Localization of Histidine Residues Responsible for Heme Axial Ligation in Cytochrome b_{556} of Complex II (Succinate:Ubiquinone Oxidoreductase) in *Escherichia coli*†

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ABSTRACT: Complex II (succinate:ubiquinone oxidoreductase) from *Escherichia coli* contains four different subunits. Two of the subunits (SDHC and SDHD) are hydrophobic and anchor the two more hydrophilic (flavin and iron-sulfur) subunits (SDHA and SDHB) to the cytoplasmic membrane. Previous studies have shown that the complex of SDHC/SDHD is required to maintain the heme B component of the enzyme and that the heme B is ligated to the protein by two histidine ligands. In the current work, the histidines within SDHC and SDHD have been systematically mutated. SDHC-His91 and SDHD-His14 were eliminated as potential ligands by these studies. SDHC-His84 and SDHD-His71 have been identified as the most likely heme axial ligands in the *E. coli* enzyme, suggesting that the heme bridges these two subunits in the membrane. Furthermore, the results show that the four-subunit Complex II assembles and retains function despite the absence of the heme B prosthetic group in the membrane. The results do not rule out completely SDHC-His30 as a candidate for heme ligation, but do show that mutation at this position prevents assembly of Complex II in the membrane.

Succinate:ubiquinone oxidoreductase, also known as Complex II, is a membrane-bound enzyme that is present in aerobically grown bacteria and in the mitochondria of eukaryotes. The enzyme plays a critical role in cellular metabolism, directly linking the tricarboxylic acid cycle to the aerobic respiratory chain. Complex II catalyzes the oxidation of succinate to fumarate (succinate dehydrogenase activity) in the Krebs cycle and subsequently transfers reducing equivalents to ubiquinone (ubiquinone reductase activity) in the aerobic electron transport chain. Detailed discussions of the enzyme may be found in review articles (*1*, *2*).

The subunit composition, structure, and function of Complex II in *Escherichia coli* are homologous to its mammalian counterparts. The bacterial and mitochondrial Complex II enzymes are often compared to one another as well as to fumarate reductase (FRD), an enzyme with strikingly similar structural and catalytic characteristics. FRD normally couples the oxidation of quinol to the reduction of fumarate to succinate in anaerobically respiring bacteria that use fumarate as the terminal electron acceptor (*3*). Both Complex II and FRD are able to catalyze either succinate oxidation or fumarate reduction.

The *sdh* operon which encodes *E. coli* Complex II has been cloned and fully sequenced (*4*, *5*). The enzyme consists of four nonidentical polypeptides. The two largest subunits (SDHA, F_p, 64 kDa, and SDHB, I_p, 27 kDa)[1] comprise the hydrophilic domain responsible for succinate dehydrogenase activity and contain one covalently bound FAD prosthetic group, three iron-sulfur redox centers, and the catalytic site for succinate oxidation. These hydrophilic subunits are associated with two hydrophobic membrane polypeptides (SDHC, 14 kDa, and SDHD, 13 kDa) to form the succinate-ubiquinone oxidoreductase complex, also termed Complex II. The small hydrophobic peptides anchor the hydrophilic domain to the membrane, allow electrons to be transferred to ubiquinone in the aerobic respiratory chain, and are the site of cytochrome b_{556}. Binding of the hydrophilic subunits

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[1] Abbreviations: F_p, flavoprotein; I_p, iron-sulfur protein; SDH, succinate dehydrogenase; PMS, phenazine methosulfate; PMSF, phenylmethylsulfonyl fluoride; DBH, 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone; DPB, 2,3-dimethoxy-5-methyl-6-pentyl-1,4-benzoquinone; DCIP, 2,6-dichlorophenolindophenol; BV, benzyl viologen; DMN, 2,3-dimethyl-1,4-naphthoquinone; $DMNH_2$, reduced form of DMN; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylene diamine.

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Table 1: *E. coli* Strains, Plasmids, and Bacteriophage

	origin	genotype	source or reference
E. coli strains			
DW35	DW31	*zjd*::Tn10 Δ(*frdABCD*)18 *sdhC*4 *araD*139 Δ(*argF-lac*) *U169 rpsl*150 *relA*1 *lbB*5301 *deoC*1 *pfsF*25 *rbsR*	(*28*)
GV141	DW35	DW35, *recA*938::*cat*	this study
GVK124	NM522	NM522, *sdhC*4 *recA*::Tn10	(*29*)
GW5180		*endA1 hsdR supE sbcB thi*-1 *strA* Δ(*lac-pro*)λ^- [F′ *traD*36 *lacI*^{q}Z Δ*M15proAB*$^+$] *recA*938::*cat*	
HB101		F$^-$ Δ(*mcrC-mrr*) *leu supEA4 ara*14 *galK*2 *lacY*1 *proA*2 *rpsL*20 (Str^r) *xyl*-5 *mtl*-1 *recA*13	(*31*)
Jmr$^-$		[F′ *traD*36 *lacI*^{q} *lacZ*Δ*M*15 *proAB*$^+$] *recA*1 *endA*1 *gyrA*96 (Nal^r) *thi hsdR*17 (r_k^- m_k^+) *supE*44e14-(mcrA$^-$) *relA*1 Δ(*lac-proAB*)	gift from Dr. M. Vandeyer
NM522		Δ*hsd*-5 Δ(*lac-proAB*) [F′ *lacI*^{q} *lacZ*Δ*M*15 *proAB*$^+$]	(*32*)
plasmids			
pBR322		Amp^r Tet^r	(*33*)
pGS133	pLG339	*sdhC*$^+$*D*$^+$*A*$^+$*B*$^+$	(*4*)
pSDH15	pBR322	*sdhC*$^+$*D*$^+$*A*$^+$*B*$^+$ Amp^r	this study
pSDH15-C30HL	pSDH15	*sdhC* codon30 histidine to leucine	this study
pSDH15-C30HN	pSDH15	*sdhC* codon30 histidine to asparagine	this study
pSDH15-C30HP	pSDH15	*sdhC* codon30 histidine to proline	this study
pSDH15-C84HL	pSDH15	*sdhC* codon84 histidine to leucine	this study
pSDH15-C84HP	pSDH15	*sdhC* codon84 histidine to proline	this study
pSDH15-C84HQ	pSDH15	*sdhC* codon84 histidine to glutamine	this study
pSDH15-C84HR	pSDH15	*sdhC* codon84 histidine to arginine	this study
pSDH15-C91HL	pSDH15	*sdhC* codon91 histidine to leucine	this study
pSDH15-C91HR	pSDH15	*sdhC* codon91 histidine to arginine	this study
pSDH15-D14HP	pSDH15	*sdhD* codon14 histidine to proline	this study
pSDH15-D71HL	pSDH15	*sdhD* codon71 histidine to leucine	this study
pSDH15-D71HN	pSDH15	*sdhD* codon71 histidine to asparagine	this study
pSDH15-D71HQ	pSDH15	*sdhD* codon71 histidine to glutamine	this study
pSDH15-D71HR	pSDH15	*sdhD* codon71 histidine to arginine	this study
bacteriophage			
M13mp19			(*34*)
SON16	M13mp8	*sdhC*′	(*35*)
M13mp19/sdhC′DA′	M13mp19	*sdhC*′*D*$^+$*A*′	this study
P1vir			(*27*)

to the integral membrane portion of the enzyme has been demonstrated for Complex II from beef heart (*6*−*8*) and *Bacillus subtilis* (*9*) and fumarate reductase of *E. coli* (*10*−*13*). Requirement of the hydrophobic subunits for interaction with quinone has been shown for both Complex II and fumarate reductase (*6*, *14*−*17*).

