Retention of Heme in Axial Ligand Mutants of Succinate-Ubiquinone Oxidoreductase (Complex II) from *Escherichia coli*®

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Succinate-ubiquinone oxidoreductase (SdhCDAB, complex II) from *Escherichia coli* is a four-subunit membrane-bound respiratory complex that catalyzes ubiquinone reduction by succinate. In the *E. coli* enzyme, heme \( \text{b}_{566} \) is ligated between SdhC His\(^{84} \) and SdhD His\(^{71} \). Contrary to a previous report (Vibat, C. R. T., Cecchini, G., Nakamura, K., Kita, K., and Gennis, R. B. (1998) *Biochemistry* 37, 4148–4159), we demonstrate the presence of heme in both SdhC H84L and SdhD H71Q mutants of SdhCDAB. EPR spectroscopy reveals the presence of low spin heme in the SdhC H84L (\( g_z = 2.92 \)) and high spin heme in the SdhD H71Q mutant (\( g_z = 6.0 \)). The presence of low spin heme in the SdhC H84L mutant suggests that the heme \( \text{b}_{566} \) is able to pick up another ligand from the protein. CO binds to the reduced form of the mutants, indicating that it is able to displace one of the ligands to the low spin heme of the SdhC H84L mutant. The \( g_z = 2.92 \) signal of the SdhC H84L mutant titrates with a redox potential at pH 7.0 (\( E_{m,7} \)) of approximately +15 mV, whereas the \( g_z = 6.0 \) signal of the SdhD H71Q mutant titrates with an \( E_{m,7} \) of approximately −100 mV. The quinone site inhibitor pentachlorophenol perturbs the heme optical spectrum of the wild-type and SdhD H71Q mutant enzymes but not the SdhC H84L mutant. This finding suggests that the latter residue also plays an important role in defining the quinone binding site of the enzyme. The SdhC H84L mutation also results in a significant increase in the \( K_m \) and a decrease in the \( k_{cat} \) for ubiquinone-1, whereas the SdhD H71Q mutant has little effect on these parameters. Over-all, these data indicate that SdhC His\(^{84} \) has an important role in defining the interaction of SdhCDAB with both quinones and heme \( \text{b}_{566} \).

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¶ The abbreviations used are: SQR, succinate-ubiquinone oxidoreductase; ALA, α-aminolevulenic acid; DCIP, 2,6-dichlorophenoldindophenol; HQNO, 2-n-heptyl-4-hydroxyquinolone-N-oxide; PES, phenazine ethosulfate; Q\(_1\), homolog of ubiquinone having one isoprenoid unit at position 6 of the quinone ring; QFR, menaquinol-fumarate reductase; TMPD, N,N,N',N'-tetramethyl-p-phenylene diamine; MOPS, 4-morpholinepropanesulfonic acid; Gpp, modulation amplitude in Guauss, peak to peak excursion.
Heme and Quinone Binding Sites in E. coli Complex II

in bovine heart complex II is not readily reducible by succinate due to its low redox potential at pH 7.0 (Em = −185 mV) (22). Bacillus subtilis complex II contains two b hemes, with the higher potential heme b1 (Em = +65 mV) reducible by succinate, whereas the lower potential heme b2 (Em = −95 mV) is not (23). Succinate is able to reduce the single heme b2sg in E. coli SQR (Em = +36 mV) (24). The varied presence and reducibility of the heme in complex IIs raises questions about whether catalysis is linked to the redox properties of cytochrome b. The heme, where present, has been shown to have an important role for proper assembly of complex II. In heme-deficient mutants of B. subtilis, the apocytochrome of SQR is made and inserted into the membrane, whereas the catalytic domain (FpIp) of the enzyme is accumulated in the cytoplasm (25). Similar results have also been reported for E. coli SQR when expressed in cells deficient in heme synthesis (26). The heme axial ligands for the two membrane anchor subunits. SdhC His 84 and SdhD His 71 were identified as the heme ligands, and it was shown that succinate-quinone reductase activity was retained in the mutant enzymes despite the apparent absence of the heme b2sg (27). Herein, we present spectral and kinetic characterization of the SdhC His 84 and SdhD His 71 mutants of E. coli SQR that retain heme. The results show that an altered heme b does assemble in the isolated mutant enzyme although with significantly lowered redox potential. The data also show that the heme moiety is near the quinone binding domain.

Materials and Methods

Bacterial Strains and Plasmids—E. coli strain DW35 (ΔfrdABC, sdhC::kan) and its recA derivative GV141 have been previously described (15, 27). The deletion in the frd operon and the insertional mutation in the sdh operon eliminates strain background expression of any enzyme capable of succinate oxidase activity. Plasmid pSDH15 (sdhC′ D′ A′ B′) and the plasmid derivatives encoding mutations in the heme histidyl ligands (pSDhC H84L and pSDhD H71Q) have been described previously (27). Plasmids pPFAS and pPGS (1) contain a frd promoter fusion to sdh (Fpr-ΔsdhC′D′A′B′) such that SQR can be expressed anaerobically in E. coli with plasmid pPFAS giving the highest expression level. In order to express the frd enzyme complex in E. coli, the 0.57-kilobase pair EcoRI-Kpn1 fragment from pSDhC H84L or the fragment from pSDhD H71Q was inserted into the equivalent site in pPFAS or pPGS to create plasmids pPFAS or pPGS SdhC H84L (Fpr-ΔsdhC H84L′D′A′B′) and pPFAS or pPGS SdhD H71Q (Fpr-ΔsdhD H71Q′D′A′B′).

