Methionine-393 Is an Axial Ligand of the Heme b_{558} Component of the Cytochrome *bd* Ubiquinol Oxidase from *Escherichia coli*[†]

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ABSTRACT: The cytochrome *bd* oxidase is one of two terminal oxidases in the aerobic respiratory chain of *Escherichia coli*. The complex is composed of two subunits (I and II) and three heme prosthetic groups (heme b_{558}, heme b_{595}, and a chlorin, called heme *d*). Both subunits are located within the bacterial cytoplasmic membrane, and each has multiple putative transmembrane helices. Heme b_{558} is a six-coordinate, low-spin heme component of the oxidase which has been shown to be contained within subunit I and has been implicated in the oxidation of the substrate, ubiquinol-8, in the cytoplasmic membrane. Previous site-directed mutagenesis studies identified His186, predicted to be near the periplasmic side of transmembrane helix D of subunit I, as one of the axial ligands of heme b_{558}. Since mutagenesis of none of the other histidines in subunit I perturbs heme b_{558}, it was concluded that this heme cannot have bis(histidine) ligation. In this work, the properties of 14 mutants are reported, including substitutions for each of 10 methionine residues within subunit I. Among this set of mutants, only the replacement of M393 perturbs heme b_{558}. Replacement of M393 by leucine results in the conversion of heme b_{558} to a high-spin state. Surprisingly, the M393L mutation does not eliminate enzymatic activity, and the mutant oxidase has sufficient turnover to support aerobic growth of the cells. The addition of imidazole to the purified M393L oxidase converts heme b_{558} back to a low-spin configuration. The data strongly suggest that the sixth axial ligand of heme b_{558} is methionine-393, and that this heme, therefore, has histidine−methionine ligation. The results are consistent with recent cryogenic near-infrared magnetic circular dichroism spectra that also indicate histidine−methionine ligation of heme b_{558}.

The cytochrome *bd* oxidase complex is one of two terminal oxidases in the aerobic respiratory chain of *Escherichia coli* (Anraku & Gennis, 1987). The cytochrome bo_3 complex predominates at high oxygen tension in the growth medium, such as in the early log phase of growth (Anraku, 1988), whereas cytochrome *bd* is the major oxidase at low oxygen tension, such as in the late log or early stationary phases of growth (Anraku & Gennis, 1987; Ingledew & Poole, 1984). The cytochrome *bd* complex catalyzes the two-electron oxidation of ubiquinol-8 in the cytoplasmic membrane, with the concomitant four-electron reduction of molecular oxygen to water (Anraku, 1988; Anraku & Gennis, 1987; Trumpower & Gennis, 1994). Cytochrome *bd* is composed of two subunits, subunit I (M_r 56K) and subunit II (M_r 43K), encoded by the genes of the *cyd* operon, *cydA* and *cydB*, respectively (Green et al., 1984a; Miller et al., 1988). The genes have been cloned and sequenced (Green et al., 1984a, 1988), and show strong homology to the more recently isolated corresponding genes from *Azotobacter vinelandii* (Kelly et al., 1990; Moshiri et al., 1991). There is also strong homology of the *E. coli* genes *appC* and *appB* with *cydA* and *cydB*, respectively (Dassa et al., 1991). These genes comprise an operon in *E. coli* of unknown function. Possibly the *appC/appB* gene product plays a role in oxygen detoxi-

fication (Dassa et al., 1991). The spectroscopic properties of the *A. vinelandii* cytochrome *bd* complex (D'mello et al., 1994; Kolonay et al., 1994; Moshiri et al., 1991) are very similar to those of the *E. coli* complex.

The *E. coli* cytochrome *bd* quinol oxidase contains three heme prosthetic groups: heme b_{558}, heme b_{595}, and heme *d*, which is a chlorin (Miller & Gennis, 1983). EPR spectroscopy has been used to characterize all three hemes. Heme b_{558} is a low-spin heme and, therefore, must have two strong endogenous axial ligands. Heme b_{595} is a high-spin heme center (Hata et al., 1985; Hata-Tanaka et al., 1987; Meinhardt et al., 1989; Rothery & Ingledew, 1989). Heme *d* is a chlorin (Sotiriou & Chang, 1988; Timkovich et al., 1985; Vavra et al., 1986) and has also been shown to be in a high-spin state (Meinhardt et al., 1989; Muller et al., 1988). A small percentage of this heme *d* is also found in a low-spin form; however, this has been proposed to be an artifact, possibly caused by freeze/thaw treatment. Heme *d* is the site of O_2 binding and reduction, and is also the site of CO binding (Kahlow et al., 1993; Lorence & Gennis, 1989; Poole, 1983; Poole et al., 1983). Recent evidence suggests the heme *d* and heme b_{595} must be very near each other within the protein, and may form a functional bimetallic center (D'mello et al., 1994; Hill et al., 1993).

Each of the genes encoding the subunits of cytochrome *bd* has been independently expressed in *E. coli* (Green, 1986; Newton & Gennis, 1991). CydA (subunit I) is clearly associated with heme b_{558}. This subunit has been biochemically purified and shown to contain heme b_{558} with electrochemical and spectroscopic properties essentially the same
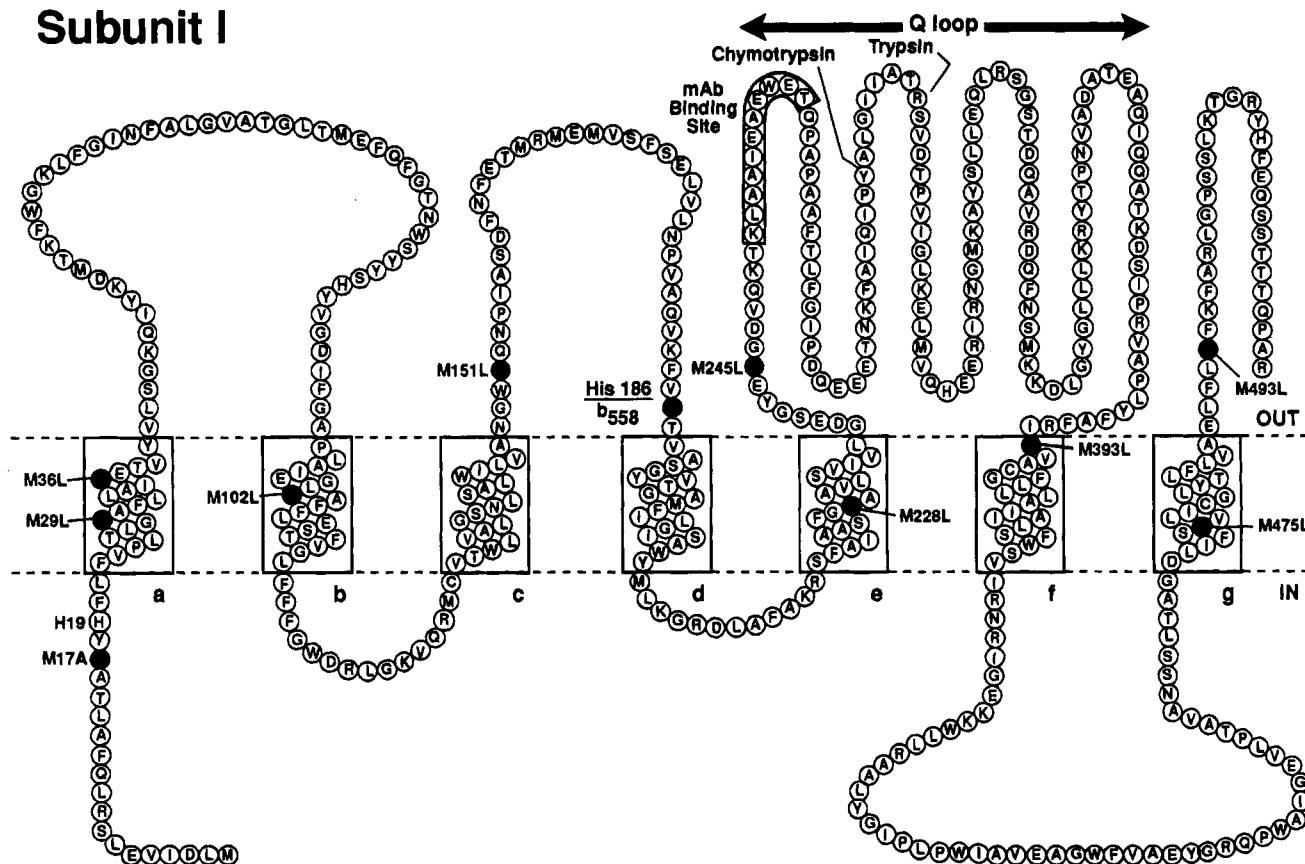
FIGURE 1: Proposed two-dimensional model of subunit I of the cytochrome d complex in the cytoplasmic membrane of *E. coli*. The 10 methionines mutagenized in this work, which are within the proposed bilayer or close to the bilayer, particularly on the periplasmic side, are shown. H186, proposed to be the fifth axial ligand to cytochrome b_{558} (Fang et al., 1989), is also shown . The Q-loop defines the periplasmic loop proposed to be important in ubiquinol oxidase activity. The binding of monoclonal antibodies to the boxed site within the Q-loop inhibits ubiquinol oxidase activity of the enzyme (Dueweke & Gennis, 1990).