The role of the B heme has long eluded investigators. The number of protoheme IX groups per complex and the redox properties of heme B within the protein vary among the characterized succino-oxidase enzymes from a wide variety of species. Complex II from *B. subtilis* (*18*) and FRD from *Wolinella succinogenes* (*19*) each contain two hemes, *Ascaris suum* (*20*) and *E. coli* (*21*) Complex II house one heme per enzyme molecule, while Complex II of *Sulfolobus acidocaldarius* (*22*) and the FRDs of *E. coli* and *Desulfovibrio mutispirans* (*23*) lack heme. Preparations of bovine heart Complex II (*24*) or its hydrophobic peptides (*14*, *15*, *25*) contain heme in substoichiometric amounts, initially leading some investigators to believe that cytochrome *b* was a contaminant in their purified protein preparations. The differing amounts of heme between some of these enzymes and the lack of heme in others raises the issue about what role cytochrome *b* plays in the function of these oxidoreductases. On the basis of the cytochrome *b* midpoint potentials of the characterized succino-oxidase enzymes, it is unclear whether the heme participates in electron transfer from succinate to quinone. While succinate ($E'_o = +30$ mV for the succinate/fumarate couple) is able to reduce cytochrome b_{556} of *E. coli* Complex II with a midpoint potential of +36 mV (*21*), it does not readily reduce cytochrome b_{560} in bovine heart Complex II with an apparent midpoint potential of −185 mV (*15*, *26*). However, prereduced cytochrome b_{560} is easily reoxidized by fumarate. Cytochrome b_{558} of *A. suum* Complex II ($E'_m = -34$ mV) is partially reduced by succinate (*20*). Of the two B hemes in *B. subtilis* Complex II, the higher potential heme b_H ($E'_m = +65$ mV) is fully reduced by succinate, while the low-potential heme b_L ($E'_m = -95$ mV) is not succinate reducible (*18*). These points prompt questions about whether catalysis is linked to the redox properties of cytochrome *b*.

In this work, site-directed mutagenesis coupled with catalytic and spectroscopic data were used to explore the location, role, and axial ligation of cytochrome b_{556} in Complex II of *E. coli*. Conclusions reached from the characterization of the bacterial mutant enzymes are critical to the understanding of cytochrome *b* in these succino-oxidase enzymes which are important in cellular respiration.

EXPERIMENTAL PROCEDURES

Materials. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs and Bethesda Research Laboratories. Calf alkaline phosphatase and polyoxyethylene (*9*) lauryl ether (Thesit) were purchased from Boehringer-Mannheim. Oligonucleotides used for site-directed mutagenesis were synthesized by the University of Illinois Biotechnology Center. Exonuclease III, T4 DNA polymerase, and the Sequenase 2.0 kit were purchased from

United States Biochemical Corporation. Deoxynucleotide triphosphates (dNTPs) and ^{5}methyl-dCTP were obtained from Pharmacia. [^{35}S]dATP was purchased from NEN-Dupont. PMS and DBH were purchased from Sigma. All other chemicals were at least reagent grade.

Media and Growth Conditions. For most experiments, cells were grown at 37 °C in Luria−Bertani (LB) media prepared as described by Silhavy (*27*). 1.5% agar was added for solid media. Antibiotics were used as required at the following concentrations: 100 μg/mL ampicillin, 34 μg/mL chloramphenicol, 50−100 μg/mL kanamycin, and 12.5−25 μg/mL tetracycline. For growth on minimal plates, the media consisted of M63 salts (*27*), 0.3% (w/v) sodium succinate or sodium DL-lactate as the carbon source, 0.03% arginine, 0.01% thiamine hydrochloride, 1 mM $MgSO_4$, and 1.5% agar. GV141 background strains carrying pBR322, pSDH15, or a pSDH15 mutant derivative were tested for their ability to grow aerobically on minimal plates.

Bacterial Strains. Table 1 describes *E. coli* strains used in these experiments. GV141 is a *recA* derivative of DW35. The parent DW35 (*13*) contains Δ*frd* and an insertional mutation in *sdh* (*sdhC*::kan^r) (*29*) that eliminate strain background expression of any enzymes capable of succino-oxidase activity. Since the mutation at the *sdh* locus is not a deletion, the *recA* mutation was introduced into the strain background to prevent the possibility of homologous recombination between DW35 chromosomal *sdh* sequences and plasmid *sdh* DNA. GV141 was constructed by P1 transduction (*27*) of *recA*::*cat* from GW5180 (*30*) into DW35, and selecting for Tet^r Kan^r and Cam^r. GV141 is UV sensitive (a *recA* characteristic), contains no covalent flavin in the membrane, and is deficient for succinate dehydrogenase and fumarate reductase activities. Strain Jmr$^-$ was the host used during site-directed mutagenesis procedures requiring F′ and *mcrA* in the background. NM522 (*32*) was used as the host strain for all other propagation of bacteriophage M13 and its derivatives. When F′ was not required, HB101 (a *recA* strain) (*31*) was the host used during the manipulation of DNA containing *sdh* point mutations.

Construction of Plasmids and Bacteriophage. Table 1 lists the plasmids and bacteriophage used in this study. Figure 1 shows the DNA segments containing parts or all of the *sdh* operon subcloned into plasmid and bacteriophage vectors and relevant restriction sites. Plasmid pSDH15 was constructed by inserting the 4.45 kb *Bam*HI fragment of pGS133 (*4*) containing the *sdh* operon into the *Bam*HI site of pBR322. To construct phage M13mp19/sdhC′DA′, pSDH15 was digested with *Ava*II, heated to 70 °C for 10 min to inactivate the endonuclease, and treated with T4 DNA polymerase plus dNTPs to generate blunt ends. The DNA was then digested with *Kpn*I, and the 1.49 kb fragment containing *sdhC*′DA′ was isolated and inserted into the *Kpn*I−*Hinc*II polylinker region of M13mp19 to generate M13mp19/sdhC′DA′.

Site-Directed Mutagenesis of Histidine Codons in sdhC and sdhD. 18-mer oligonucleotides were used to generate the point mutations. Primers used to change SDHC-His30, SDHC-His84, SDHC-His91, SDHD-His14, and SDHD-His71 codons are listed in Table 2. Single-stranded M13mp19/sdhC′DA′ served as the template for mutagenesis of the histidine codons in *sdhC* and *sdhD*, except for SDHC-His30. (M13mp19/sdhC′DA′ does not contain the coding sequences for SDHC-His30; *sdh* gene sequences in this vector are downstream from the SDHC-His30 codon.) Single-stranded SON16 DNA (*35*) was used as the template for mutagenesis of SDHC-His30. Site-directed mutagenesis was performed with the methylated nucleotide incorporation protocol of Vandeyar et al. (*36*). Mutants were confirmed by dideoxynucleotide sequencing (*37*) using the United States Biochemical Corporation Sequenase 2.0 kit. Mutations were initially identified by single-stranded sequencing of the M13 phage. For SDHC-His30 mutants, the 0.2 kb *Hpa*I−*Kpn*I DNA fragment from double stranded SON16 containing the *sdhC* mutation was subcloned into pSDH15. Mutants at SDHC-His84, SDHC-His91, SDHD-His14, or SDHD-His71 were obtained by replacing the 1.43 kb *Kpn*I−*Bst*XI DNA fragment in pSDH15 with the corresponding double stranded mutant fragment from M13mp19/sdhC′DA′. All pSDH15 point mutant plasmid constructs were confirmed by restriction digestion analysis and double-stranded DNA sequencing of both strands in the region of the mutation.

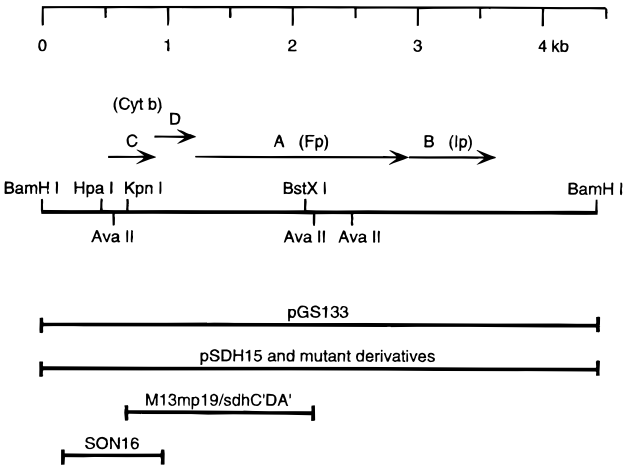

FIGURE 1: Physical map of the *sdh* genes in *E. coli* and summary of the subcloned regions on plasmid and bacteriophage vectors. The organization of the *sdh* operon and direction of the transcribed genes are indicated by the thin lines with arrows. DNA sequences are represented by the thick lines. Left to right orientation correlates to the clockwise direction in the genetic linkage map of *E. coli*. Sequence, orientation, and transcription information were previously determined in the laboratory of Dr. John R. Guest (*4*, *5*, *35*). Relevant restriction sites are indicated on the map.