Growth Conditions—Anaerobically grown cultures were grown overnight in Luria-Bertani (LB) medium with appropriate antibiotics, and 150 ml was used as inoculum for a 10-liter fermentor (New Brunswick Scientific, Edison, NJ) containing the medium previously described (1) supplemented with 0.05% (v/v) casamino acids, 0.2% (v/v) tryptone, 0.1% (v/v) yeast extract, 0.2 mM MgCl2, 5 μM CaCl2, 20 μM MgSO4, and 50 mM sodium succinate. Ampicillin (35 μg/ml) and kanamycin (50 μg/ml) were included in all media. Cultures were grown with high aeration and harvested at late exponential phase. Anaerobically grown cultures were grown overnight in the same medium described above (minus succinate) with 50 mM glycerol and 50 mM fumarate as electron donors and acceptor, respectively (1). The heme-deficient strain, E. coli SASK41B (HfrP20a hfa111 metB1 relA1), a δ-aminolevulinic acid (ALA) auxotroph (28), was transformed with appropriate plasmids and grown in 50 ml of LB medium in the presence of ampicillin (50 μg/ml) and ALA (50 μg/ml). Cells were collected by centrifugation (10 min at 500 × g) and gently resuspended in 20 ml of LB medium without ALA, and 1 ml was used to inoculate 1 liter of LB medium. Cultures were then grown aerobically in LB with ampicillin (35 μg/ml) or anaerobically in the same medium with 50 mM glucose. When necessary, the medium was supplemented with 100 μM CaCl2. Preparation of Membrane Fractions and Enzyme Purification—Cells were collected by centrifugation, and the membrane fraction enriched in SQR was isolated as previously described (1) with the exception that the cells were disrupted by one passage with an EmulsiFlex-C5 homogenizer (Avestin, Inc., Ottawa, Ontario, Canada) at 18,000 p.s.i. at 4 °C. Membranes containing wild type or mutant SQR were resuspended in 50 mM potassium phosphate, 0.2 mM EDTA (pH 7.2) to −30 μg of protein/ml and frozen at −70 °C. To purify wild type and mutant SQR, the membranes were extracted with 2% (w/v) of the nonionic detergent Thesit (polyoxyethylene 9-dodecyl ether) (Roche Molecular Biochemicals) as previously described (1). The solubilized extract was then applied to a HiLoad 26/10 Q-Sepharose Fast Flow column (Amersham Pharmacia Biotech) and eluted using a 600-ml linear gradient of 0.1–0.25 mM NaCl in 10 mM potassium phosphate (pH 7.2) with 0.05% Thesit according to published procedures (24, 27). The brownish fractions containing succinate dehydrogenase activity were pooled and concentrated with Centriprep-30 concentrators (Amicon Inc., Beverly, MA). The enzyme was washed with 100 mM potassium phosphate (pH 7.0), and concentrated to 15–20 μg of protein/ml and stored at −70 °C.

Measurement of Enzyme Activity—Activity measurements for the succinate oxidase reaction were measured in the presence of 10 mM succinate in the assay cuvette as previously described (29). The succinate-phenazine ethosulfate (PES) reaction in the presence of dichlorophenolindophenol (DCIP) (ε500 = 21.8 mM−1 cm−1, pH 7.8) was measured with 1.5 mM PES and 50 μM DCIP. To measure kinetic parameters of succinate-quinone reductase activity of wild type and mutant SQR, 30 μM Wurster’s Blue (ε412 = 12 mM−1 cm−1) was used as final electron acceptor with varied amounts of quinone as previously described (29, 30).

Spectrophotometric Measurements—Absorption spectra were recorded at 25 °C with a Hewlett Packard 8453 diode array spectrophotometer (Palo Alto, CA) in a 2-ml anaerobic cuvette. The spectrum and molar extinction coefficient of cytochrome c were determined. The molar extinction coefficients of purified and membrane-bound SQR enzymes was determined as previously described (1, 24, 27). Spectra were routinely recorded of membranes suspended in 50 mM potassium phosphate (pH 7.0), 0.2 mM EDTA at a protein concentration of 0.25 mg/ml. Anaerobiosis was achieved by vacuum evaporation and saturation of the buffer with oxygen-free argon.