as in the intact complex (Green, 1986; Green et al., 1984a,b). In contrast, CydB (subunit II) on its own is not associated with heme. The two high-spin heme components, which appear to exist within a common binding pocket in the enzyme, require both subunits for stable binding and may be located at the subunit interface.

In a previous effort to identify the amino acids ligated to each of the 3 heme components of the oxidase, all 10 of the histidines in the complex were altered by site-directed mutagenesis (Fang et al., 1989). Only two of the histidines were found to be likely heme ligands, H19 and H186, both in subunit I. Alteration of H19 in subunit I to leucine or arginine generates a complex that is lacking both of the high-spin components, heme b_{595} and heme d, as evidenced by optical spectroscopy. Subsequent ENDOR spectroscopy has indicated that the proximal ligand of heme d is probably not nitrogenous, thus ruling out histidine as a likely candidate for the heme d axial ligand (Jiang et al., 1993). Hence, H19 is important for the stable binding of the two high-spin hemes in the putative bimetallic center in the oxidase and could be the axial ligand of heme b_{595}.

Replacement of H186 by leucine selectively eliminates heme b_{558}, leaving intact the heme d—heme b_{595} bimetallic center. It was concluded that H186 is a likely ligand of heme b_{558}. Figure 1 shows a two-dimensional schematic of subunit I (Newton et al., 1991), indicating the seven putative transmembrane spans and H186 located near the periplasmic side of helix D. The location of heme b_{558} near the periplasmic surface has been indicated by spectroscopic

studies (Rothery & Ingledew, 1988), and is also consistent with the role of heme b_{558} in the oxidation of ubiquinol. The current functional model of the oxidase is that quinol oxidation results in the release of the two substrate protons to the periplasmic space, and the integrity of the interhelical connection between helices E and F is critical for quinol oxidation. This interhelical region, called the Q-loop, has been clearly shown to be on the periplasmic side of the membrane and may be directly involved in quinol binding (Dueweke & Gennis, 1990, 1991). Presumably, heme b_{558} is at or in the immediate vicinity of the quinol binding site near the periplasmic surface of the enzyme.

The purpose of the research presented here is to determine the second axial ligand of heme b_{558} . The low-spin character of this heme indicates a second strong axial ligand which, based on the mutagenesis studies, cannot be histidine. Recent cryogenic near-infrared magnetic circular dichroism studies (Spinner et al., 1995) have indicated that heme b_{558} is likely to have histidine—methionine ligation. Consequently, methionines in subunit I that are modeled to be either within or near the bilayer, particularly on the periplasmic side of the membrane, were targeted for site-directed mutagenesis. Four of these methionines, M36, M151, M393, and M493, are conserved in the three sequences of cytochrome bd and its homologues. In addition, four other residues were altered by site-directed mutagenesis (N153T, K505L, H86L, and H314A), including two new alterations of histidines (H86L and H314A) designed to verify the previous study. N153 and K505 were considered as possible

Table 1: Strains of *E. coli* Used in This Work

strains	relevant genotype	source/reference
RG129	*cyo*123, *cydA*2, *srl* −300::Tn*10*, *recA*	Au & Gennis (1987)
JC7623	Δ*sbcA, sbcBC, recB*	Horii & Clark (1973)
GK101	Δ*cydAB::cam* , Δ*sbcA, sbcBC, recBC*	this work; isogenic to JC7623
GO104	Δ*cyo::kan*	Calhoun et al. (1993b)
GO102	F-, *cyo*123, *zbg*-2200::*kan*, Δ(*cydAB′*)445	Oden & Gennis (1991); Calhoun et al. (1993b)
GK100	Δ*cydAB::cam*,Δ*cyo::*kan	this work
GO105	Δ(*cydAB′*)455, *zbg* − 2200::Km^R *cyo,recA,srl*-300::Tn10	Calhoun et al. (1993a)
Jmr⁻	*mcrA*, F′ [*lacI*^{q}Δ(*lacZ*)M15, *proAB*$^+$]	Vandeyar et al. (1988)
HB101	F⁻, *recA*13, *lacY*1	Lacks & Greenberg (1977)
NM522	*supE*, Δ(*lac -proAB*), F′ [*lacI*^{q}Δ(*lac Z*)M15,*proAB*$^+$]	Gough & Murray (1983)
TG1	Δ(*lac -proAB*), F′[*traD*36, *lac*^{q}Δ(*lacZ*)M15,*proAB*$^+$]	Amersham
GR84N	F-, *nadA*50, *cydA*-2, *recA*	Green et al. (1984a)

alternate amine ligands, since the corresponding residues present in the homologous proteins are potential heme axial ligands, histidine or tyrosine.

All of these mutants are functional in a genetic complementation assay, conferring the ability to grow aerobically on nonfermentable substrates to a strain lacking the chromosome-encoded oxidases. However, optical spectroscopy shows that in one mutant, M393L, heme b_{558} is in a high-spin state, strongly suggesting that methionine-393 is the second axial ligand of heme b_{558}. This is consistent with the near-infrared magnetic circular dichroism results. In the current model of subunit I (Newton et al., 1991), M393 is located at the periplasmic boundary of transmembrane helix F, which is in agreement with the periplasmic location of heme b_{558} and with the presumed proximity of heme b_{558} to the Q-loop connecting helices E and F. It is concluded that heme b_{558} is ligated to H186 and M393 and is sandwiched between helices D and F near the periplasmic side of the membrane.