Preparation of Membranes for Optical Spectra, Measurement of Enzymatic Activity, and Protein Purification. Strains were inoculated and grown by shaking overnight at 37 °C in 5 mL of LB broth containing ampicillin, kanamycin, tetracycline, and chloramphenicol at the concentrations listed above. The following day, 25 μL of the cell culture was transferred into 25 mL fresh LB plus antibiotics and allowed to reach stationary phase. 10 mL of the stationary culture was then inoculated into 1 L of fresh media plus antibiotics and grown with shaking for 12 h at 37 °C. The cells were harvested by centrifugation for 20 min at 8000*g*, the pellet was resuspended and washed twice in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and the final cell pellet was frozen at −80 °C until use. Membrane vesicles were prepared by resuspending thawed cells in three times their weight of buffer containing 10 mM Tris-HCl (pH 8.0), 65 mM $MgSO_4$, 10 μg/mL DNase, 0.5 μg/mL leupeptin, and a saturating amount of PMSF. The cell suspension was kept cold and passed through a French pressure cell twice at 20 000 psi. Two 20 min centrifugations at 12 000*g*, at 4 °C were

Table 2: Primers for Site-Directed Mutagenesis of Histidine Residues in *E. coli* SDHC and SDHD

subunit	residue number	primer name		primer nucleotide sequence 5′−3′		nucleotide change	mutant residue
SDHC	30	R-SDHC-H30	1187	AAACGCGAXGGAGAATGG	1170	X = A	leucine
						X = C	arginine
						X = G	proline
SDHC	30	R-SDHC-30HN	1186	AACGCGATXGAGAATGGA	1169	X = T	asparagine
SDHC	84	SDHC-H84	1333	GGCGTATCXCGTCGTCGT	1350	X = C	proline
						X = G	arginine
						X = T	leucine
SDHC	84	SDHC84HQ	1334	GCGTATCAXGTCGTCGTA	1351	X = G	glutamine
SDHC	91	SDHC-H91	1354	TATTCGCCXCATGATGAT	1371	X = C	proline
						X = G	arginine
						X = T	leucine
SDHD	14	SDHD-H14	1506	TGGCGTACXTGATTTCAT	1523	X = C	proline
						X = G	arginine
						X = T	leucine
SDHD	71	SDHD-H71	1677	CTTGATCCXTGCCTGGAT	1694	X = C	proline
						X = G	arginine
						X = T	leucine
SDHD	71	SDHD71HN	1676	TCTTGATCXATGCCTGGA	1693	X = A	asparagine
SDHD	71	SDHD71HQ	1678	TTGATCCAXGCCTGGATC	1695	X = G	glutamine

performed to remove unbroken cells and cell debris. The supernatant was recovered and centrifuged at 150000*g* for 2 h. The translucent light greenish-tan to dark reddish brown (the color depended on the type of *sdh* mutation) upper layer of the pellet containing the cytoplasmic membrane fraction was collected, a tissue homogenizer was used to suspend the membranes in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and the membranes (∼50 mg/mL final protein concentration) were stored at −80 °C until analysis.

Quantitation of Covalent Flavin and Protein Concentration. Protein concentration was measured by the method of Lowry et al. (*38*) in the presence of 0.1% SDS. Quantitative analysis of 8α-*N*(3)-histidylflavin was performed as previously described (*39*, *40*).

Measurement of Enzymatic Activities. A Yellow Springs Instrument Co. (YSI) model 53 oxygen monitor was used to determine *in situ* succinate oxidase activity. In this assay, oxygen consumption is dependent on the ability of the endogenous membrane components to transport electrons from succinate to molecular oxygen (i.e. through the aerobic respiratory chain). The assays were performed at 38 °C with air-saturated buffer. A 2 mL aliquot of 50 mM Tris-SO_4, 0.1 mM EDTA (pH 7.5 at 38 °C) was placed into the oxygen electrode chamber and warmed to the proper temperature, and 50 μL of crude membrane sample was added and allowed to equilibrate with the buffer for 7 min. To start the reaction, 1 M sodium succinate was added to a final concentration of 12.5 mM and the rate of oxygen consumption was observed.

Succinate oxidation using PMS or DBH as the primary electron acceptor was measured as previously described (*16*, *41*), except that DBH was used in place of DPB in the quinone reduction assays. Activities were measured at six substrate concentrations and V_{max} was determined by extrapolation from a Lineweaver-Burk plot. Prior to each series of assays involving a particular sample, bulk activation of the enzyme was performed by incubating the sample in 20 mM succinate, 2 mM KCN, at 38 °C for 7 min. This procedure removes inhibitory oxaloacetate bound to the enzyme (*41*). After preactivation, the enzyme was kept on ice until ready to be assayed. Substrate concentrations used for the PMS assays were (in mg/mL) 0.33, 0.22, 0.14, 0.11, 0.09, and 0.067. For the DBH assays, substrate concentrations were (in μM) 40, 20, 12.5, 8, 6, and 4. Both assays were started by the addition of preactivated enzyme to the cuvette, and the reaction rates were determined by the DCIP absorbance change at 600 nm. The extinction coefficient used for DCIP was 19.1 mM^{-1} cm^{-1}.

Fumarate-dependent oxidation of reduced BV and $DMNH_2$ was determined as described by Cecchini et al. (*16*, *17*). For the BV assay, substrate concentration was 100 μg/mL, and reaction rates were determined by monitoring the absorbance change of BV at 550 nm using 7.8 mM^{-1} cm^{-1} as the extinction coefficient. The DMN assays were carried out according to the menaquinol-6 oxidase assay of Cecchini et al. (*17*), with 2 mM fumarate and 100 μg/mL $DMNH_2$, but omitting octyl β-D-glucopyranoside. Oxidation of the menaquinol analogue was monitored at 272 nm using 16.4 mM^{-1} cm^{-1} as the extinction coefficient.

Optical Spectrophotometric Analysis. Absorption spectra were recorded at room temperature with an Aminco DW-2 spectrophotometer. The spectrum of succinate reducible heme attributed to cytochrome b_{556} of Complex II in *E. coli* membranes was determined as follows. In a 1 mL cuvette, 800 μL of 100 mM potassium phosphate (pH 6.8), 100 μL 50% glycerol, 100 μL membrane sample (typically 30−60 μg/mL before dilution), 10 μL 50 mM TMPD, and 10 μL 500 mM sodium ascorbate (pH 7.0) were gently stirred while argon was flushed through the solution for 20 min. The TMPD/ascorbate reduced spectrum was recorded. A 20 μL amount of a 1 M sodium succinate solution (pH 7.0) was added to the cuvette, the mixture was constantly stirred an additional 20 min under argon, and the succinate reduced spectrum was recorded. A dithionite reduced spectrum was also taken. Measurements were performed both with and without incubation of carbon monoxide.

Quantitative Analysis of Cytochrome b_{556}. In a previous study (*42*), we established a method for quantitative determination of the composition and the amount of cytochromes in the aerobic respiratory chain of *E. coli*. For the quantitation of cytochrome b_{556} from membranes of GV141/pSDH15 and its mutant derivatives in this work, slight modifications were made to the older protocol. Cells were grown 8 h to late-log phase, harvested, and washed once

with 30 mM Tris-HCl, pH 8.0. One gram of cell pellet was suspended in 20 mL of 30 mM Tris-HCl (pH 8.0), 20% sucrose. Lysozyme and EDTA (pH 7.5) were then added to respective final concentrations of 0.1 mg/mL and 10 mM. Cells were placed on ice for 30 min and were then disrupted by the French press, and the lysate was centrifuged 10 min at 6000*g* to remove unbroken cells and debris. The supernatant was centrifuged 1 h at 110000*g*, the membrane precipitate was homogenized in 30 mM Tris-HCl (pH 8.0), recentrifuged at 110000*g*, and the final pellet was homogenized in a minimum volume of 30 mM Tris-HCl. Crude membranes were solubilized in 3% Sarkosyl, 30 mM Tris-HCl (pH 8.0), 0.6 M NaCl, and the solution was centrifuged at 330000*g* for 30 min; more than 95% of the protein from the inner membrane is solubilized under these conditions. For HPLC analysis, solubilized membranes were loaded and eluted in 0.05% Sarkosyl, 30 mM Tris-HCl (pH 8.0), 0.6 M NaCl with a Shimadzu LC 10AD apparatus and cytochrome absorption was determined at 412 nm. Complete recovery of cytochromes from HPLC is obtained when the elution buffer contains the above NaCl concentration. Analysis of peak area provides rapid and sensitive detection of cytochromes. The relative ratios of Peak I (containing cytochrome bo_3) and Peak III (containing cytochrome b_{556}) were estimated from the peak areas. Correlation between peak area and cytochrome content determined by hemochromogen spectra has been confirmed previously (*42*). The recovery of mutant cytochromes was also confirmed in the present study.

