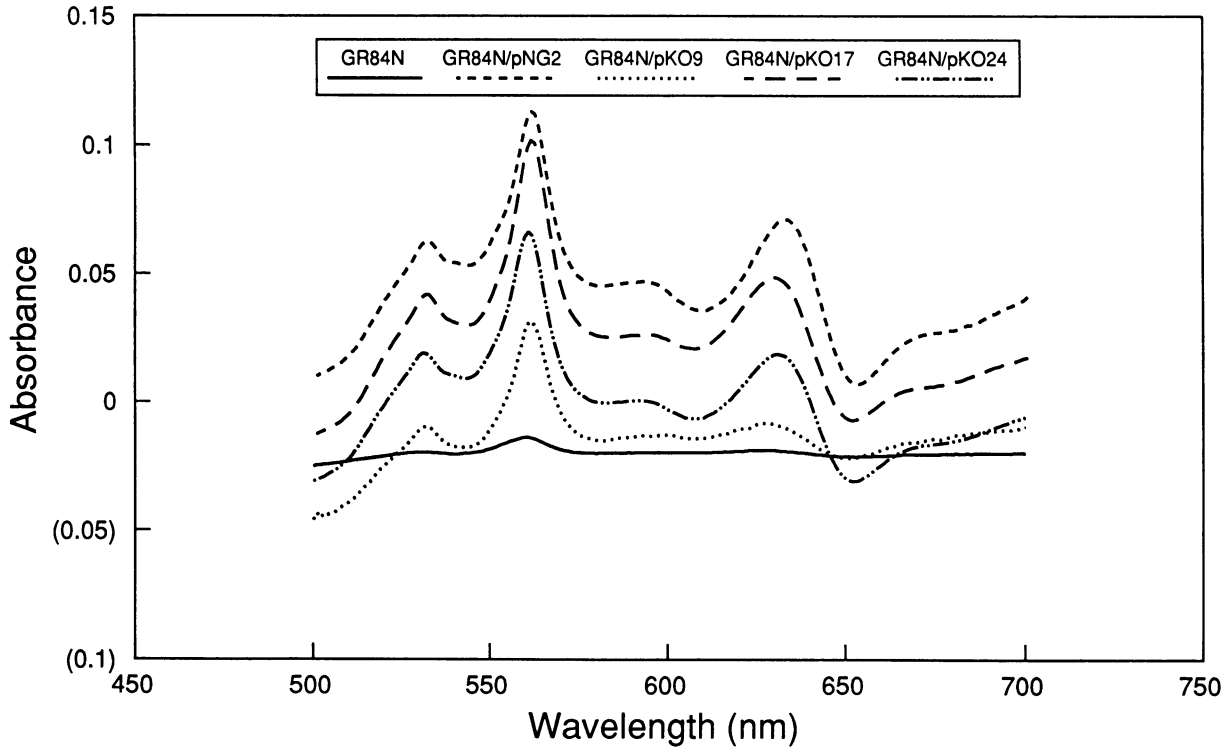


FIG. 6. Reduced-minus-oxygenated spectra of membranes containing wild-type and mutant cytochrome *d* terminal oxidases from cells grown anaerobically in the presence of fumarate and glucose. All spectra were normalized to a protein concentration of 1 mg/ml.

anaerobically grown derivatives of GO102 expressing the mutant *cyd* alleles were also examined for ubiquinol-1-oxidase activity. Surprisingly, the membranes of GO102/pKO24 exhibited substantial quinol oxidase activity, comparable to that of a control strain expressing the single-copy chromosomal *cyd* allele. It would appear, therefore, that the

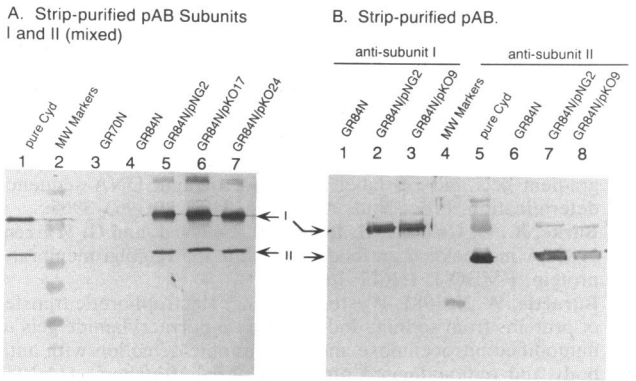


FIG. 7. Western blot of membranes from strains grown anaerobically. (A) Blot probed with strip-purified polyclonal antibodies to subunit I and subunit II. Protein amounts are as follows: pure cytochrome *d* oxidase 100 ng; GR84N membranes, 20 μg; all others, 5 μg. (B) Lanes: 1 through 3, probed with strip-purified polyclonal antibodies to subunit I; 4 through 8, probed with strip-purified polyclonal antibodies to subunit II. Protein amounts were 2.5 μg for pure cytochrome *d* oxidase and 40 μg for the rest. The polyclonal antibodies to subunit II cross-react weakly with subunit I in lane 5, serving as a standard for subunit I. Arrows indicate subunits I and II.

cydB24 allele results in an enzyme that is inactive under aerobic growth conditions but has partial activity under anaerobic growth conditions.