MATERIALS AND METHODS

Materials. The restriction enzymes *Eco*RI, *Hind*III, and *Msp*I were purchased from Bethesda Research Laboratories. *Hha*I, T4 DNA ligase, and T4 DNA kinase were purchased from New England Biolabs. T4 DNA polymerase, exonuclease III, and Sequenase kits were obtained from U.S. Biochemical Corp. The nucleotides dATP, dGTP, dTTP, and 5-Me-dCTP, used in the mutagenesis procedures, were purchased from Pharmacia LKB Biotechnology Inc. ATP and phenylmethanesulfonyl fluoride were purchased from Sigma. SeaPlaque low melting point agarose was obtained from FMC Bioproducts. Geneclean kits were purchased from Bio101. All oligonucleotides used to sequence or to generate mutations were produced by the Biotechnology Center at the University of Illinois, Urbana, IL. 5-Bromo-4-chloro-3-indolyl-β-D-galactoside and isopropyl-β-D-thiogalactopyranoside were obtained from Sigma. Alkaline phosphatase conjugated goat anti-rabbit IgG was purchased from Bio-Rad. Leupeptin was obtained from Boehringer Mannheim. [α-^{35}S]dATP was acquired from New England Nuclear.

Bacterial Strains. Bacterial strains used in this research are described in Table 1. GK100 and GK101 were constructed in this work as described below. Strain GO105 is a partial deletion of the *cyd* operon with a corresponding insertion of a kanamycin-resistance cartridge (Calhoun et al., 1993b; Oden & Gennis, 1991). This strain contains an undefined mutation in the *cyo* operon preventing production of cytochrome *bo₃*. The *recA* mutation in GO105 is required

to prevent homologous recombination of plasmid-borne *cydAB* with the chromosomal *appCB* genes (see below). This strain allows expression of a plasmid-encoded cytochrome *bd* complex in the absence of chromosomal expression of both the wild-type cytochrome *bd* and cytochrome *bo₃* complexes. Therefore, GO105 was used for characterizing the plasmid-encoded cytochrome *bd* oxidase complexes carrying point mutations.

If the plasmid-borne oxidase is not functional, GO105 carrying the plasmid will be unable to grow aerobically on nonfermentable substrates. These mutants can be grown anaerobically, however, and therefore the spectroscopic properties of the mutant oxidase in cell membranes can be analyzed without interference from the cytochrome *bo₃* complex and background wild-type cytochrome *bd* complex.

Plasmids and Phage. Several of the mutations (M228L, M102L, and M393L) were generated using single-stranded M13 phage containing *cydA* as the template (M13mp18spsp; Figure 2). This phage was constructed previously (Fang et al., 1989) and contains the subunit I coding region subcloned into *Sph*I-digested M13mp18. To express the mutant complex for analysis, the *cydA* fragment must be moved back into the entire operon in the plasmid pFH101 by *Sph*I digestion. Ligation products must then be analyzed by restriction digestion to determine those ligated in the correct orientation for expression. To facilitate the subcloning of subunit I, two unique sites were engineered into pFH101: a *Hind*III site before the start of *cydA* and a *Sal*I site between *cydA* and *cydB* (Figure 2). *cydA* was then subcloned into the phagemid pT7T319u using the unique *Hind*III and *Sal*I sites generated. The resultant phagemid (pZGI) and plasmid (pTKI, a pFH101 derivative) were used for the generation of the remaining mutants. The alterations imposed to generate the restriction sites were shown not to alter expression of the oxidase. These sites also allow the convenient subcloning of *cydB* by digestion with *Sal*I and *Eco*RI for mutagenesis of subunit II (pZH1).

Production of single-stranded phagemid for use as template in mutagenesis was as described by the manufacturer (Pharmacia), using the helper phage M13KO7.

Mutagenesis. Two oligonucleotide-directed mutagenesis procedures were utilized for these studies. A few mutations were generated using the *in vitro* mutagenesis system of Amersham Corp. according to the given protocol. The majority of the mutations were generated using the protocol of Vandeyar (Vandeyar et al., 1988). Strains Jmr⁻, TG1, HB101 and NM522 were used in these procedures (see Table 1). Oligonucleotides designed for this method which contained either an *Msp*I or an *Hha*I site within the sequence
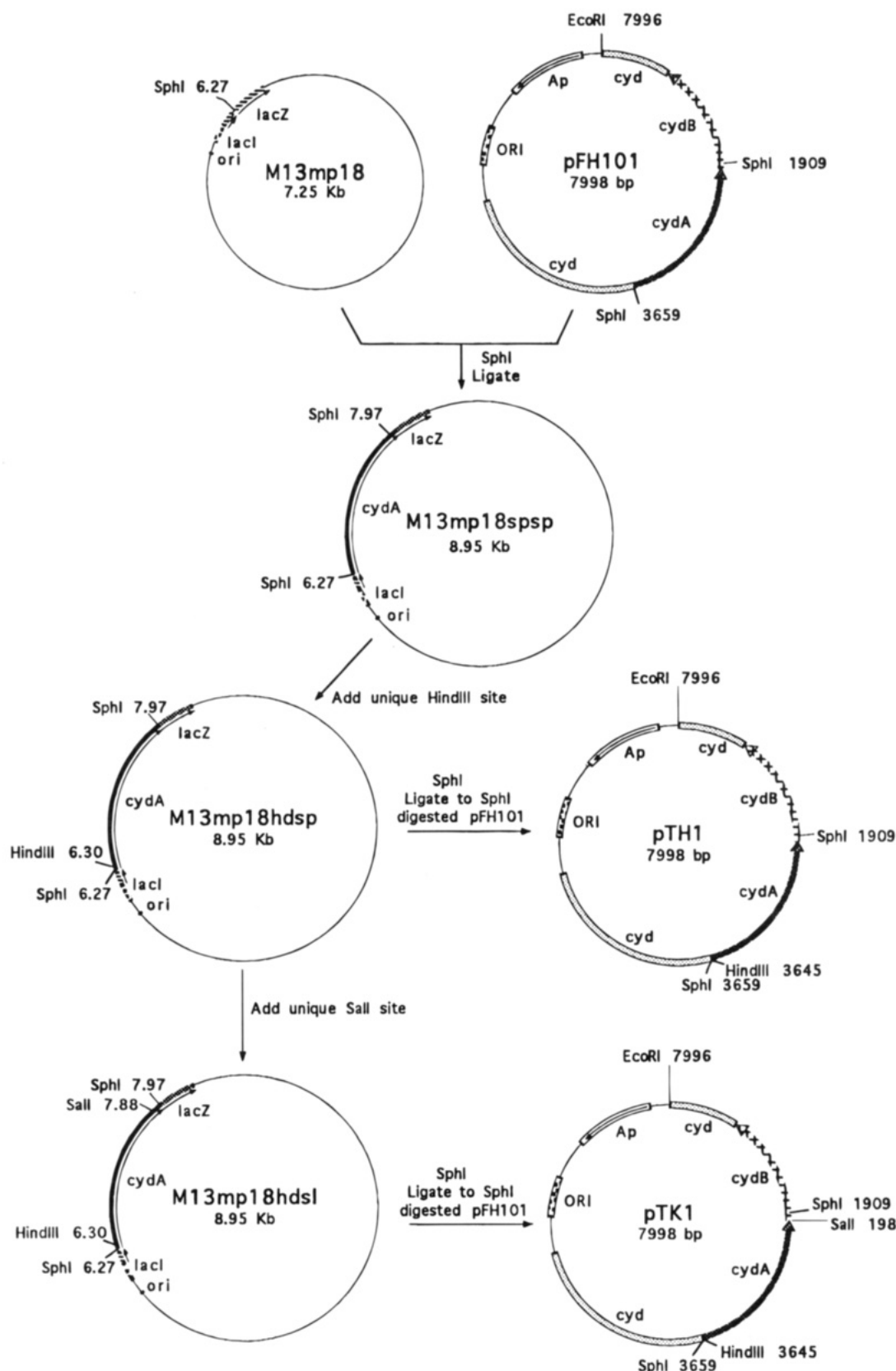
FIGURE 2: Schematic of the construction of plasmids and phagemids used in the mutagenesis and expression studies in this work. The unique restriction sites *Hin*dIII and *Sal*I were introduced into M13mp18spsp and pFH101 by site-directed mutagenesis for efficient subcloning of *cyd*A between mutagenesis and expression vectors.

were synthesized methylated at the appropriate deoxycytidine triphosphate residues to avoid restriction within the primer. Mutations were detected by sequencing the single-stranded M13 template or the double-stranded pT719u derivative plasmid, as appropriate, using the USB Sequenase kit.