FPLC Purification and Heme Analysis of Wild-Type and Mutant E. coli Complex II. GV141/pSDH15, GV141/pSDH15-C84HL, and GV141/pSDH15-D71HQ were used for these experiments. The Complex II derivatives chosen for these experiments (i) appeared to lack cytochrome b_{556} (by analysis of membranes) and (ii) displayed the highest PMS turnover among the series of mutants made at that respective position. For each strain, a single colony was inoculated into 25 mL of LB with antibiotics, and grown by shaking overnight at 37 °C. A 5 mL aliquot of this culture was transferred into 500 mL of fresh media plus antibiotics and grown to stationary phase. The 500 mL culture was used to inoculate a 20 or 30 L fermenter, where the final culture was grown for 16 h with high agitation and aeration. Cells were harvested by filtration and low-speed centrifugation, washed two times in 800 mL of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and stored at −80 °C. Membrane vesicles were prepared as described earlier, except that large-scale cell disruption was performed by passing the cell suspension three times through a Microfluidics M110 microfluidizer. FPLC purification of *E. coli* Complex II was carried out using the following procedure adapted from that of Kita et al. (*21*). Membranes were solubilized in cold, freshly prepared buffer containing 20 mM Tris-HCl (pH 7.5), 4% (w/v) Thesit, 2 mM PMSF, and 2 mg/mL leupeptin; the final protein concentration was 5 mg/mL. The suspension was stirred for 1.5 h at 4 °C and was then centrifuged for 2 h at 150000*g* in a Beckman Ti 60 or Ti 45 rotor. The supernatant was applied at a rate of 0.5 mL/min to a 45 mL bed volume (2.5 cm diameter × 9 cm height) DEAE-Sepharose CL-6B column equilibrated with 20 mM Tris-HCl (pH 7.5), 1% (w/v) Thesit, and 2 mM PMSF. The column was washed with 50 mL of the same buffer. Bound protein was eluted by a step gradient of 0.25 M NaCl in the same buffer applied at the rate of 0.5 mL/min. Fractions eluted from the brown (SDHC84HL or SDHD71HQ mutants) or reddish-brown (wild-type) band were pooled, diluted 3-fold in buffer, passed though a low-protein-binding polyethylene filter, and loaded onto an equilibrated Waters DEAE−5PW (8.0 × 75 mm) Protein Pak FPLC column. A flow rate of 0.5 mL/min was used for all loading, wash, and elution steps on the FPLC column. The loaded column was washed for 40 min with 0.1 M NaCl in the same buffer. A linear 0.1−0.25 M NaCl gradient in the same buffer was run for 2 h. Complex II eluted in the middle of the gradient in the first highly colored sharp peak. A second, smaller colored peak trailed the first, but these fractions had much lower DBH specific activity. Composition of the second peak was not explored, but it is likely that these fractions may contain partially degraded or dissociated Complex II. Peak fractions from the first sharp peak were pooled, concentrated in a Centricon-30 (Amicon), diluted in buffer to reduce the NaCl concentration below 50 mM, and reconcentrated. The samples were frozen in liquid nitrogen and stored at −80 °C.

Extraction and analysis of heme from purified wild-type, SDHC-84HL, and SDHD-71HQ Complex II was based on the procedure described by Puustinen and Wikström (*43*) omitting the DEAE-Sepharose step. Purified enzyme, 0.29−1.75 mg, was brought to a volume of 3 mL with H_2O and then combined with 15 mL of a cold 9:1:1 mixture of acetone/concentrated HCl/H_2O. The solution was vortexed vigorously for 1 min and centrifuged at 15000 rpm in a Sorvall SS-34 rotor for 2 min at 4 °C. The supernatant was transferred to a new tube and extracted with 15 mL of ethyl ether. The organic phase was extracted twice with equal volumes of HPLC grade H_2O. After the aqueous washes, the organic phase was transferred to a new tube and evaporated under a stream of nitrogen. The dried samples were resuspended in 50−100 μL of a 70:17:13 mixture of 95% ethanol/glacial acetic acid/H_2O (HPLC grade). The sample was filtered and applied to a reverse phase Rainin Microsorb-MV column equilibrated with the above solvent. A Milton Roy CM4000 solvent delivery system maintained the HPLC flow rate at 0.5 mL/min. Heme was detected by absorbance at 405 nm using a Milton Roy Spectro-Monitor 3100.

RESULTS

Amino Acid Residues Targeted for Site-Directed Mutagenesis. Hydropathy plots from Kyte−Doolittle (*44*) and Rao−Argos (*45*) membrane-spanning helix algorithms were generated with the SEQANAL computer program of Dr. A. Crofts (University of Illinois). The profiles were used to predict the positions of the putative membrane-spanning regions of the hydrophobic subunits as shown in the proposed topological model of SDHC and SDHD in Figure 2. This work investigates the location, function, and axial ligation of the B heme in cytochrome b_{556} of *E. coli* Complex II. Since histidine commonly participate as ligands which bind B hemes to proteins, the five histidine residues in SDHC and SDHD were the most reasonable targets for the site-specific mutagenesis experiments of *E. coli* cytochrome b_{556}. Furthermore, the choice of histidine residues was validated by EPR and MCD analysis of cytochrome *b* in Complex II

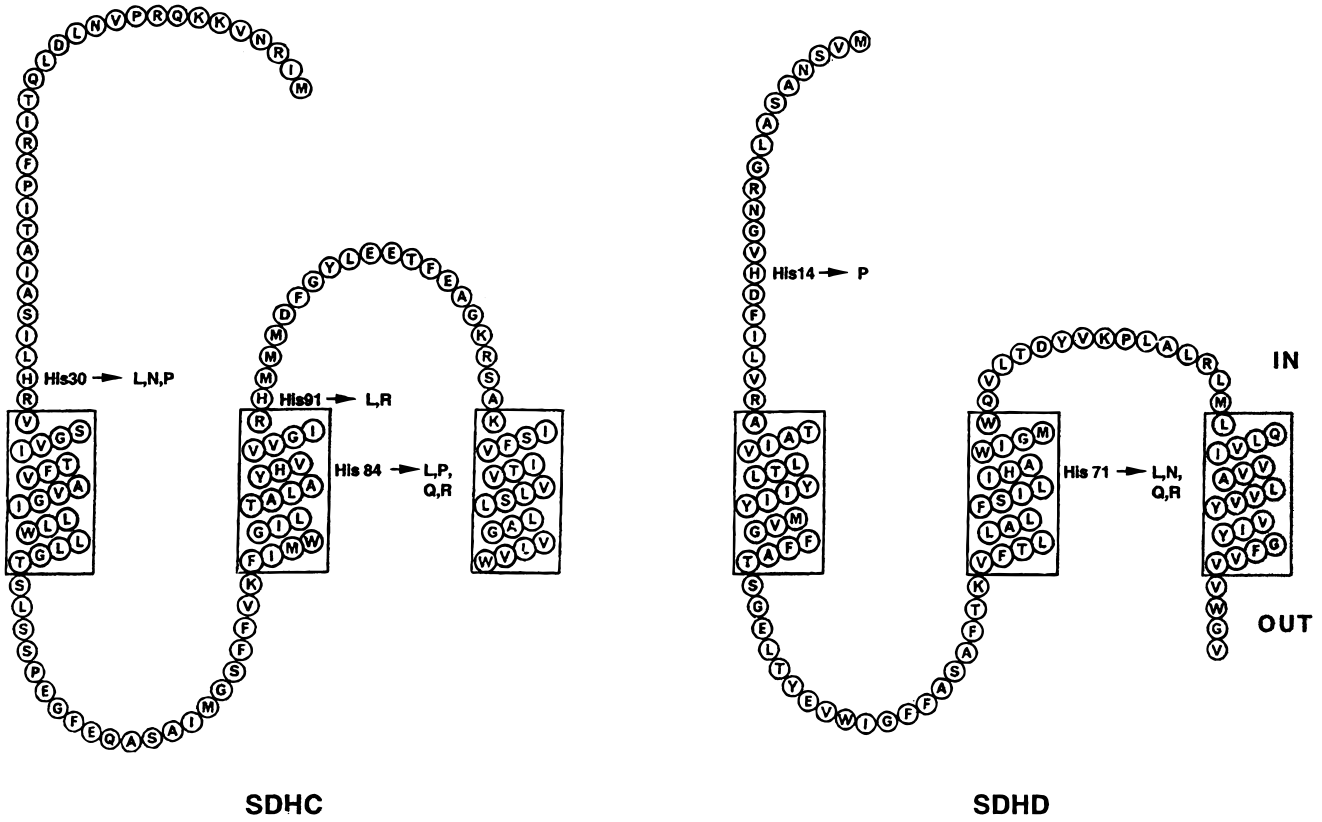

FIGURE 2: Proposed topological model of the hydrophobic subunits in Complex II of *E. coli* showing positions and changes of histidine residues. The locations of putative membrane-spanning helices are based on information from the hydropathy and membrane helix profiles. The position of the five histidine residues are shown along with the amino acid substitutions made at these locations.

in *E. coli* (*46*), *B. subtilis* (*18*, *47*), and bovine heart (*48*) where bishistidyl ligation have been reported. Point mutations were generated at each of the histidine loci in SDHC (positions 30, 84, and 91) and SDHD (positions 14 and 71). Figure 2 indicates the amino acid substitutions made. Initially, mixed oligonucleotides were used for mutagenizing the histidine to leucine, proline, and arginine. Additional substitutions to asparagine and glutamine, which more closely match the hydrogen bonding character of the original histidine residues, were generated at positions where mutations resulted in loss of heme.

