The Quinone Binding Site in *Escherichia coli* Succinate Dehydrogenase Is Required for Electron Transfer to the Heme b*

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We have examined the role of the quinone-binding (QP) site of *Escherichia coli* succinate:ubiquinone oxidoreductase (succinate dehydrogenase) in heme reduction and reoxidation during enzyme turnover. The SdhCDAB electron transfer pathway leads from a cytosolically localized flavin adenine dinucleotide cofactor to a QP site located within the membrane-intrinsic domain of the enzyme. The QP site is sandwiched between the [3Fe-4S] cluster of the SdhB subunit and the heme b$_{556}$ that is coordinated by His residues from the SdhC and SdhD subunits. The intercenter distances between the cluster, heme, and QP site are all within the theoretical 14 Å limit proposed for kinetically competent intercenter electron transfer. Using EPR spectroscopy, we have demonstrated that the QP site of SdhCDAB stabilized a ubisemiquinone radical intermediate during enzyme turnover. Potentiometric titrations indicate that this species has an $E_m$ of ~60 mV and a stability constant ($K_{STAB}$) of ~1.0. Mutants of the following conserved QP site residues, SdhC-S27, SdhC-R31, and SdhD-D82, have severe consequences on enzyme function. Mutation of the conserved SdhD-Y83 suggested to hydrogen bond to the ubiquinone cofactor had a less severe but still significant effect on function. In addition to loss of overall catalysis, these mutants also affect the rate of succinate-dependent heme reduction, indicating that the QP site is an essential stepping stone on the electron transfer pathway from the [3Fe-4S] cluster to the heme. Furthermore, the mutations result in the elimination of EPR-visible ubisemiquinone during potentiometric titrations. Overall, these results demonstrate the importance of a functional, semiquinone-stabilizing QP site for the observation of rapid succinate-dependent heme reduction.

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‡1 Escherichia coli encodes a tetrameric enzyme, succinate dehydrogenase (succinate:ubiquinone oxidoreductase; SQR)$^2$, that is responsible for the oxidation of succinate to fumarate during aerobic growth. As a constituent of the bacterial inner membrane, SQR also interacts with the membrane-intrinsic quinone pool and couples the essential tricarboxylic acid cycle step of succinate oxidation to the respiratory electron transport chain (1). The structure of *E. coli* SQR has been solved by x-ray crystallography (2), and it displays remarkable structural and inferred functional similarity to the structures of the chicken (3) and pig (4) enzymes. As a model system for mitochondrial Complex II, *E. coli* SQR has the advantages of facile genetic manipulation, conservation of critical amino acid residues, and high level protein overexpression (5).

*E. coli* SQR comprises four subunits, two of which, SdhA and SdhB, are hydrophilic and attached to the inner (cytoplasmic) surface of the plasma membrane via interactions with two hydrophobic membrane-intrinsic subunits, SdhC and SdhD (6). SdhA contains a redox active flavin adenine dinucleotide (FAD) at its dicarboxylic acid binding site that cycles via an EPR-visible flavin semiquinone between the FAD and FADH$_2$ redox states during catalytic succinate oxidation (7). The electrons from this reaction are sequentially transferred through SdhB, the iron-sulfur protein subunit, via a series of cofactors, a [2Fe-2S] cluster, a [4Fe-4S] cluster, and a [3Fe-4S] cluster, to a quinone binding site (QP site) located at the interface of the SdhB, SdhC, and SdhD subunits. Between them, the SdhC and SdhD subunits form a membrane anchor domain comprising a total of six transmembrane helices, three from each subunit. A single low spin b-type heme (heme b$_{556}$) is coordinated by two His residues, one from SdhC and the other from SdhD (8, 9). This heme is distal to the QP site in the electron transfer pathway, and its role in catalysis remains unresolved.

Among members of the Complex II superfamily, it is in the membrane extrinsic region where most sequence identity and structural similarity are retained. There is much greater sequence variation between the different orthologs within the membrane domain. An example of this divergence is a comparison of *E. coli* SQR and its paralog menaquinol:fumarate oxi-

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2 The abbreviations used are: SQR, succinate:ubiquinone oxidoreductase; FAD, flavin adenine dinucleotide; [Fe-S], iron-sulfur; Sdh, succinate dehydrogenase; SQ, semiquinone; MOPS, 4-morpholinepropanesulfonic acid; Tricine, N-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine.
doreductase (FrdABCD). The two enzymes have very similar overall architectures (7, 10, 11), including the conservation of a two-subunit, six-transmembrane helix membrane anchor domain. However, menaquinol:fumarate oxidoreductase lacks heme and has been proposed to contain two quinone binding sites, a distal site (Qp site) in addition to the proximal Qp site (10, 11). Menaquinol:fumarate oxidoreductase has been demonstrated to complement a ΔsdhCDAB mutant in vivo (5, 12), catalyzing the succinate:ubiquinone oxidoreductase reaction in the absence of heme.