Double-stranded DNA for sequencing was prepared using Magic miniprep columns of Stratagene. Two individual clones of each mutation were isolated and analyzed separately to detect phenotypic characteristics due to spurious secondary mutations. Mutant fragments from each were sub-

Table 2: Mutations Generated in the Investigation of Axial Ligation to Heme b_{558}

mutant generated	mutagenic oligomer sequence[a]	template	parent vector	name of clone
Met17Ala	5'-GGAAGTGGTAC<u>G</u>CCGCGGTC-3'	pZG1	pTK1	pM17A
Met29Leu	5'-GGAACGCCAXA<u>CC</u>GAGCG-3'	pZG1	pTK1	pM29L
Met36Leu	5'-GACCGTTTCC<u>A</u>AAAATGGCCAG-3'	pZG1	pTK1	pM36L
Met102Leu	5'-GGAAGAAGGC<u>C</u>AXCAGACC-3'	m13mp18spsp	pFH101	pM102L
Met151Leu	5'-GGGTTTTGCAX<u>C</u>C<u></u>AGCCG-3'	pZG1	pTK1	pM151L
Met228Leu	5'-CAGCAGCCAX<u>A</u>CCGAAGC-3'	m13mp18spsp	pFH101	pM228L
Met245Leu	5'-CGTCGCCCAG<u>T</u>TCGTAGC-3'	pZG1	pTK1	pM245L
Met393Leu	5'-CACGCCACC<u>A</u>XGATACGG-3'	m13mp18spsp	pFH101	pM393L
Met475Leu	5'-CAGCACCAXT<u>G</u>AGAAGATG-3'	pZG1	pTK1	PM475L
Met493Leu	5'-AACTTGAAC<u>A</u>XTAAGAACAA-3'	pZG1	pTK1	pM493L
His86Leu	5'-CCCTACATAG<u>A</u>GGGAATAG-3'	pZG1	pTK1	pH86L
His314Ala	5'-GCGTTCTTCA<u>G</u>CCTGCACCA-3'	pZG1	pTK1	pH314A
Asn153Thr	5'-CGATTGGGGT<u>TT</u>TGCATCC-3'	pZG1	pTK1	pN153T
Lys505Leu	5'-GCGACCGGT<u>TT</u>AGCAGGCTGC-3'	pZG1	pTK1	pK505L

[a] The underlined residue(s) indicates the site of mismatch for mutation. An X in the primer sequence indicates that the oligomer was constructed with a mixture of C, G, and A at the mismatch position.
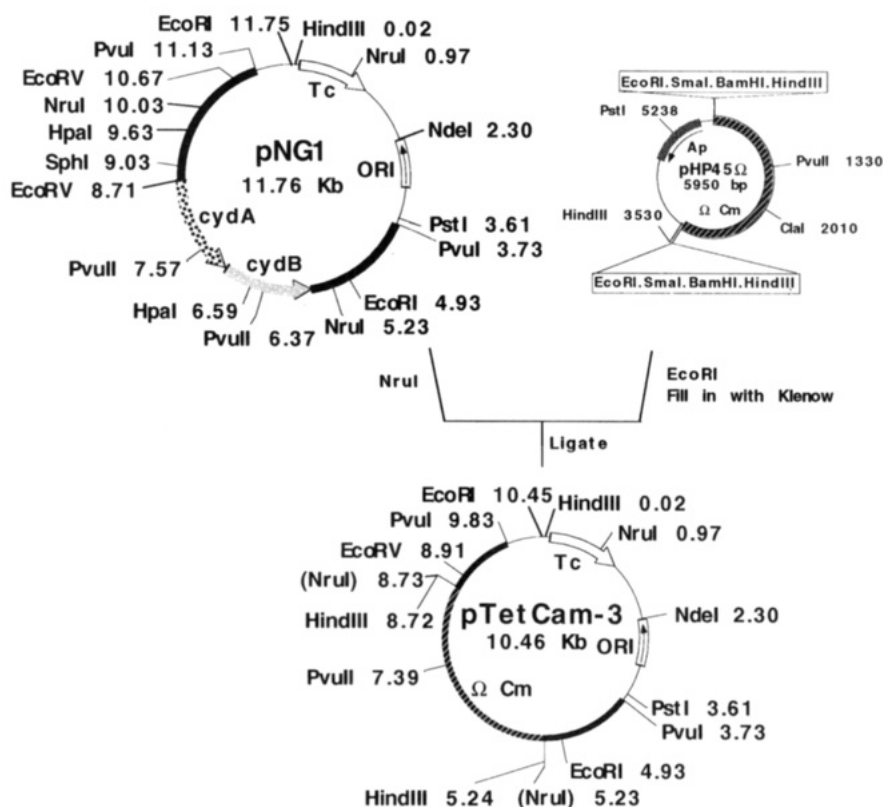

FIGURE 3: Construction of plasmid pTetCam-3 used in the production of the *cyo*, *cyd* double-operon deletion strain GK100. pNG1, a pBR322 derivative plasmid containing the *cyd* operon, was digested with *Nru*I to remove the entire *cyd*AB coding sequence along with 970 bp upstream of the *cyd*A translational start site and 148 bp downstream of the *cyd*B stop codon. The chloramphenicol cartridge (ΩCm) was isolated from pHP45Ω by *Eco*RI digest. The chloramphenicol cartridge was blunt-ended by filling in with Klenow enzyme and ligated to the 2.70 and 4.26 kb tetracycline resistance gene containing *Nru*I fragments of pNG1. The clone pTetCam-3 contains the chloramphenicol resistance cartridge gene in place of the deleted *cyd* operon.

cloned into either pFH101 or pTK1, depending on the template for mutagenesis (M13mp18spsp or pZG1, respectively). Table 2 describes the oligonucleotides used to generate each mutant. Each mutation was confirmed after subcloning by directly sequencing the double-stranded plasmid.