Assembly and Expression of Complex II in Membrane Fractions. Complex II and fumarate reductase are the only two membrane bound proteins of *E. coli* which are known to contain covalently bound flavin. Strain GV141 (Δ*frd sdh recA*) lacks both of these enzymes and was used as the background strain in most of the experiments involving the study of Complex II point mutants expressed from plasmid DNA. Hence, in the GV141 strain, protein expressed from plasmid DNA is the only source of covalently bound flavin in the membrane. The quantitative determination of histidyl-FAD in the membrane fraction provides a measure of SDHA subunit associated with the cytoplasmic membrane and can be used to assess Complex II assembly in the membrane. As indicated by the histidyl flavin contents listed in Table 3, mutations made at SDHC-His30 resulted in no Complex II mutant enzyme associated with the cytoplasmic membrane. Only background levels of covalent flavin are found in membranes when histidine 30 in SDHC was mutated.

Whole cell lysates of GVK124 (*sdh*) containing SDHC-His30 mutant derivatives of pSDH15 were assayed for PMS activity immediately after cell breakage; they contained the same background activity levels as GVK124 without plasmid. The reason for the lack of SDH or Complex II activity in these mutants was investigated further. During the growth cycle of *E. coli* strain GV141 (*sdh* Δ*frd*) containing the SDHC-His30 mutant plasmid derivatives, a fluorescent band corresponding to SDHA (histidyl-FAD containing subunit) was synthesized but present in a degraded form (data not shown). This was observed by SDS−polyacrylamide gel electrophoresis of whole cell extracts. This result, along with the lack of heme in the SDHC-His30 mutants (see Figures 3 and 4), suggests that the SDHC-His30 substitutions result in enzyme which is expressed but rapidly degraded due to lack of assembly into the membrane. To further ensure that the mutations at this position had not resulted in a nonsense or frame shift mutation, all SDHC 30 mutant derivatives were sequenced. Sequences of the entire inserted region, as well as the ligation junctions, were verified to be correct, confirming that the desired point mutations were the only genetic alterations in this segment of the operon.

At SDHC position 84, the proline mutant fails to assemble, although all other amino acid substitutions made at this position resulted in mutant Complex II able to bind the membrane. As in the SDHC 30 mutants, it is likely that SDHC-His84 → Pro causes a perturbation in subunit conformation which disrupts the structural integrity of the entire enzyme complex. As shown by the measurements of covalent flavin in Table 3, all other mutants made at SDHC-His84, SDHC-His91, SDHD-His14, and SDHD-His71, were expressed from the plasmid and are able to assemble to the membrane. The results demonstrate that *E. coli* apocyto-

Table 3: Effect of Amino Acid Substitutions on Assembly, Succinate Oxidation, and Fumarate Reduction

membrane sample	covalent flavin (nmol of flavin)/(mg of membrane protein)	succinate-reducible cytochrome b_{556}	succinate oxidation				fumarate reduction	
			succinate: oxidase in situ	succinate:PMS oxidoreductase turnover[a]	succinate:DBH oxidoreductase turnover[a]	DBH/PMS activity ratio	BV-fumarate: oxidoreductase turnover[a]	$DMNH_2$-fumarate: oxidoreductase turnover[a]
sdh- strain	0.002	−	−	0	0	−	ND[b]	ND
wild-type	0.264	+	+	27,500	13,600	0.50	1523	2020
SDHC 30 His → Leu	0.002	−	−	0	0	−	ND	ND
SDHC 30 His → Asn	0.002	−	−	0	0	−	ND	ND
SDHC 30 His → Pro	0.002	−	−	0	0	−	ND	ND
SDHC 84 His → Leu	0.148	−	+	20,500	8,300	0.40	1158	736
SDHC 84 His → Pro	0.003	−	−	0	0	−	ND	ND
SDHC 84 His → Gln	0.057	−	+	16,600	10,500	0.63	920	911
SDHC 84 His → Arg	0.103	−	+	7,500	3,300	0.44	ND	440
SDHC 91 His → Leu	0.449	+	+	16,400	7,000	0.43	ND	649
SDHC 91 His → Arg	0.283	+	+	25,800	13,400	0.52	1297	1028
SDHD 14 His → Pro	0.105	+	+	22,400	14,300	0.64	950	1320
SDHD 71 His → Leu	0.203	−	+	9,500	5,700	0.60	ND	379
SDHD 71 His → Asn	0.294	−	+	10,900	6,300	0.58	557	356
SDHD 71 His → Gln	0.224	−	+	21,400	12,300	0.58	880	1083
SDHD 71 His → Arg	0.094	−	+	300	200	0.67	ND	ND

[a] Activities are expressed as turnover/minute based on 1 mol of histidyl-FAD/mol of enzyme. [b] ND, not determined.

chrome b_{556} is able to bind SDH to the membrane. Insertion of the heme is not crucial for the assembly or catalytic function of Complex II point mutants. SDHC-His84 and SDHD-His71 Complex II mutants lack heme, are expressed in the membrane, and are enzymatically active. Thus, it appears that the heme in cytochrome b_{556} does not assume a required structural role in Complex II of *E. coli*.

Aerobic Growth and Enzymatic Activity of Mutants. Cells were tested for their ability to grow on nonfermentable substrates. Strains capable of growth on succinate minimal media (i) contained membrane-associated Complex II and (ii) were able to catalyze succinate oxidation, as discussed below. Cells lacking membrane-bound Complex II were unable to grow on succinate minimal media. All strains were able to grow on lactate, indicating that the cells were competent in aerobic respiration. In Complex II deficient cells, electron transport occurs via pathways that circumvent Complex II.

To elucidate a possible role for cytochrome b_{556} in the electron pathway between succinate and quinone, Complex II mutants were examined for their ability to perform the interconversion of succinate and fumarate in various succinate oxidation and fumarate reduction reactions. As shown in Table 3, all mutant enzymes that assembled in the membrane were catalytically active. These enzymes were capable of performing electron transport functions in series with the endogenous *E. coli* membrane components of the aerobic respiratory chain as determined by monitoring oxygen consumption upon the addition of succinate. Though membrane-associated Complex II activity can be quantitated, it is difficult to accurately measure and compare reaction rates between samples using only the endogenous membrane components since quantities of quinone and respiratory enzymes may vary from one membrane preparation to another. The use of artificial electron acceptors permits more uniform measurement of catalytic activity, and allows more direct comparisons between wild-type and mutant Complex II, as well as related enzymes from the same or different species. Electron-accepting dyes and relatively hydrophilic synthetic quinone analogues were used to examine the following catalytic functions of wild-type and mutant Complex II of *E. coli*: (i) succinate dehydrogenase activity (PMS assay), (ii) succinate:ubiquinone oxidoreductase activity (DBH assay), (iii) fumarate reductase activity (BV assay), and (iv) menaquinol:fumarate oxidoreductase activity (DMN assay). Flavin content was used to normalize all activities measured with artificial electron accepting dyes; thus, different membrane expression levels of the various mutants are taken into account. Results of the PMS and DBH assays (Table 3) reflect the degree to which the amino acid substitutions affected succinate dehydrogenase and quinone reductase activities. The most dramatic change in enzyme activity was demonstrated in the SDHD-His71 → Arg mutation where both succinate dehydrogenase and quinone reductase activities are drastically reduced. One may speculate that placing a charged residue in this portion of the putative membrane spanning region is not tolerated very well. Upon inspection of the assembled SDHC-His84