EPR Spectroscopy and Redox Potentiometry—EPR spectra were recorded using a Bruker ESP300 spectrometer equipped with an Oxford Instruments ESR-900 flowing helium cryostat. Samples were prepared as described in Fig. 4. Potentiometric titrations were carried out as previously described with 150-μl samples being extracted from the titrations into 3-mm internal diameter quartz EPR tubes (31, 32). Titrations were carried out on membranes enriched in wild-type and mutant SQRs with the redox titrators DCIP and pMBAP (pMBAP and 5 mM EDTA (pH 7.0). EPR spectra were recorded as described in Fig. 6 legend.

Analytical Methods—Protein content in membranes was determined by the Biuret method and in isolated enzymes by the method of Lowry in the presence of 1% (w/v) SDS with bovine serum albumin as a standard. The proteinase IX context of cytochrome b was determined from the pyridine hemochromogen difference spectra (dithionite-reduced minus oxidized) (ε556−540 = 23.98 mM−1 cm−1) as described (33). The histidyl-flavin concentration in purified SQR enzymes was determined as follows. Purified protein (0.5–0.7 mg) was precipitated with 1 ml of cold acetone (8 μl of 6 M HCl per 1 ml of acetone) to remove proteinase IX and the iron-sulfur clusters and then centrifuged for 30 s in a microcentrifuge. The yellowish pellet was washed three more times with the same volume of cold acetone and suspended in 0.5 ml of 0.1 M sodium phosphate (pH 7.0) with 1% (w/v) SDS, and the precipitated protein was solubilized after 2 h at 38 °C. The spectrum of the resulting solution shows two peaks at 354 and 445 nm attributed to histidyl-riboflavin. The covalent flavin concentration was determined using ε445 = 12.0 mM−1 cm−1 for histidyl-riboflavin (34).

Results

Anaerobic Expression of SQR Mutants—It has been previously shown that aerobic overexpression of SQR can be achieved in E. coli from plasmids that encode wild type SQR (24). Nevertheless, it has been shown that anaerobic expression of SQR driven from the frd promoter (FrdIfrd) enables even higher levels of SQR to be produced in the membranes of E. coli (1). Therefore, to facilitate expression of site-directed mutant forms of SQR and to aid in purification of the enzymes, constructs were cloned into plasmid pPGS so expression could be driven by the Pfr promoter. E. coli strain GW35, which expresses pPGS, is capable of growth under anaerobic conditions on glycerol-fumarate minimal medium, indicating that wild type SQR can replace fumarate reductase in the anaerobic respiratory chain (1). It has been shown that E. coli GW141 expressing SdhC H84L or SdhD H71Q mutant enzyme is able to grow aerobically on succinate minimal medium, indicating a
functional complex II is formed (27). To test whether anaerobic growth is possible with these mutant SQR enzymes, DW35 containing either pFGS SdhC H84L or pFGS SdhD H71Q was grown anaerobically on glycerol-fumarate minimal medium as previously described (1). Both mutants supported anaerobic growth on glycerol-fumarate minimal medium in E. coli DW35 with a doubling time of 3.8 h for pFGS SdhC H84L and 3.2 h for pFGS SdhD H71Q as compared with 3.0 h for wild-type SQR (data not shown). These results indicate that a functional complex competent in catalysis for the menaquinol-fumarate reductase reaction in vivo is expressed from both the wild-type and mutant plasmids.

Properties of Isolated Mutant Membranes—Previous studies with aerobically grown E. coli cells encoding the SdhC H84L and SdhD H71Q SQR mutants suggested that these substitutions resulted in formation of catalytically active membrane-associated complexes that lacked heme (27). Therefore, it was surprising that membranes isolated from anaerobically grown DW35 cells transformed with both mutants plasmids had an intense color. Membranes from SdhC H84L were brownish red in color, similar to those from cells transformed with wild type SQR plasmids. The membrane fraction from the SdhD H71Q mutant was brownish green in color. The absorption spectra of membranes from strain DW35 enriched in wild-type or mutant SQR complex show a significant absorbance at the Soret region compared with membranes obtained from an E. coli control strain (MC4100) transformed with pBR322. MC4100, the parent strain of DW35 (15), contains chromosomal copies of the sdh and fdr operons and under the anaerobic conditions used for growth does not express SQR. Under the anoxic conditions used for growth, the heme-containing bd-oxidase is expressed (35) and contributes somewhat to the absorbance at the Soret region in the membrane fraction. Fig. 1 shows the dithionite-reduced minus air-oxidized difference spectra of membranes enriched in wild-type and mutant SQR complexes as well as membranes from anaerobically grown cells of E. coli MC4100. The spectra show a significant \( \alpha \)-absorption at 558 nm and a broader \( \beta \)-absorption between 526 and 528 nm as well as the Soret absorption (~425 nm) characteristic of room temperature difference spectra for cytochrome \( b_{580} \) from SQR (36). Analysis of protophe IX content of the membranes by the pyridine hemochromogen method showed similar cytochrome \( b \) concentrations for wild-type SQR and both mutant SQRs (Table I). Mutant forms, however, show lower amplitudes at the Soret absorption and also changes in the line shape of the spectrum (Fig. 1). Both mutants demonstrate succinate-PES reductase and succinate-Q reductase activity; however, SdhC H84L showed at least two times lower succinate-PES reductase activity than the wild-type or SdhD H71Q mutant membranes. Moreover, the succinate-quinone reductase activity was even lower in the SdhC mutant, and the ratio of Q\(_1\)/PES activities indicated that this mutant is significantly impaired in its ability to interact with quinones.