Construction of Strains GK100 and GO105. Complementation analysis of mutant cytochrome *bd* species encoded on plasmids has generally been studied in one of two host strains: RG129 (*cyd*, *cyo*) or GO102 (Δ*cydAB'*, *cyo*). The *cydAB* deletion is a well-defined partial deletion generated by allelic exchange, but the *cyo* and *cyd* alleles are spontaneous, undefined mutations, and occasional reversion of the *cyd* allele has been observed in RG129. In order to avoid

the occasional problem of reversion and to work in a genetically better defined host strain, at the initial phase of this project it was decided to construct a strain from which both the *cyd* and *cyo* operons have been cleanly deleted. Furthermore, since this would remove the possibility of homologous recombination with the chromosomal *cyd* allele, there would be no need to work in a *recA* background, in principle. Since *recA* strains are substantially less robust than their *recA*+ counterparts, this is also advantageous. The construction of the double-deletion strain GK100 was as follows. A plasmid, pTetCam-3, was constructed containing a complete deletion of *cydAB* by *Nru*I digestion of pNG1 (Figure 3). The two fragments of 2.70 and 4.26 kb,

containing between them the complete resistance gene, were isolated, and the deleted locus was replaced with a chloramphenicol cartridge upon religation of the three fragments. Both tetracyline and chloramphenicol resistance were selected and the plasmids isolated. Restriction digestion was used to verify correct ligation orientations. pTetCam-3 was used to transform strain JC7623 (Horii & Clark, 1973; Oden et al., 1990) to chloramphenicol resistance and tetracycline sensitivity, implicating a double-crossover event. Plasmid-linearization was required to get sufficient double-crossover events. A P1 lysate was generated from this strain (GK101) by established techniques, and was used to transduce strain GO104 (Δcyo::kan) to chloramphenicol resistance. Southern blot analysis was performed to verify integration of the chloramphenicol cartridge in place of the deleted *cyd* locus. Although this strain has proved useful for a variety of purposes, the discovery of the *AppCB* operon has rendered GK100 inappropriate for the analysis of plasmid-borne *cyd* alleles because of apparent homologous recombination between these closely related genes. GO105 was, therefore, constructed and used for the complementation analysis. This strain contains the partially deleted chromosomal *cyd* operon of GO102 (Calhoun et al., 1993b; Oden & Gennis, 1991) but is also *recA*, thus preventing homologous recombination. This strain was constructed by transducing *recA* into GO102 (Calhoun et al., 1993b; Oden & Gennis, 1991).

Complementation Analysis of the Mutant Plasmids. The ability of the plasmid-encoded mutant oxidases to complement a strain unable to grow under aerobic conditions was tested using the strain GK100 or GO105. Ultimately, due to the ability of the plasmid-borne *cyd* alleles to recombine with the GK100 genome and form a functional oxidase, all mutations were retested in the strain GO105 to verify results. GO105 grown anaerobically was transformed to ampicillin resistance with the mutant plasmids using the method of Hanahan (1985), and cells were grown anaerobically for selection of ampicillin resistance without selective pressure on the plasmid-borne oxidase operon. Those strains exhibiting ampicillin resistance were restreaked and grown anaerobically to obtain single colonies. Four colonies from each of these plates were checked for aerobic growth on M63 minimal medium (Cohen & Rickenberg, 1956); 0.3% lactate, 0.3% succinate plates supplemented with 100 μM ampicillin to maintain the plasmid; and 50 μM kanamycin or 12.5 μM tetracycline to maintain the strain, as required by the chromosomal mutation. Incubation for 48−72 h at 37 °C was required to define true complementation by demonstrating aerobic growth.

Cell Growth and Preparation of Membranes. If the oxidase was found to complement the anaerobic strain, then the cells were grown aerobically. Colonies were grown in 5 mL of LB medium supplemented with 0.2% glucose, 100 μg/mL ampicillin, and 50 μg/mL kanamycin overnight. These cells were then used to inoculate 100 mL of the same medium. After growth for 5 h at 37 °C with shaking, 25 mL of this culture was used to inoculate a 1-L flask of the same medium, except that the glucose was replaced with 0.3% lactate. The cells were grown for 18 h before harvesting. Cells were washed 1 time with 100 mM potassium phosphate, 1 mM EDTA, pH 7.4, and then frozen at −80 °C until use.

If the mutant oxidase was determined not to complement the anaerobic strain, the cells were grown anaerobically on

LB, 0.2% glucose, 100 μg/mL ampicillin, and 50 μg/mL kanamycin. The protocol for inoculation is as described above, except that the cultures were grown in 2-L stoppered flasks without shaking at 37 °C for 48 h.

Membranes were prepared from harvested cells resuspended in 100 mM potassium phosphate, 1 mM EDTA, pH 7.4, containing 0.5 μg/mL leupeptin. The cells were disrupted by passing 3 times through a Microfluidizer (Microfluidics Corp., Newton, MA), and unbroken cells were removed by centrifugation at low speed. The membranes were then pelleted by ultracentrifugation at 100000g for 2 h at 4 °C. Membranes were resuspended in a minimal amount of the same buffer as described above. Protein was determined using the BCA protein assay reagent (Pierce) with 1% SDS added to the diluted membranes to solubilize proteins.

Protein Purification. Purification of the wild-type enzyme was performed as described previously (Miller & Gennis, 1986) but with minor modifications. For preparation of the wild-type oxidase, either MR43L/F1 (Shipp, 1972) or GO102/pFH101 was used, both of which overexpress the cytochrome *bd* complex. After being washed, the cells were frozen at −70 °C until use. Seventy-five grams of frozen cells was suspended, using a blender, in 300 mL of a buffer consisting of 100 mM Tris/HCl, 15 mM EDTA, 15 mM benzamidine, and 1 mM phenylmethanesulfonyl fluoride (PMSF), pH 8.3. The suspension was then passed through a Microfluidizer (Microfluidics) 3 times to ensure thorough breakage of cells. Unbroken cells were pelleted by centrifugation for 20 min at 10000g. The supernatant was then centrifuged at 200000g for 1 h to pellet the membranes. Subsequent membrane solubilization and fractionation of the protein by DEAE and hydroxyapatite chromatography was as described previously (Miller & Gennis, 1986), with the exception that the flow rate for the DEAE column was 75 mL/h instead of 200 mL/h, and the elution gradient volume for this column was 2000 mL instead of 1600 mL.

The same protocol was used to try to purify the two mutants, H186L and H19L (Fang et al., 1989). These mutants were expressed in strain GR84N (*cyd, cyo*$^+$). One problem that was encountered was the persistence of cytochrome *bo*$_3$ as a contaminant in the final preparation of both H186L and H19L. This was evaluated by Western immunoblotting. By manipulating the detergent concentration (up to 1% in the buffer used for DEAE chromatography), this problem was significantly, though not completely, eliminated.

The mutant M393L was purified according to the same procedure as for wild-type oxidase, except that the KCl concentration was decreased to 100 mM in the DEAE chromatography loading buffer to ensure strong binding of the oxidase to the column. The purified mutant protein was initially dialyzed into sarcosyl, as for the wild-type enzyme. However, upon analysis, the mutant oxidase was found to have minimal activity in this detergent. As a result, the concentrated stock of dialyzed enzyme was diluted into a buffer containing β-dodecyl maltoside, and the protein was reconcentrated by ultrafiltration using an Amicon PM-50 membrane. This effected a reasonable exchange of detergents.

Western Immunoblotting Analysis. SDS−PAGE analysis of membranes and purified proteins was carried out according to Laemmli (1970). The Western blotting procedure was performed as described elsewhere (Harlow & Lane, 1988)

using affinity-purified polyclonal antibodies against the individual subunits of the complex. Goat anti-rabbit secondary antibodies conjugated to alkaline phosphatase were used to detect the primary antibodies.

Visible Spectroscopy. Visible spectra were obtained with a DW2 spectrophotometer (SLM-Aminco), modified by On-Line Instrument Systems (OLIS). Membranes were diluted for spectroscopic studies in 100 mM potassium phosphate, 1 mM EDTA, pH 7.4. Purified protein was diluted in 10 mM sodium phosphate, 5 mM EDTA, and 0.025% sarcosyl, pH 8.0, unless otherwise specified.