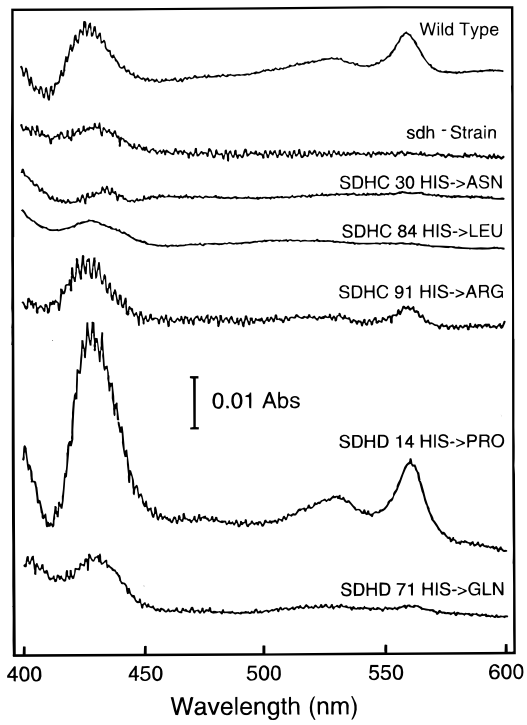

FIGURE 3: [Succinate reduced] − [TMPD/ascorbate reduced] difference spectrum of *E. coli* membranes containing wild-type or selected mutants of Complex II. Shown are the spectra of the mutants displaying the highest PMS turnover number among the series of derivatives at their respective residue position. Other mutants generated at these respective positions showed similar characteristics in the optical spectra. The spectra were taken at room temperature under the reducing conditions described in the text. Each spectrum is the average of five scans. For every scan, five readings per data point per 0.5 nm were recorded. Except for the *sdh* background strain and SDHC-His 30 → Asn, all spectra are normalized to 1 nmol of covalently bound flavin. Spectra of the *sdh* background strain and SDHC-His30 → Asn are normalized to the same protein concentration as the wild-type sample.

mutants, the arginine substitution also caused the greatest reduction of catalytic activity at that position also predicted to be in the membrane. The DBH/PMS ratios indicate that each mutation affected quinone reductase and succinate dehydrogenase activities to relatively the same degree (Table 3). When compared to wild-type Complex II, these ratios reflect the intactness of the mutant enzyme complexes.

Membranes were examined for fumarate reduction capabilities. Table 3 shows that mutant enzymes that catalyzed succinate oxidation were also able to carry out fumarate reduction. Trends in the effect of amino acid substitution on fumarate reductase and menaquinol:fumarate oxidoreductase activities were similar to the effect on succinate dehydrogenase and succinate:ubiquinone oxidoreductase activities, respectively.

Putative Heme Axial Ligand Identification. Optical spectra and cytochrome *b* analysis of *E. coli* membranes containing mutant Complex II suggest which histidine residues are most likely to participate in heme axial ligation. Wild-type *E. coli* Complex II cytochrome b_{556} is fully reducible by succinate (*21*). However, it was imperative to define conditions under which Complex II-associated cytochrome *b* could be specifically observed when studying membrane samples since numerous *b* cytochromes with overlapping spectra exist in *E. coli* membranes. For the optical spectra of membranes, this selection was achieved by using the redox mediators TMPD and ascorbate to reduce the high potential cytochromes (*49*). Lower potential membrane components which are reducible by succinate but not TMPD/ascorbate could then be identified more easily. [Succinate reduced] − [TMPD/ascorbate reduced] difference spectra were determined in membrane samples of GV141 containing pBR322, pSDH15, pSDH15 mutants, or no plasmid. For each altered histidine position in SDHC or SDHD, the spectrum of the mutant with the highest PMS activity is shown in Figure 3. Spectra of the other mutants at the same position (data not shown) were similar to those represented. Optical spectra of membranes containing Complex II mutants SDHC-His91 and SDHD-His14 display characteristics typical of *b* cytochromes, and the maxima of the α-peaks in these mutants are identical with those found in wild-type. The [succinate reduced] − [TMPD/ascorbate reduced] difference spectra of SDHC-His30, SDHC-His84, and SDHD-His71 Complex II mutants show a loss in absorbance characteristic of cytochrome b_{556} seen in the wild-type enzyme (Figure 3 and Table 3). A minute amount of [dithionite reduced] − [succinate reduced] cytochrome *b* was detected in all samples (data not shown), including the negative control (*sdh* strain with pBR322 or without plasmid). Withers and Bragg (*50*) identified two previously unrecognized cytochrome b_{556} species expressed in aerobically grown *E. coli*. It is possible that one of these species is the low-potential cytochrome observed in the [dithionite reduced] − [succinate reduced] spectra. Consistent with the findings that heme B in Complex II is six-coordinate, incubation of the membranes with CO did not change the optical spectra of wild-type or mutant Complex II.

As shown in Table 4, analysis of membrane cytochrome *b* content is consistent with results from the difference spectra. Strains which contained membrane cytochrome b_{556} as determined by HPLC analysis, were identical with those which displayed [succinate reduced] − [TMPD/ascorbate reduced] difference spectra. A minor cytochrome *b* component appeared in the b_{556} elution peak of GV141/pBR322. The difference spectrum of this minor cytochrome was determined to be different from that of cytochrome b_{556} of Complex II. Taking this species into account, cytochrome b_{556} content was determined by calculating the relative ratios of the elution peak areas of cytochrome b_{556} (Peak III) and cytochrome bo_3 (Peak I) (*42*). Figure 4 shows the membrane cytochrome elution profiles of representative mutant samples. Calculated cytochrome *b* content values are found in Table 4.

Because mutations at SDHC-His91 and SDHD-His14 still contain cytochrome b_{556} and display difference spectra with characteristics similar to that of wild-type, it is reasonable to rule out these residues as the axial ligands to the heme iron. Data from the [succinate reduced] − [TMPD reduced] difference spectra and the analysis of cytochrome *b* content support SDHC-His30, SDHC-His84, and SDHD−His71 as possible residues which ligate the ferric heme. From theoretical comparison of a vast database of membrane hemoproteins, Degli Esposti (*51*) previously predicted the above-mentioned residues as potential heme ligands in cytochrome b_{556} of *E. coli* Complex II. The experimental