Spectral Properties of Isolated SdhC H84L and SdhD H71Q Mutants—Previous data had suggested that the SdhD H71Q SQR complex was less stable during purification. In the present studies, however (using slight modifications of the original protocol; see “Materials and Methods”), the chromatographic profiles for both SQR mutants and the wild-type enzyme were identical. A single brownish peak with succinate dehydrogenase activity appears at the end of the 0.1–0.25 M NaCl gradient (data not shown). On the basis of SDS-PAGE analysis, the purity of the wild-type and mutant SQRs also appear identical (data not shown). Table II indicates the protophe IX content of the isolated SQR enzymes. Comparing the ratio of covalent FAD to protophe IX content of the purified enzymes, it appears that the SQR mutants show a 10–15% deficiency in protophe IX compared with the ratio of wild-type enzyme.

The absorption spectra of the wild-type and mutant SQR enzymes isolated from the anaerobically grown E. coli cells are shown in Fig. 2. The oxidized cytochrome in wild-type SQR shows a broad absorption at the \( \alpha \)- and \( \beta \)-regions with the Soret absorption at 412 nm. Incubation of wild-type enzyme with sodium dithionite reduces the cytochrome completely within half a minute, and an \( \alpha \) absorption at 558 nm and a broad \( \beta \) absorption at 528 nm appear along with a sharp symmetrical Soret absorption at 424 nm. The air-oxidized spectrum for the SdhC H84L mutant SQR shows a broad absorption at 540 and 580 nm unlike wild-type SQR; however, the Soret absorption shows a maximum at 411 nm with similar intensity to the wild-type cytochrome \( b_{580} \). Complete reduction of the cytochrome by dithionite in this mutant takes 4–5 min. The reduced enzyme displays an \( \alpha \) absorption at 559 nm and a broad \( \beta \) absorption at 528 nm. The Soret absorption in the SdhC H84L mutant enzyme exhibits a maximum at 426 nm and a discernible shoulder at 445 nm, and its absorption intensity is some 2-fold lower compared with wild-type SQR (Fig. 2). The SdhD H71Q mutant SQR differs noticeably in color from wild type or the SdhC H84L mutant SQR; it is less reddish and more green-brown. As shown in Fig. 2, the oxidized spectrum of the SdhD H71Q mutant SQR shows no pronounced peak in either
the \( \alpha \) or \( \beta \) regions, whereas the Soret absorption is similar to wild-type and the SdhC mutant SQR, although the maximum is shifted to 407 nm. The SdhD H71Q enzyme could be slowly reduced with dithionite, similar to the results with the SdhC mutant. The spectrum of the reduced SdhD mutant shown in Fig. 2 shows an \( \alpha \) absorption at 560 nm, a broad absorption at the \( \beta \) region, and a Soret absorption at 423 nm similar in intensity to that of SdhC H84L.

Reduced cytochrome \( b_{556} \) from \textit{E. coli} SQR or the isolated SdhCD domain does not react with carbon monoxide (36), typical of low spin hexacoordinated hemes. As shown in Fig. 3, carbon monoxide causes noticeable alterations in the absorption spectra of both the SdhC and SdhD mutants. The Soret absorption is shifted to 422 nm in SdhC H84L and 423 nm in the SdhD H71Q mutant with a comparable increase in the absorption intensity and a more symmetrical shape to the Soret absorption. The \( \alpha \) absorption was unaffected in the SdhC mutant, whereas a decrease in absorption intensity is found in the SdhD mutant similar to that seen in the isolated cytochrome domain of beef heart succinate dehydrogenase (22). One interpretation of this data is that both mutations result in a change of ligation of the heme \( b_{556} \) from hexa- to pentacoordinate (\( uiz. \), from low to high spin.