Air-oxidized spectra were recorded under conditions where cytochrome *bd* remains oxygenated. A small amount of solid potassium dithionite was used to reduce the enzyme. To obtain the reduced + CO spectra, carbon monoxide was blown over the reduced samples for approximately 1 min. The purified M393L protein and membranes containing this enzyme (GO105/pM393L) were more difficult to reduce. These samples were made anaerobic by passing oxygen-depleted nitrogen over the sample in a stoppered cuvette for approximately 30 min. Aliquots of an anaerobic solution of dithionite were then added until reduction was complete, as monitored by visible spectroscopy, and CO was then passed over the sample in the stoppered cuvette.

The imidazole-bound form of the purified M393L protein was prepared by adding 0.5 M imidazole (pH 7.5) to the enzyme, as purified, and incubating for 30 min. The procedure is modified from Brautigan (Brautigan et al., 1977). The buffer contained 10 mM sodium phosphate, 5 mM EDTA, and 0.025% sarcosyl at pH 7.5. A spectrum was then obtained of the oxidized imidazole-bound state before reduction of the hemes with a small amount of dithionite. The spectrum of the reduced imidazole-bound form was then recorded.

EPR Spectroscopy. EPR spectra were obtained on a Bruker ESP300 X-band EPR spectrometer. The temperature was regulated by an Oxford Instruments cryostat and liquid helium transfer system. All experiments were performed at about 7 K.

Oxidase Activity Assays. Either membranes containing overexpressed oxidase or purified protein was used in the activity assay; 5 μL samples at varying concentrations were diluted into 1.8 mL of 25 mM Tris, 1 mM EDTA, and 2 mM dithiothreitol or dithioerythritol, pH 7.5, equilibrated to 37 °C in a Clark-type oxygen electrode apparatus (Yellow Springs Instrument Co., Yellow Springs, OH). For routine measurements with the pure oxidase, 0.05% Triton X-100 was added; however, other detergents were used to determine the detergent sensitivity of some pure samples. Ubiquinol-1 (Q_1) (kindly provided by Hoffmann-LaRoche) was added to a final concentration of 245 μM. Activities were determined assuming a value of 237 μmol of O_2/L for saturated buffer at 37 °C.

Pyridine Hemochromagen Assay. The concentrations of total heme *b* in the purified wild-type and M393L mutant proteins were determined by the pyridine hemochromagen method (Berry & Trumpower, 1987; Fuhrop & Smith, 1985). The concentration of pure wild-type cytochrome *bd* oxidase was 60 μM, and that of the pure M393L protein was 50 μM, both determined by the BCA protein assay. The concentration of heme *d* in the stock solutions of the enzymes was determined to be 56 μM and 27 μM for wild-type and M393L mutant, respectively, with the use of the extinction

coefficient 14.8 mM^{-1} cm^{-1} for the wavelength pair 628−607 nm. This extinction coefficient is twice that reported previously (Lorence et al., 1986), since the original report assumed 2 equiv of heme *d* per oxidase molecule, not one heme *d* as currently believed.

RESULTS

The goal of this work was to determine the second axial ligand to the heme b_{558} component of the cytochrome *bd* oxidase. Fourteen residues were altered, including 10 methionine residues, 2 histidines, a lysine, and an asparagine residue. The positions of the residues on the proposed topological model are shown in Figure 1. These residues were chosen to be within or close to the membrane bilayer, as heme b_{558} is expected to reside near or within the membrane boundaries. Four of these residues, M36, M151, M393, and M493, are conserved in the *cyd*A sequence of *A. vinelandii* (Moshiri et al., 1991) and the *app*C sequence of *E. coli* (Dassa et al., 1991), both of which shown strong homology to the *E. coli cyd*A sequence.

Strain GK100, which lacks both the *cyd* and *cyo* coding regions, was constructed for genetic complementation analysis of these mutant oxidases. As a control, plasmids encoding mutants in cytochrome *bd*, H19L and H186L, known to be lacking heme groups and oxidase activity (Fang et al., 1989), were introduced into GK100. Surprisingly, after a slight lag time, both of these strains grew on a nonfermentable substrate, whereas GK100 did not grow under these conditions. The same plasmids introduced into RG129 (*cyd, cyo, recA*) did not confer aerobic growth. The implication of these results is that a recombination event can occur between the plasmid-encoded mutant *cyd*AB genes and the chromosomal DNA such that a functional oxidase is generated. The homology of *app*CB to *E. coli cyd*AB (Dassa et al., 1991) indicates that homologous recombination may likely be occurring with in the *app*CB locus. All subsequent complementation analyses were performed with GO105 (*cyo*, Δcyd, *recA*), which is recombination deficient. All of the mutants generated in this work conferred aerobic growth to this strain. The mutants were then analyzed to determine if the heme groups were perturbed.

In order to analyze the mutant oxidases by spectroscopic means, GO105 containing the plasmid-borne mutant *cyd* alleles was grown aerobically to induce overexpression of the oxidases. Spectroscopic analysis of the mutants can be done without enzyme purification (Fang et al., 1989). Figure 4 shows the room temperature dithionite-reduced-*minus*-oxygenated visible spectra of the methionine mutants. All of the mutants except M393L exhibit spectra similar to the wild type control, showing all three heme groups (heme b_{558} at 558 nm, heme b_{595} at 595 nm, and heme *d* at 628 nm). The spectrum of the M393L mutant has an absorbance band at 558 nm which is broader and less intense than the control. The spectrum of the M393L mutant is distinctly different from that of H186L (Fang et al., 1989), which lacks heme b_{558}, and suggests that rather than missing this heme component, heme b_{558} has shifted to a high-spin configuration in M393L. To further examine this, M393L, H186L (lacking heme b_{558}), and H19L (lacking heme b_{595} and heme *d*) were each purified and examined.

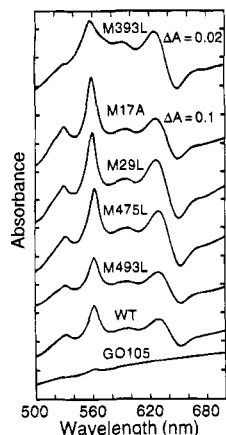SDS−PAGE and Western immunoblot analyses of the purified M393L oxidase show that both subunits are present

FIGURE 4: Representative dithionite-reduced-*minus*-oxygenated optical spectra from 500 to 700 nm of membranes containing the methionine mutants generated in this study, expressed in the strain GO105. The spectrum of membranes containing the wild-type cytochrome *bd* (GO105/pTK1) and the GO105 background spectrum are shown for comparison. Cells were grown aerobically to stationary phase and harvested, and membranes were isolated. "As-isolated" oxygenated spectra were taken, and a small amount of dithionite was added to obtain the reduced form. For M17A, M475L, WT, and GO105 membranes, the protein concentration was approximately 4 mg/mL. For M393L and M493L membranes, the protein concentration was approximately 1 mg/mL. For M29L membranes, the protein concentration was approximately 7 mg/mL. Spectra were taken at room temperature. All of the spectra (including those not shown for M36L, M102L, M151L, M228L, and M245L) have wild-type maxima for the three heme prosthetic groups except M393L, which has a very broad, low-intensity absorbance at 560 nm.
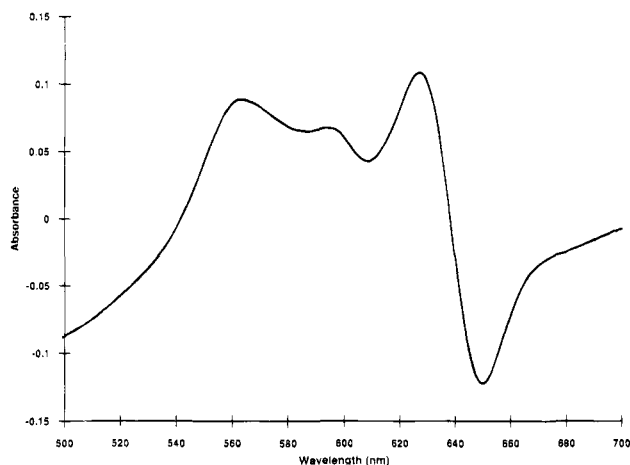