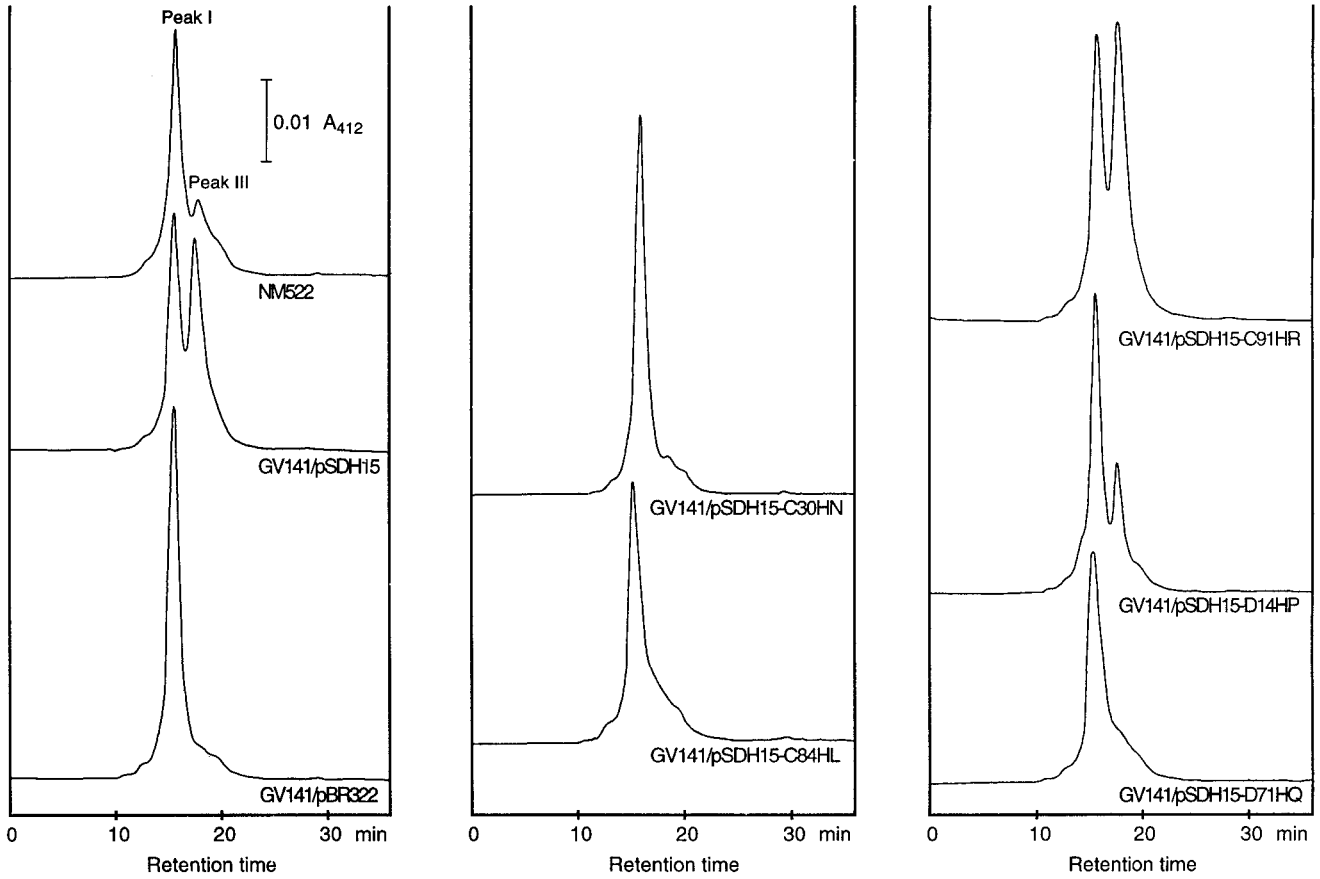

FIGURE 4: Cytochrome analysis of *E. coli* membranes. HPLC profiles of *E. coli* membranes extracted with Sarkosyl are shown. See text for detail. Cytochrome bo_3 elutes in Peak I. Cytochrome b_{556} appears in Peak III. Cytochrome b_{556} content was determined by calculating the relative ratios of Peak III/Peak I.

Table 4: Effect of Amino Acid Substitution on Cytochrome *b* Content in *E. coli* Membranes

membrane sample	cytochrome *b* contents (nmol/mg of protein)	cytochrome b_{556} peak HPLC	Peak III/Peak I relative ratio (b_{556}/bo)
NM522	0.80	+	0.25
GV141/pBR322	0.88	−	0
GV141/pSDH15	1.66	+	0.78
GV141/pSDH15-C30HL	ND[a]	ND	ND
GV141/pSDH15-C30HN	0.95	−	0.01
GV141/pSDH15-C30HP	ND	ND	ND
GV141/pSDH15-C84HL	0.97	−	0.03
GV141/pSDH15-C84HP	ND	ND	ND
GV141/pSDH15-C84HQ	1.07	−	0.02
GV141/pSDH15-C84HR	0.83	−	0.01
GV141/pSDH15-C91HL	ND	ND	ND
GV141/pSDH15-C91HR	2.19	+	0.89
GV141/pSDH15-D14HP	1.03	+	0.36
GV141/pSDH15-D71HL	ND	ND	ND
GV141/pSDH15-D71HN	1.10	−	0.02
GV141/pSDH15-D71HQ	0.97	−	0.03
GV141/pSDH15-D71HR	0.91	−	0.01

[a] ND, not determined.

Table 5: Activity of DEAE-FPLC Purified Wild-Type and Mutant Complex of *E. coli*

purified enzyme	heme HPLC analysis	succinate:PMS oxidoreductase turnover[a]	succinate:DBH oxidoreductase turnover[a]	DBH/PMS activity ratio
wild-type	+	8800	7000	0.80
SDHC84HL	−	5500	2700	0.50
SDHD71HQ	−	2200	800	0.37

[a] Activities are based on turnover/minute based on 1 mol of histidyl-FAD/mol of enzyme.

evidence presented here not only agrees with the theoretical predictions but attempts to address possible roles of the cytochrome.

FPLC Purification and Characterization of SDHC84HL and SDHD71HQ Complex II Mutants. Purification and characterization of selected mutant Complex II enzymes were carried out in order to address whether the drastic alterations in absorbance spectra were caused by (i) a loss of heme, (ii) the loss of one axial ligand resulting in a change from a 6-coordinate to 5-coordinate heme, or (iii) a shift in the heme potential causing the cytochrome to be either reducible by TMPD/ascorbate or not reducible by succinate. A purification protocol modified from that of Kita et al. (*21*) was used for the FPLC procedure. The SDHD71HQ mutant appeared to be less stable than the wild-type or SDHC84HL enzymes. Omission of potassium phosphate from the original protocol seemed necessary for binding the SDHD71HQ mutant to the DEAE column. Furthermore, attempts to purify the SDHD71HQ mutant using only standard DEAE columns were unsuccessful. Purification of the SDHD71HQ mutant enzyme by FPLC was far more effective. Although it was not pursued, it is possible that SDHD71HQ Complex II was less stable than the wild-type or SDHC84HL enzymes due to differences in electrostatic interactions and/or an increased susceptibility to oxidation of the mutant protein. Table 5 summarizes the PMS and DBH activities determined for the purified wild-type, SDHC84HL, and SDHD71HQ Complex II enzymes. On the basis of SDS−PAGE analysis, subunit

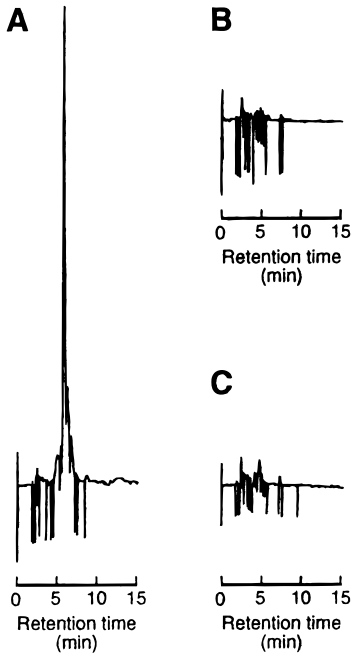

FIGURE 5: Reverse-phase HPLC profiles of heme extracted from purified wild-type, SDHC84HL mutant, and SDHD71HQ mutant Complex II of *E. coli*. Heme was extracted as described in the text and eluted from a Rainin Microsorb-MV column. Heme was detected by absorbance at 405 nm. For each mutant or wild-type Complex II, extraction and detection of heme were performed on at least two different enzyme preparations. The profiles are normalized to the same concentration of covalent flavin in the wild-type and mutant samples. Traces are labeled as follows: (A) wild-type Complex II; (B) SDHC84HL mutant Complex II; (C) SDHD71HQ mutant Complex II.

stoichiometry of the purified SDHC84HL and SDHD71HQ Complex II enzymes appear normal (data not shown).

Heme extraction from the purified enzymes and analysis by reverse phase HPLC was performed (Figure 5). As expected, there was a large heme peak found in the extracted wild-type Complex II sample. There was no heme-containing peak observed in either of SDHC84HL or SDHD71HQ Complex II. These results confirm that heme is absent in these point mutants. The data clearly verify that heme is not required for enzymatic activity.

Taken together, the results demonstrate that the Complex II activity does not require the presence of heme in the enzyme. SDHC-His84 and SDHD-His71 mutants lack heme yet are still able to carry out catalysis. Thus, the heme in cytochrome b_{556} is not essential for the membrane assembly or catalytic function of *E. coli* Complex II.

DISCUSSION

This study addresses questions regarding the cytochrome *b* component of succinate:ubiquinone oxidoreductase (Complex II). The physiological role of cytochrome *b* in this enzyme and in the similar fumarate reductase complex has not yet been established. Researchers have suggested possible structural, redox, and catalytic roles for the heme but have had difficulty reaching concrete conclusions regarding functions of the cytochrome. Part of the ambiguity stems from the observation that cytochrome *b* is present in many, but not all, Complex II and fumarate reductase enzymes isolated from vastly different phylogenetic species. In addition, a wide range of midpoint potentials is found in the characterized cytochromes of the succino-oxidase enzymes carrying heme. In this work, site-directed mutagenesis was used to substitute histidine residues in the SDHC and SDHD peptides to localize the heme axial ligands of the cytochrome b_{556} of *E. coli* Complex II. A combination of biophysical and biochemical analyses of the wild-type and mutant derivatives were used to gain information about possible functions of heme B in the enzyme.