In order to further investigate potential spin state changes elicited by the SdhC H84L and SdhD H71Q mutations, we subjected oxidized membranes enriched in these mutant enzymes to EPR analysis. Fig. 4A shows EPR spectra around \( g = 2 \) that arise primarily from [3Fe-4S] clusters. Comparison of the spectrum shown in Fig. 4A (i) (background strain, DW35) and those of Fig. 4A (ii–iv) (overexpressing wild-type and mutant enzyme) indicates that high levels of the SdhB [3Fe-4S] cluster can be detected in the overexpressed wild-type and mutant enzymes. Fig. 4B shows equivalent spectra recorded around \( g = 6 \). Noticeable in the spectrum of the background strain (Fig. 4B (i)) is a typical high spin heme spectrum that probably arises from pentacoordinate hemes such as those found in cytochromes \( b_{556} \) (37) and \( bd \) (28). In membranes containing overexpressed wild-type SQR (Fig. 4B (ii)), there is a diminution of the \( g = 6 \) signal compared with that observed in the background strain. This is likely to be due to the dilution of the proteins responsible for the background signal of Fig. 4B (i) by the overexpressed wild-type SdhCDAB, which contains no high spin heme. The spectrum of membranes enriched in SdhC H84L (Fig. 4B (iii)) is essentially identical to that of membranes containing wild-type SdhCDAB. The spectrum of membranes enriched in SdhD H71Q, however, has an intense signal at \( g = 6 \), indicative of the presence of elevated amounts of pentacoordinate heme. Given that wild-type SdhCDAB contains hexacoordinate heme \( b_{556} \), it is likely that the intense \( g = 6 \) signal arises from the loss of one of the histidine imidazole ligands of this heme, in agreement with the optical data presented herein. Given that no significant increase in \( g = 6 \) signal intensity is observed in the SdhC H84L mutant, we also looked for low spin heme spectra in samples containing wild-type and mutant enzymes (Fig. 4C). Noticeable in the spectra of membranes lacking overexpressed enzyme (\textit{E. coli} DW35, Fig. 4C (i)) is a peak at \( g_s = 3.3 \) similar to that assigned to heme \( b_{558} \) observed in spectra of membranes containing the cytochrome \( bd \) ubiquinol oxidase (28, 38). A broad peak is observed between \( g = 3.65 \) and \( g = 3.50 \) in spectra of membranes containing overexpressed wild-type enzyme (Fig. 4C (ii)). This feature appears to be essentially identical to the spectrum reported for the \( g_s \) feature of low spin heme \( b_{558} \) in purified \textit{E. coli} SQR (36). The spectrum of the SdhC H84L mutant lacks the \( g = 3.33 \) and \( g = 3.76-3.50 \) features and instead contains a distinct peak at \( g_s = 2.92 \), indicating that in this mutant the heme remains low spin in its oxidized state but has a significantly altered environment compared with the wild type.

**Kinetic Properties of Isolated Enzymes**—The ratio of quinone reductase activity to that with artificial electron acceptors such as PES and DCIP has been shown to indicate the ability of complex II to interact with quinones (2). Both mutants showed catalytic activity with PES and Q1, although the SdhC H84L mutant showed a 4-fold lower turnover number in its ability to reduce Q1 (Table II). There was also an increase in the \( K_m \) for Q1 in the SdhC H84L mutant, whereas the \( K_m \) was similar to wild-type enzyme for the SdhD H71Q mutant. Interestingly, the quinone site competitive inhibitor pentachlorophenol (PCP) (29) showed an increased \( K_m \) for the SdhC mutant, and there was no change of the \( K_m \) for the SdhD mutant.

The diheme cytochrome \( b \) of the \textit{B. subtilis} SQR complex has its absorption spectrum perturbed by the addition of the quinone site inhibitor 2-n-heptyl 4-hydroxyquinoline-N-oxide (HQNO) (39). Although HQNO is a potent inhibitor of \textit{B. subtilis} SQR and \textit{E. coli} QFR, it does not inhibit \textit{E. coli} SQR (29). The effects of quinone site inhibitors on the absorption spectra of \textit{E. coli} SQR cytochrome \( b \) have not been reported, so it was of interest to determine if an inhibitor like PCP affected the

### Table I

**Cytochrome b content and analysis of activity in membranes from anaerobically grown \textit{E. coli}**

| Membrane sample     | Cytochrome b contents | Succinate:PES reductase | Succinate:Q1 reductase | Ratio Q1/PES activity |
|---------------------|-----------------------|-------------------------|------------------------|-----------------------|
| GV141/pFGS          | 3.4                   | 22.1                    | 19.6                   | 0.88                  |
| GV141/pFGS SdhC H84L| 2.6                   | 9.5                     | 3.8                    | 0.4                   |
| GV141/pFGS SdhD H71Q| 3.3                   | 17.3                    | 13.1                   | 0.76                  |
| MC4100/pBR322       | 0.6                   | ND                      | ND                     | ND                    |

* Parent strain, containing only a chromosomal copy of \( sdhCDAB \).
* ND, not determined.

### Table II

**Cofactor content and kinetic parameters of purified wild-type and mutant SQR of \textit{E. coli} (pH 7.8, 30 °C)**

| Purified enzymes | Prothione IX | FAD/prothione IX ratio | Succinate:PES turnover | Succinate:Q1 turnover | Q1/PES activity ratio | \( K_m \) (μM) | \( K_{TCP} \) (μM) |
|------------------|--------------|------------------------|------------------------|------------------------|------------------------|----------------|------------------|
| Wild type        | 5.9          | 1/0.69                 | 82                     | 78                     | 0.95                   | 2.5            | 17               |
| SdhC H84L        | 5.4          | 1/0.83                 | 44                     | 18                     | 0.38                   | 40             | 83               |
| SdhD H71Q        | 5.2          | 1/0.87                 | 67                     | 54                     | 0.8                    | 5              | 20               |

* Based on cytochrome b content.
* Determined with Wurster’s Blue as final electron acceptor.