FIGURE 5: Dithionite-reduced-*minus*-oxygenated spectrum from 500 to 700 nm of the purified M393L mutant oxidase. An "as-isolated" oxygenated spectrum was taken, and then the protein was deoxygenated by stirring under a flow of nitrogen in a stoppered cuvette for approximately 30 min. Small aliquots of an anaerobic dithionite solution were then added to the closed cuvette until subsequent spectra no longer showed a change in peak intensity. The reduced spectrum was then taken, and the difference of the two spectra was obtained. Protein concentration was 1 mg/mL. Spectra were taken at room temperature.

and full length (not shown). The dithionite-reduced-*minus*-oxygenated spectrum of the pure M393L oxidase is shown in Figure 5. The difference spectrum of the purified M393L protein does not manifest the characteristics of low-spin heme *b*, i.e., the major narrow α-band near 560 nm and the 531 nm β-band. The absorption band near 560 nm is broad and has low intensity. The visible difference spectrum of the M393L mutant in the 500−580 nm region does not resemble
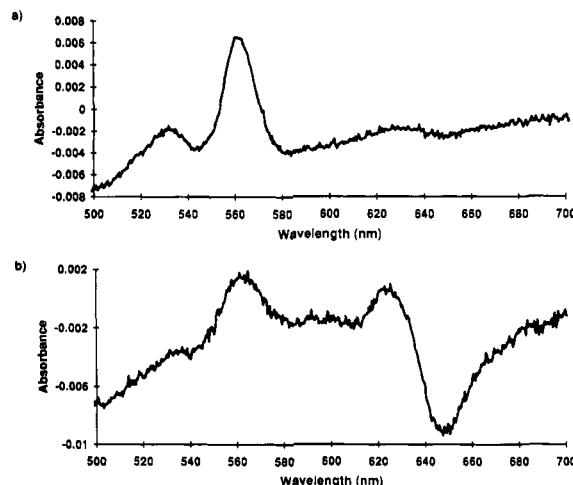

FIGURE 6: Dithionite-reduced-*minus*-oxygenated optical spectra from 500 to 700 nm of purified mutants, H19L and H186L (**a** and **b**, respectively), generated previously (Fang et al., 1989). The "as-isolated" oxygenated spectra were taken, and then a small amount of dithionite was added to generate the reduced form of the enzyme. The purified H19L mutant (**a**) appears to be similar to the enzyme expressed in membranes, lacking both heme b_{595} and heme *d*, which usually have absorption maxima at 595 and 628 nm, respectively. The small broad absorbance at 630 nm may indicate a small amount of the chlorin is still incorporated into the enzyme. The H186L mutant (**b**) lacks the intense band near 558 nm, indicative of heme b_{558}, and appears also to have lost intensity at the 628 nm chlorin band through the purification process. Spectra were taken at room temperature.

the spectrum of either the purified wild-type oxidase (not shown) or the purified cytochrome b_{558} (not shown), or the partially purified H186L or H19L mutants (Figure 6).

The EPR spectrum of the purified M393L mutant is consistent with the interpretation that heme b_{558} has been converted to a high-spin form (not shown). The spectrum shows a high-spin axial signal at $g_{x,y} = 6$, $g_z = 2$, and the rhombic signal at $g_{y,z} = 5.5$, 6.3 assigned to heme b_{595} in wild-type oxidase (Meinhardt et al., 1989; Muller et al., 1988). The spectrum shows no evidence of the $g = 3.3$ signal previously assigned to heme b_{558} (Meinhardt et al., 1989; Muller et al., 1988). The high-spin form of heme b_{558} in this mutant appears to be adding to the intensity of the axial $g = 6$ signal, but this was not quantified.

In an attempt to convert the high-spin heme b_{558} to a low-spin form, an imidazole-bound derivative of the purified protein was produced. After a period of incubation with imidazole at pH 7.5, dithionite was added to the protein to reduce the heme groups. Figure 7 shows the dithionite-reduced spectra of purified M393L with and without imidazole. The imidazole-bound form has a sharper, more intense 560 nm band and has regained a 531 nm band, both indicating conversion to a low-spin heme *b*. The spectrum of the derivative is very similar to that of the wild-type oxidase. There also appears to be loss of intensity of the heme *d* band at 628 nm, which might be due to some denaturation induced by the high concentration of imidazole. The data clearly demonstrate that M393L is not missing heme b_{558}, but rather that heme b_{558} is in a high-spin form.

To verify the presence of 2 equiv of heme B in M393L, a pyridine hemochromagen assay was performed. This analysis yields a ratio of heme B to heme D of 1.7 for both the mutant and wild-type oxidase. This is consistent with the conclusion that heme b_{558} is present, but has altered
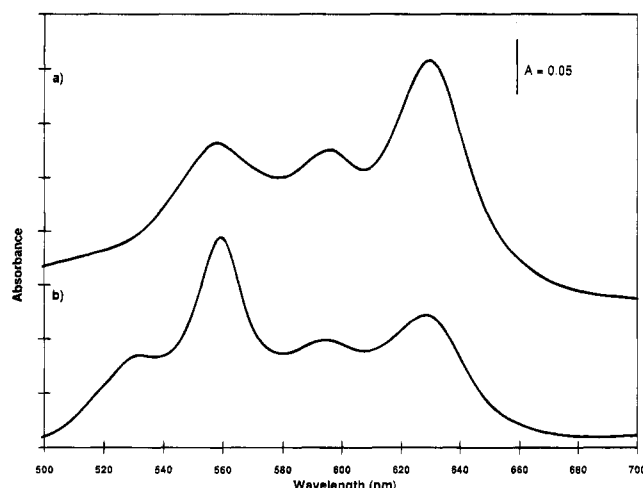
FIGURE 7: Dithionite-reduced spectra of the pure M393L mutant in the presence (**b**) or absence (**a**) of imidazole. (**a**) Pure M393L oxidase reduced with dithionite. The protein was monitored throughout the reduction to ensure the use of a minimal amount of dithionite. The spectrum of the fully reduced protein was then taken. Protein concentration was 1 mg/mL. (**b**) Pure M393L reduced in the presence of imidazole. A sample of pure protein at the same concentration as in panel **a** was brought to 0.5 M imidazole (pH 7.5) from a 10 M stock solution, and the solution was incubated for 1 h. The protein was then reduced with a minimum amount of dithionite, and the fully reduced spectrum was taken. The increase in the intensity and the sharpening of the band at 560 nm, in addition to the formation of a new 531 nm peak, indicate the formation of low-spin heme b_{558}.

spectroscopic features attributed to its conversion to a high-spin configuration. It is noted that the total heme content of M393L is about half the expected value, suggesting that the mutant is labile and probably partially denatured during isolation.