DISCUSSION

The cytochrome *d* complex is a heterodimer containing three cytochrome components: b_{558}, b_{595}, and *d*. Previous genetics studies have been valuable in providing structural information about the enzyme. Mutants of *E. coli* have been characterized in which the subunit II of the oxidase is totally lacking but the cytochrome b_{558} component is present in the membrane (18). Cytochrome b_{558} is clearly located within subunit I (15). Site-directed mutagenesis studies have implicated His-186 in subunit I as one of the axial ligands of cytochrome b_{558} (Fig. 3) (11). Substitution of this histidine results in the specific loss of cytochrome b_{558} from the membrane-bound oxidase, but both cytochromes b_{595} and *d* are still present (11). In contrast, substitutions of His-19 in subunit I result in the loss of both cytochromes b_{595} and *d*, but cytochrome b_{558} is unaffected (11). It is likely that cytochromes b_{595} and *d*, both 5-coordinate cytochromes (34), are physically close together and that His-19 is either an axial ligand to one of these cytochromes or important for maintaining the conformation required for stable binding of both hemes.

The current effort was motivated by the expectation that random mutagenesis might reveal additional amino acid residues essential either for heme binding or for the interaction with ubiquinol. Such mutants would lack enzymatic activity but might retain selective or full heme binding. The first set of mutations characterized in this work affect the binding of hemes associated with cytochromes b_{595} and *d*.

All three mutants that were examined in detail contain missense mutations in which glycines are replaced by either glutamate or aspartate. All three mutations are located within the N-terminal third of subunit II, and in all three cases the mutant oxidase is unable to support aerobic growth on nonfermentable substrates. All three mutations result in altered spectroscopic properties of the oxidase, and therefore the mutants are not simply defective in ubiquinol binding.

The simple spectroscopic analysis of the membranes containing the oxidase mutants is useful because the plasmid-encoded cytochrome *d* complex variants dominate the optical properties of these membranes (Fig. 4 and 6). One surprising result is that the heme binding of the mutant oxidases and, in one case, the subunit stability are clearly dependent on growth conditions. This is most dramatically illustrated by mutant *cydB9* (Gly-31→Glu). In this case, subunit II is not apparent in a Western blot when membranes from aerobically grown cells are examined (Fig. 5) but is present when the strain is grown anaerobically (Fig. 7). Also, the amount of subunit I produced under aerobic conditions is about 60% of that produced from the wild-type gene as estimated from densitometry (data not shown). This suggests that the overall stability of the protein is also affected. However, under anaerobic conditions, the amount of subunit I produced appears to be comparable for both the wild type and the *cydB9* mutant. The amount of cytochrome *b* associated with the *cydB9* mutation also increases under anaerobic growth conditions but is still less than that associated with the wild-type protein. The amounts of cytochrome *b* associated with the mutant proteins produced by the *cydB17* and *cydB24* alleles are at wild-type levels when cells are grown under anaerobic conditions. Also, the relative amounts of cytochrome *d* are enhanced for all three mutants when the cells are grown anaerobically. Both *cydB17* (Gly-69→Asp) and *cydB24* (Gly-126→Asp) have close to the same heme content as the control, which overproduces the wild-type *cyd* allele (Table 2). In the case of *cydB17*, however, the spectrum of the cytochrome *d* component is clearly perturbed (Fig. 6).

Two conclusions are warranted from these data. (i) All three mutations affect the heme binding properties of the oxidase, but none of the mutations represents a substitution of an axial ligand. Hence, the glycine substitutions in subunit II influence the local and/or global conformation of the enzyme. (ii) When expressed under anaerobic conditions, *cydB17* and *cydB24* have nearly normal levels of both cytochromes *b* and *d*. Hence, Gly-69 and Gly-126 in subunit II cannot be essential for heme binding. However, the change of Gly-69 to Asp (*cydB17*) causes a perturbed spectrum of the cytochrome *d* component, and both mutants have reduced levels of cytochrome *d* when the cells are grown aerobically. It is likely, therefore, that the lack of enzymatic activity of these mutants is due to perturbations in the heme binding domain for cytochrome *d*, where oxygen is reduced to water. Presumably, cytochrome b_{595}, which is difficult to quantify, is near cytochrome *d*.

Taken with other data from this laboratory showing that substitutions for His-19 in subunit I also cause selective loss of cytochrome *d* and cytochrome b_{595} (11), it is evident that these two hemes reside in a locus that is sensitive to alterations within either of the two subunits. One reasonable hypothesis is that these hemes are located at or close to the subunit interface. This would also provide a rationale for how amino acid substitutions that are probably on opposite sides of the membrane (Fig. 3) result in perturbations at the presumed single cytochrome *d*-cytochrome b_{595} locus, which is probably located near the cytoplasmic surface of the bilayer (30). This is also consistent with the observation that both cytochromes b_{595} and *d* require the presence of both subunits (37a). In contrast, cytochrome b_{558} requires only subunit I to be bound stably (15).

If the cytochrome *d*-cytochrome b_{595} locus is located at the subunit interface, one would expect many mutations to cause perturbations in this locus due to conformational changes resulting from changes in the subunit interactions (Fig. 3). The fact that all three mutations characterized in this work are grouped in the first third of subunit II suggests that this portion is somehow critical to subunit interactions. Substitutions for glycines could certainly create packing problems if these residues were located at the interface.

In summary, the three missense mutations isolated by random mutagenesis point to the N-terminal portion of subunit II as being of particular importance in maintaining an active conformation of the cytochrome *d* complex. The His-19 mutations (11) in subunit I also appear to be implicated in maintaining this active conformation. The predominant effect of these substitutions appears to be at the presumed cytochrome *d*-cytochrome b_{595} locus. The most attractive hypothesis is that this heme binding domain is at the subunit interface and that these mutations perturb the subunit interaction. The extent of b_{595} and *d* heme binding observed in *cydB17* and *cydB24* suggests that the subunits must still form a complex, albeit an altered complex. This may not be the case for *cydB9*.

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