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**Note:** The text continues with further details and analysis related to the experimental results and discussions.
with PCP, the isolated wild-type SdhCD subunits show differently from the soluble SdhAB domain (36). When incubated in the b
the inhibitor. The value obtained of 30 absorbance changes (564–556 nm) induced upon the addition of b
The SdhCD cytochrome
mitochondrial cytochrome bc
shifts in change in the Soret region induced by PCP was accompanied by
B. subtilis have been seen after treatment of
5 mg of solid sodium dithionite after incubation for 5 min. The spectra were recorded following reduction of the enzyme with 1–2
m protein concentration of 2.65
a 1-ml cuvette in 50 mM potassium phosphate, 0.05% (w/v) Thesit at a
line
. The spectra of the oxidized enzymes were scanned at 25 °C in
dashed line
is shown by the
; 3.8 mM
564 nm and a significant increase in the extinction (\( \Delta e = 8.7 \text{ mM}^{-1} \text{ cm}^{-1} \)). There was only a very slight \( \beta \) absorption, but the \( \alpha \) absorption was blue-shifted like wild type with a \( \lambda_{\text{min}} = 562 \text{ nm} \) (\( \Delta e = 1.9 \text{ mM}^{-1} \text{ cm}^{-1} \)). It was not possible to reliably determine the \( K_{i, \text{PCP}} \) for the SdhD H71Q enzyme due to the instability of the mutant enzymes in the reduced state (see below). By contrast to wild-type and SdhD H71Q SQR, PCP had very little effect on the difference spectrum of the SdhC H84L enzyme (Fig. 5). There was a very broad absorbance with a minimum at 425 nm and a minor absorbance change at 560–565 nm. These data along with the changes in \( K_\text{i} \) and \( K_{\text{m}} \), (Table II) suggest that the association of PCP with the enzyme has been affected in SdhC H84L.

**Redox Properties of the Heme**—The favorable redox potential of cytochrome \( b_{562} \) of wild type E. coli SQR (\( E_m = +36 \text{ mV} \)) allows complete reduction by succinate under anaerobic conditions (36). By contrast, the cytochrome in the mutant SQRs investigated in this study are only partially reduced by succinate under anaerobic conditions. The SdhC H84L heme is only reduced some 20% (compared with dithionite reduction) after 12 min (pH 7.2, 25 °C), and the SdhD H71Q mutant is reduced about 30% using the same conditions (data not shown). Overall, these results suggest that there is a thermodynamic or kinetic

![Fig. 2. Visible light absorption spectra of purified wild-type and mutant SQR complex.](http://www.jbc.org/)

![Fig. 3. Effect of carbon monoxide on the visible absorption spectra of purified SdhC H84L and SdhD H71Q SQR enzymes.](http://www.jbc.org/)
In order to determine if the heme present in the two mutants does have a lower midpoint potential \( E_{m,7} \) than the low spin heme of the wild-type enzyme, we subjected the two mutant and wild-type enzymes to potentiometric analysis in combination with EPR spectroscopy. Fig. 6A shows that the high spin heme signal of membranes containing wild-type SdhCDAB titrates with an \( E_{m,7} \) of \( \pm 210 \) mV, consistent with this signal arising from high potential pentacoordinate hemes present in either cytochrome \( b_o \) (37) or cytochrome \( bd \) (28). The \( g = 6.0 \) signal from membranes enriched in SdhD H71Q titrates as a single species with an \( E_{m,7} \) of approximately \(-97 \) mV. Potentiometric titration of the \( g = 2.92 \) signal of the SdhC H84L mutant reveals that its \( E_{m,7} \) is approximately \(+15 \) mV. Similarly, analysis of the \( g = 6.0 \) signal of membranes enriched in this mutant reveals that it titrates with two components, one major one at \( E_{m,7} = \pm 210 \) mV and a minor one at \( E_{m,7} = \pm 15 \) mV. However, the low concentration of high spin heme in membranes enriched in this mutant, it is unlikely that the high spin \( E_{m,7} = \pm 15 \) mV component contributes significantly to the analyses reported herein (cf. Figs. 4B and 6B). Overall, the data suggest that the heme \( b_{556} \) is overwhelmingly in a low spin hexacoordinate state in membranes enriched in the SdhC H84L mutant.

In order to investigate the possibility that changes in the coordination of heme \( b_{556} \) have any effect on the properties of the \([3Fe-4S]\) cluster located in SdhB, we also determined the \( E_{m,7} \) of the \([3Fe-4S]\) cluster in membranes enriched in wild-type, SdhC H84L, and SdhD H71Q SQR. The \( E_{m,7} \) values for the \([3Fe-4S]\) cluster were determined to be \( +75, +65, \) and \(+83 \) mV for the wild type and SdhC H84L and SdhD H71Q mutants, respectively (data not shown). These values are in reasonable agreement with those previously reported for the SdhB \([3Fe-4S]\) cluster \( (E_m = +65 \) mV (41)).