Cytochrome b_{556} is one of the major *b* cytochromes expressed in the membranes of aerobically grown *E. coli*. This cytochrome has been isolated (*52*) and the first 24 residues from N-terminal amino acid sequence data of the cytochrome (*53*) are identical with residues 4−27 deduced for the *sdhC* gene product. Recent studies using plasmids carrying portions of the *E. coli sdh* operon have demonstrated that both SDHC and SDHD are essential for heme B ligation in Complex II (*54*). EPR and near-infrared magnetic circular dichroism (NIR-MCD) studies on the purified wild-type enzyme are consistent with bishistidyl ligation (*46*). In addition, bishistidine coordination has been reported from NIR-MCD and EPR studies for Complex II in *B. subtilis* (*47*) and beef heart (*48*). A low-spin ferric heme in *E. coli* Complex II is further supported by the inability of the native enzyme to bind CO. Together, these data imply that heme B in *E. coli* Complex II is likely to bridge both hydrophobic subunits of the enzyme, and the heme is ligated to one histidine residue from each of SDHC and SDHD.

Spectroscopic analysis of membranes containing Complex II mutants rule out SDHC-His91 and SDHD-His14 as axial ligands. Membranes containing these mutants were determined to contain cytochrome b_{556}, and [succinate reduced] − [TMPD/ascorbate reduced] difference spectra were similar to wild-type samples. Mutations at the other positions resulted in a loss of absorbance in the difference spectra and the absence of cytochrome b_{556} in membrane samples. Thus, SDHC-His30, SDHC-His84, and SDHD-His71 are possible candidates for heme coordination.

Although all substitutions of His30 in SDHC and the SDHC-His84 → Pro mutant resulted in the inability to assemble Complex II, as well as the loss of membrane-associated cytochrome, the other three SDHC84 mutants and all the SDHD71 mutants were able to assemble Complex II into the membrane fraction despite the lack of heme. These results demonstrate that bound heme is not structurally essential for assembly of *E. coli* Complex II.

In contrast, a structural role has been demonstrated for cytochrome b_{558} in *B. subtilis* Complex II (*55*−*57*). Blocking heme synthesis in 5-α-aminolevulinic acid (5-ALA) auxotrophs resulted in an accumulation of unassociated F_p (flavoprotein) and I_p (iron−sulfur protein) subunits in the cytoplasm. Restoring heme permitted insertion of the prosthetic group into the hydrophobic apocytochrome b_{558} and assembly of the three subunit and two heme *B. subtilis* Complex II in the membrane (*55*, *56*). Mutations made at four of the six histidines in *B. subtilis* cytochrome b_{558} result in partial heme deficiency and prevent binding of F_p and I_p to the membrane subunit (*57*). Thus, heme insertion affects the assembly of *B. subtilis* Complex II. By analogy, site-directed mutations made at the axial ligand residues of the cytochrome b_{559} subunits of Photosystem II in *Synechocystis* 6803 caused destabilization of the complex (*58*); although mutant mRNA transcripts were abundant, functional enzyme

did not form. Therefore, while there is evidence for the structural significance of heme in the cytochrome *b* component of proteins, the heme in cytochrome b_{556} does not appear to be required for assembly of the functional membrane bound Complex II in *E. coli*.

Previous work demonstrated the effect of heme deficiency upon *E. coli* Complex II assembly (*54*). In a *hemA* mutant strain transformed with a plasmid carrying the *sdh* operon, SDH activity was localized in the cytoplasmic fraction when the strain was grown in the absence of 5-ALA. Growth in the presence of 5-ALA resulted in membrane associated SDH activity and F_p binding to the membrane. However, unlike the case of *B. subtilis* Complex II where heme appears to be structurally important, this present work provides strong evidence that heme does not play an essential structural role in the *E. coli* enzyme. Membrane bound SDH and succinate:ubiquinone oxidoreductase activities are maintained in various *E. coli* Complex II mutants lacking heme. The inability of a strain to synthesize heme may have an indirect effect upon the assembly of numerous *E. coli* proteins, including Complex II.

Possible redox and catalytic roles of the cytochrome *b* in Complex II are points to consider. It is not clear whether the B heme is involved in the electron transfer path between succinate and quinone. As mentioned earlier, the cytochrome *b* components of Complex II preparations from different organisms vary greatly in midpoint potential and reducibility by succinate. The cytochrome b_{560} of bovine heart Complex II is a curious case. Cytochrome b_{560} has an apparent $E'_m = -185$ mV in the enzyme complex and $E'_m = -144$ mV in preparations of the isolated hydrophobic subunits, often called QPs (ubiquinone-binding protein component of succinate:ubiquinone oxidoreductase) (*26*). As one would expect from the redox potentials, cytochrome b_{560} is not readily reducible by succinate. However, fumarate or ubiquinone-2 has been shown to reoxidize the cytochrome prereduced by dithionite (*15*). It was also demonstrated that the succinate dehydrogenase inhibitor, mersalyl, prevents reoxidation of the cytochrome by fumarate but not by ubiquinone. These results suggest that cytochrome b_{560} may be linked in some way to electron transfer between succinate and ubiquinone. A number of researchers have successfully reconstituted bovine QPs with succinate dehydrogenase to form succinate:ubiquinone oxidoreductase (*6*, *14*, *15*). These preparations all contained cytochrome *b*, though in less than stoichiometric amounts. Yu and Yu purified QPs with very little B heme; this preparation conferred quinone reductase activity with SDH although higher activity was obtained with preparations containing greater amounts of the cytochrome (*6*). Recently, Yang et al. reconstituted active *E. coli* Complex II from its SDH and QPs constituents (*59*). When the isolated membrane-anchoring protein fraction (SDHC + SDHD) was combined with bovine mitochondrial SDH, cytochrome b_{556} reduction by succinate correlated with the reconstitutive activity of the bovine SDH. Reduced cytochrome *b*, however, is unable to reduce ubiquinone in these preparations (*59*). These data and the work reported here suggest that the heme in cytochrome b_{556} is not essential for succinate:ubiquinone reductase activity in the *E. coli* enzyme.

Site-directed mutagenesis has commonly been used to change heme coordination or to change the environment of the heme pocket in various proteins (*60*). Perturbation of catalytic function can be an effective method to examine mechanisms of enzymes. Here, mutagenesis served to destroy heme coordination in order to address questions regarding the role of the cytochrome b_{556} in *E. coli* Complex II. Biochemical characterization of the histidine mutants reveal that the heme is not necessary for enzymatic activity. All mutants that assembled into the membrane fraction were enzymatically active. Under steady state assay conditions, it does not appear that the heme in Complex II contributes significantly to catalysis. The SDHD71HQ mutant exhibited ubiquinone reductase activity close to that of wild-type. The individual substitutions made in Complex II affected turnover rates to different extents. However, if one examines succinate oxidation and fumarate reduction rates in the membrane sample of a particular mutant, the turnover was influenced to about the same degree.

Characterization of the purified Complex II mutants SDHC84HL and SDHD71HQ confirm that cytochrome *b* was eliminated from the enzyme. HPLC analyses of heme extracted from purified wild-type, SDHC84HL, and SDHD71HQ Complex II indicate that loss of absorbance in the difference spectra of membrane samples containing these mutants was indeed due to the loss of heme and not by alterations of the midpoint potential in cytochrome b_{556} (possibly rendering it reducible by TMPD/ascorbate or not reducible by succinate). Both membrane samples and purified enzyme preparations of these mutants contained succinate dehydrogenase and succinate:ubiquinone activities, clearly verifying that heme B was not essential for catalysis.

Data from the expression of *sdhC* and *sdhD* genes from plasmids indicate that SDHC and SDHD are both part of the cytochrome *b* component in *E. coli* Complex II (*54*). Previous studies indicate bishistidyl coordination (*46*) in the *E. coli* enzyme. In this study, SDHC-His91 and SDHD-His14 are eliminated as possible axial ligands because substitutions at these positions at did not result in a loss of B heme. Although SDHC-His30 cannot be ruled out as a participant in heme coordination, SDHC-His84 and SDHD-His71 appear to be the strongest candidates for heme axial ligation, in agreement with a recent structural model that has been proposed for heme coordination in Complex II enzymes (*61*, *62*). Mutants at these positions assemble in the membrane and are catalytically active, yet they do not contain cytochrome. The results from this study suggest that the heme bridges the two hydrophobic subunits with SDHC-His84 and SDHD−His71 as the axial ligands to the cytochrome *b*, and insertion of heme is not required for assembly or catalysis of membrane bound Complex II in *E. coli*. The definitive role of cytochrome *b* in Complex II has yet to be resolved, but one may speculate that the heme may participate in some regulatory mechanism not revealed by the analysis of the enzyme under steady-state conditions.

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