Remarkably, the M393L oxidase is sufficiently active to support robust aerobic growth. After purification, the mutant has a turnover which is less than 1% of the wild type. This residual activity is very sensitive to the detergent present in the assay buffer, reminiscent of the detergent effects on heme b_{558} in the wild-type oxidase (Lorence et al., 1984). Quite possibly the enzyme turnover in the membrane is higher than after purification. However, this was not quantified.

DISCUSSION

The cytochrome *bd* quinol oxidase is the only well-characterized respiratory oxidase that is clearly not a member of the heme—copper oxidase superfamily (García-Horsman et al., 1994; Trumpower & Gennis, 1994). The enzyme has a remarkably high affinity for dioxygen, and predominates when *E. coli* is grown under microaerophilic conditions. Cytochrome *bd* is a coupling site in the aerobic respiratory chain of *E. coli*, and the reduction of dioxygen to water generates a transmembrane voltage (Miller & Gennis, 1985) and results in the net translocation of 1 H^+/e^- across the *E. coli* cytoplasmic membrane (Puustinen et al., 1991). It has been proposed that the mechanism by which proton translocation is accomplished is by scalar chemistry, with protons being released to the periplasm upon the oxidation of ubiquinol and protons being consumed from the cytoplasm by the reduction of dioxygen to water (Dueweke & Gennis, 1990, 1991; Lorence et al., 1988; Trumpower & Gennis, 1994). This suggests that the site of oxidation of quinol may be located near the periplasm, whereas the site where the

oxygen chemistry is catalyzed may be on the opposite side of the membrane, near the cytoplasmic surface (Trumpower & Gennis, 1994). One way to examine this question is to locate the ligands of the heme prosthetic groups which are associated with these reactions. There are three heme prosthetic groups in the enzyme: heme b_{558}, heme b_{595}, and heme *d*.

Heme b_{558} is the low-spin component, thought to be at or near the site where quinol is oxidized (Hata-Tanaka et al., 1987; Hill et al., 1994; Jünemann & Wrigglesworth, 1994; Poole et al., 1983). Previous work has established that heme b_{558} is contained entirely within subunit I (*cyd*A) of the oxidase (Green, 1986; Green et al., 1984b). One of the axial ligands to heme b_{558} has been identified as H186 (Fang et al., 1989; Newton et al., 1991) which, in the current topology, is located near the periplasmic surface of transmembrane helix D within subunit I (Figure 1). Substitutions by mutagenesis for each of the remaining histidines within subunit I have no influence on heme b_{558}, so it is clear that this heme does not have bis(histidine) ligation. The effort to locate the second axial ligand of this 6-coordinate heme has involved a parallel effort of spectroscopy and mutagenesis. The combination of techniques has now provided a strong case that the second axial ligand of heme b_{558} is methionine, and that the specific residue is M393.

As described in the current work, the mutagenesis effort has focused on residues at or near the periplasmic surface of subunit I. The project has emphasized methionines as a reasonable guess for the identity of the sixth ligand of heme b_{558}, but spectroscopic verification of methionine ligation was obtained only recently, long after the mutagenesis experiments were in progress. Cryogenic near-infrared magnetic circular dichroism has now demonstrated that heme b_{558} has histidine—methionine ligation (Spinner et al., 1995), justifying, after the fact, the decision to target methionines in the mutagenesis project.

Progress in the mutagenesis study was made difficult by the unexpected complication of the apparent homologous recombination of the plasmid-borne *cyd*AB alleles with the chromosomal *app*CB operon. Hence, use of the strain GK100, constructed for use in this project, was abandoned in favor of a recombination-deficient strain, similar to that used in the previously reported histidine mutagenesis study (Fang et al., 1989). In addition, the assumption that mutagenesis of the second axial ligand to heme b_{558} would eliminate the heme and, hence, enzymatic function turned out to be incorrect. Although the substitution of a leucine for H186 does eliminate heme b_{558} and enzymatic function, this is not true of the M393L mutation.

All of the mutants in the current study are able to confer aerobic growth to host strains lacking the chromosomal-encoded respiratory oxidases. Further examination of the reduced-*minus*-oxidized difference spectra of the membranes containing the mutant oxidases revealed that one of the mutants, M393L, has perturbed spectroscopic properties (Figure 4). Hence, M393L was purified for more detailed examination. The spectroscopic features of M393L are distinct from those of isolated *cyd*A, which contains heme b_{558} but not the other heme components of the oxidase (Green, 1986), and are also distinct from two other mutants with perturbed hemes, H186L (lacking heme b_{558}) and H19L (lacking heme b_{595} and heme *d*) (Fang et al., 1989) (Figure 6).

The reduced-*minus*-oxidized absorption difference spectrum (Figure 5) and the EPR spectrum (not shown) suggest that heme b_{558} has been converted to a high-spin configuration in M393L. Quantitation of the heme content confirms that M393L contains two heme B per heme D, just as does the wild-type control. Hence, the altered spectroscopic characteristics are not due to loss of heme. The addition of imidazole to ferrous M393L results in the reappearance of a low-spin heme b_{558} optical spectrum. This suggests that the substitution of leucine for M393 leaves the heme intact and results in a cavity in the protein that can be filled by an exogenous ligand (Figure 7).

The data indicate that M393 is the second axial ligand to heme b_{558}, but also indicate that this ligand is not essential to maintaining the heme in a functional form in the protein. Remarkably, the enzymatic activity of M393L is sufficient to support robust aerobic growth, suggesting either that heme b_{558} can be bypassed in the enzymatic reaction or, more likely, that the electrochemical properties of the high-spin form of heme b_{558} are still compatible with its presumed function in quinol oxidation. After purification, M393L retains about 1% of the quinol oxidase activity of the wild-type control, and this activity is very dependent on detergents present in the assay buffer.

It is been observed that the Met80 axial ligand of horse cytochrome c is labile and can be displaced by exogenous ligands (Shao et al., 1995). Similar lability of the M393 ligand to heme b_{558} may be responsible for the perturbations observed in the presence of different detergents, and to the sensitivity to different glassing agents observed in the cryogenic near-infrared magnetic circular dichroism study (Spinner et al., 1995). The formation of a cavity by elimination of the normal heme axial ligand (M393) and the ability to substitute an exogenous imidazole ligand are not unprecedented in other heme proteins. For example, exogenous ligands can be used to replace the proximal ligand in mutants of myoglobin (Barrick, 1994; Decatur & Boxer, 1995; DePillis et al., 1994), cytochrome c peroxidase (McRee et al., 1994), and cytochrome c (Lu et al., 1993).

Finally, it should be noted that M393 is conserved in the sequences of the homologues of *E. coli* cytochrome *bd*. This residue is proposed to be located (Newton et al., 1991) near the periplasmic surface of putative transmembrane helix F (Figure 1). Hence, the position is consistent with the postulate that heme b_{558} is located near the periplasmic surface. The data strongly suggest that heme b_{558} is suspended between two transmembrane helices (D and F) and ligated to H186 (helix D) and M393 (helix F). Furthermore, the region of subunit I connecting helix E and helix F, called the Q-loop, has been definitively shown to be facing the periplasm and to be involved in the interaction with the quinol substrate. The data, summed together, indicate that quinol is oxidized by a site within subunit I (*cyd*A) at or near the periplasmic surface of cytochrome *bd* and that heme b_{558} is part of that active site. The expected release of protons resulting from the oxidation of quinol at this site is the most reasonable explanation for the protons released to the periplasm concomitant with the quinol oxidase activity catalyzed by cytochrome *bd* (Puustinen et al., 1991).

ACKNOWLEDGMENT

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