**Stability of the Mutant Enzymes**—Both the SdhC H84L and SdhD H71Q mutant enzymes in the oxidized state and neutral pH remain catalytically active for several days at 4 °C; however, incubation at 30 °C and pH 7.8 results in inactivation of both mutants (Fig. 7A). As seen in Fig. 7, the quinone reductase activity is lost at about twice the rate of the succinate oxidase activity. The succinate oxidase activity measured with PES/DCIP decreased with higher pH and temperature. Incubation of the SdhD H71Q mutant enzyme with 10 mM succinate increased the rate of inactivation of the quinone reductase activity some 10-fold; however, succinate oxidase activity was affected to a lesser extent (Fig. 7B). The inset in Fig. 7B shows the amplitude of the dithionite-reduced signal attributable to the heme during incubation of the SdhD H71Q enzyme with succinate. The decrease of the signal of the reduced heme \( b_{556} \), but not its spectral nature, indicates the release of protoheme IX from apocytochrome. The rate of release of heme \( b \) is more rapid in aerobic than anaerobically incubated enzyme, and the loss of heme directly correlates with the decrease of succinate-Q\(_1\) reductase activity. The correlation of the rapid loss of quinone reductase activity with the decrease in the spectral signal for cytochrome \( b_{556} \) suggests that the dissociation of SdhAB caused the loss of protoheme IX from SdhCD.

**Effect of Growth Conditions on Heme Assembly**—The data reported above indicates that pentacoordinated heme is assembled in the SdhC H84L and SdhD H71Q mutants when grown anaerobically in strains DW35 or GV141. Previous studies using
these same mutants expressed in GV141 had suggested that heme was not assembled in the mutants, although SQR assembled in the membrane and was functionally active (27). In these studies, SQR was expressed aerobically, conditions that are different from those in the current paper. Therefore, wild-type SQR and the SdhC H84L and SdhD H71Q mutant enzymes were expressed aerobically in minimal medium with succinate in strain GV141 or DW35. The content of cytochrome b in membranes isolated from the aerobically grown cells was 1.1 nmol/mg of protein for the SdhC H84L and 1.5 nmol/mg of protein for the SdhD H71Q mutant for strain GV141 (data not shown). This is less than half the amount found in anaerobically grown GV141 used in the current studies (see Table I). Even higher levels of heme were found in the membranes from aerobically grown DW35 containing the mutant plasmids, consistent with previous results showing higher expression levels for SQR in this strain (1). The enzyme isolated from membranes of aerobically grown cells was found to have identical redox characteristics as to the enzyme from anaerobically grown cells (25). The recent crystal structure of the diheme W. succinogenes QFR shows that heme b14 (equivalent to the single heme of E. coli SQR) has amino acid side chains from four of the five a-helices in the membrane domain that aid in binding the heme (12). This suggests the importance of the heme in the assembly and structure of the complex. The results obtained with mutants of the histidyl ligands of the heme in E. coli SQR that suggested that the enzyme assembled and functioned in the membrane, in the absence of heme, were therefore not entirely consistent with the other available data (27). The results reported in this study show that heme is indeed assembled in the SdhC H84L and SdhD H71Q enzymes. In the case of the SdhC H84L mutant, the heme appears to become pentacoordinate and high spin with a gxy at 6.0. For the latter mutant, the \( E_m \),7 is also significantly lowered to approximately –97 mV. In the case of the SdhD H71Q mutant, it is not surprising that the high spin heme is unable to bind CO. However, CO binding also occurs in the SdhC H84L mutant, indicating that it is able to displace the ligand that presumably replaces the imidazole nitrogen of His84. The results reported here are in agreement with those of others that show that the heme (26), when present, is important for proper assembly of complex II.

The retention of low spin heme in the SdhC H84L mutant suggests that His84 might not be the heme ligand or that another residue within SQR can serve as an axial ligand for heme b556. Given the results of sequence alignments of complex
small subunit (26, 43). In this study, the observation of a g at 2.92 (Fig. 4) is also typical for a heme with bis-histidine ligation with a small interplanar angle between the planes of the ligating imidazoles (44, 45). This suggests that the replacement ligand is another residue, possibly His91 or His30 from SdhC or alternatively His14 of SdhD. Future site-directed mutagenesis experiments will address this question. Such ligand displacement has also been observed in the CO-sensing CooA protein from Rhodospirillum rubrum (46).

The reason for the discrepancy between the results reported here and the previous results (27) with the SdhC His64 and SdhD His71 mutants is not entirely clear. In the case of the SdhD H71Q mutant, the succinate-reduced – TMPD/ascorbate-reduced difference spectrum would not be expected to show the presence of the heme because, as the present studies document, the heme is of much lower potential. In the case of the SdhC H84L mutant, there may be a blockage in electron transfer from the [3Fe-4S] cluster to the heme, possibly as a result of disruption of the quinone binding site. More difficult to explain is the reported inability to detect heme extracted from purified SdhC H84L and SdhD H71Q mutant SQR (27). As shown in this paper (Fig. 7) and as noted in the previous study (27) the mutant SQR complexes are less stable than wild type and are particularly sensitive to temperature and aeration. Therefore, it is conceivable that in the previous study the heme could have been lost during the purification procedure, resulting in the inability to detect it in the final samples obtained from the chromatographic column. Nevertheless, as shown in the current study, heme is present in the purified mutant enzymes whether it is produced from anaerobically or aerobically grown E. coli.

The results in Table II and Fig. 5 show that PCP affects the ability of SQR to interact with quinones and perturbs the heme environment. In Table II, it can also be seen that the SdhC H84L enzyme is much more severely affected in its ability to interact with Q1 than is the SdhD H71Q mutant. Also, the inhibitor PCP shows a 5-fold increase in its Ki in the SdhC H84L mutant, whereas the SdhD H71Q enzyme shows no change in inhibition. These results are consistent with SdhC His84 being part of the quinone binding site, in addition to being a ligand of the b heme of SQR. Alternatively, the apparent ligand displacement observed might result in the disruption of the quinone binding site being a secondary effect; i.e. whichever residue replaces SdhC His84 may in fact be essential for ubiquinone binding and oxidation. Azidoquinones have been used to label the SdhC subunit of SQR and have implicated Ser27 and Arg31 of SdhC as being part of a quinone binding site in the enzyme (16). Although the primary sequence of the membrane anchor subunits of complex IIs are not highly conserved, the available structures and models (11, 12, 20, 21) all suggest a very similar transmembrane topology. In two subunit membrane anchors, like E. coli SQR, this would place SdhC His84 in helix II on the cytoplasmic membrane face and on the opposite side of a pocket from SdhC Ser27 and SdhC Arg31 as previously suggested (16). The structure of QFR from E. coli shows two quinone binding pockets on the opposite side of the membrane (11), and SdhC His84 would be localized near the Q1 (quinone-proximal) binding site. PCP perturbs the heme environment in E. coli SQR similar to that seen with HQNO in B. subtilis SQR, and it has been suggested for B. subtilis SQR that the cytochrome participates in binding and stabilization of the semiquinone generated during electron transfer in the enzyme (39). The semiquinone radical attributed to the Qp site shows the presence of the heme because, as the present studies document, the heme is of much lower potential. In the case of the SdhC H84L mutant, there may be a blockage in electron transfer from the [3Fe-4S] cluster to the heme, possibly as a result of disruption of the quinone binding site. More difficult to explain is the reported inability to detect heme extracted from purified SdhC H84L and SdhD H71Q mutant SQR (27). As shown in this paper (Fig. 7) and as noted in the previous study (27) the mutant SQR complexes are less stable than wild type and are particularly sensitive to temperature and aeration. Therefore, it is conceivable that in the previous study the heme could have been lost during the purification procedure, resulting in the inability to detect it in the final samples obtained from the chromatographic column. Nevertheless, as shown in the current study, heme is present in the purified mutant enzymes whether it is produced from anaerobically or aerobically grown E. coli.

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that they are near to the $Q_p$ site (47). The need for proper assembly of the heme to maintain the integrity of the quinone binding sites is also supported by the data in Fig. 7 showing that alteration of the histidyl ligands affects the stability of the enzyme. The more rapid loss of quinone reductase activity, as compared with succinate-PES reductase activity, suggests that if the heme is lost upon incubation of the mutant enzymes then the catalytic SdhAB subunits dissociate from the enzyme. Taken together, these data are consistent with Q$_p$ being located near the [3Fe-4S] cluster in the SdhB subunit and near the SdhC Hig$^\text{B}$ histidyl ligand of cytochrome b of SQR.

These studies do not directly address the role of the heme in electron transfer in complex II. QFR from *E. coli* catalyzes with similar efficiency the same reactions as SQR and does so in the absence of heme. This suggests that heme is not essential for electron transfer, but the results presented demonstrate that heme is present in SdhC Hig$^\text{B}$ and SdhD Hig$^\text{B}$ mutants and that the mutations also have an effect on quinone reductase activity. This leaves open the possibility that heme is directly involved in reaction with quinone/quinol. A mutation in the human SdhD gene equivalent to the *E. coli* SdhC Hig$^\text{B}$ residue results in hereditary paraganglioma (48). As shown in Fig. 6, such a mutation may drop the redox potential of the heme b by more than 100 mV. If SQR heme acts as a sensor for the redox state of the quinone pool or as an oxygen sensor, the hypoxic phenotype observed (48) would be consistent with a lowered potential of human heme b. Structural information for SQR and further characterization of the cytochrome b should help resolve these issues.

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Retention of Heme in Axial Ligand Mutants of Succinate-Ubiquinone Oxidoreductase (Complex II) from *Escherichia coli*

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