The electrons from succinate oxidation are ultimately transferred via the [3Fe-4S] cluster to reduce a bound ubiquinone at the Qp site. Still, the electron transfer pathway through the enzyme is highly ambiguous. Limitations in electron tunneling efficiency dictate that electrons must travel in a non-deviant pathway from the [3Fe-4S] cluster to the Qp site and the nearest heme propionate other [Fe-S] clusters. The edge-to-edge distances from the heme can participate in fast electron equilibration between the [3Fe-4S] cluster and quinone in the electron transfer pathway, as electrons are fully capable of traveling directly to a ubiquinone bound at the Qp site. It has been determined that the heme performs a structural role in stabilizing the holoenzyme (14), but the question of its role in the electron transfer pathway has not been adequately addressed. It should be noted that using pulse radiolysis it has been suggested that the heme may have in the electron transfer pathway, as electrons are fully capable of traveling directly to a ubiquinone bound at the Qp site. Enzyme turnover and subsequent quinone reduction efficiencies are a key step in the elucidation of the electron transfer mechanisms between key areas within the membrane domain of Complex II.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—Expression of wild-type and mutant SdhCDAB was carried out using the E. coli strain DW35 (ΔfrdABCD, sdhC::kan), laboratory collection) as the host that has been previously described (23). Wild-type SdhCDAB was expressed from plasmid pFAS (pREPsdhC::D+A+B+) under the control of the fumarate reductase promoter (5). All cloning procedures were performed in TG1 cells (K12, Δ(lac-proAB), supE, thi, hsd Δ5 F’[traD36 proA+B’ lacP lacZ ΔM15]) (Amersham Biosciences).

Site-directed Mutagenesis of Q-site Mutants—The 3.7-kb Sphi/KpnI fragment of pFAS was excised and placed into vector pTZ18R [AmplacZ′] (Pharmacia), and this construct was used as a template for the mutagenesis reaction. Mutants were created with the QuikChange site-directed mutagenesis kit from Stratagene using 20-mer oligos as the mutagenic primers. All mutants were verified by sequencing (DNA core facility, University of Alberta). Mutagenized fragments were subcloned back into pFAS and transformed into E. coli DW35 for expression. All cloning procedures were performed in accordance with methods described in Sambrook and Russell (24).

Growth Conditions—E. coli DW35 expressing the appropriate SdhCDAB enzymes from recombinant plasmids were grown under microaerobic conditions. A 25-ml culture of Terrific Broth containing 100 μg/ml ampicillin, 100 μg/ml streptomycin, and 50 μg/ml kanamycin was grown overnight under standard aerobic conditions. This was used to inoculate 2-liter cultures (in 4-liter Erlenmeyer flasks), which were then grown overnight at 37 °C with moderate shaking, each with antibiotics identical to those of the inoculum. The cells were harvested and washed once in 100 mM MOPS/5 mM EDTA, pH 7, buffer before freezing in liquid nitrogen and storing at −70 °C.

Growth Studies—The ability of mutant SQR to support growth in the presence of succinate was determined by growing E. coli DW35 expressing each of the mutants in minimal medium containing 20 mM succinate at 37 °C and monitoring cell turbidity with a Klett-Summerson spectrophotometer (red filter number 66). Growth studies were also done in glycerol-fumarate minimal medium as previously described (25).

Preparation of Membranes—Cells were lysed by two passages through a French press at 15,000 psi. The bacterial inner membrane fraction was concentrated by subjecting the cell lysate to a low speed centrifugation at 15,000 × g followed by high speed centrifugation steps at 150,000 × g as previously described (26). A 52% sucrose gradient was used to separate the inner membrane from the outer membrane, and following this step the E. coli SQR within the membranes was activated with 1 mM malonate at 30 °C for 20 min to release an oxaloacetate molecule bound to the active site FAD (27). Final membrane preparations were aliquotted into 300-μl batches, frozen in liquid nitrogen, and stored at −70 °C.

Analytical Methods—Protein concentrations were determined by a modified Lowry assay (28), and SdhCDAB concentration was determined through a covalently bound flavin assay (29).
Measurement of Enzyme Activity—Enzyme activity was assayed in 100 mM MOPS/5 mM EDTA, pH 7, degassed buffer at 23 °C.

Succinate-Q0 Assay—This assay was performed anaerobically using a combination of 20 mM glucose in the buffer and the addition of 25 units of glucose oxidase/catalase (Sigma) as an oxygen scavenger. The absorbance decrease at 410 nm was measured in the presence of 10 mM succinate and 0.7 mM Q0 (ε_{110} = 0.73 mM^{-1} cm^{-1}).

Plumbagin-Fumarate Assay—This assay was performed, as described (30), anaerobically in the presence of 10 mM fumarate and 0.35 mM plumbagin (5-hydroxy-2-methyl-naphthalene-1,4-dione) (ε_{570} = 17 mM^{-1} cm^{-1}). A combination of 20 mM glucose in the assay buffer and 25 units of glucose oxidase/catalase was used as well.

PMS-MTT Assay—The ability of the enzyme to pass electrons from succinate to 3-(4,5-dimethylthiazolyl-2-)-2,5-di-phenyltetrazolium bromide was assayed as previously described (31) by monitoring the absorbance increase at 570 nm in the presence of 4 mM succinate, 0.75 mM phenazine methosulfate, and 0.075 mM 3-(4,5-dimethylthiazolyl-2-)-2,5,di-phenyltetrazolium bromide (ε_{570} = 17 mM^{-1} cm^{-1}). All spectroscopic measurements were performed with a Hewlett Packard 8453 UV-VIS diode array spectrophotometer.

Heme Reduction Assay—The ability of the heme to be reduced in the presence of succinate was monitored spectrophotometrically. Membrane vesicles were injected into a cuvette containing degassed 100 mM MOPS/5 mM EDTA/20 mM glucose buffer to a final concentration of 0.5 mg ml^{-1}. 25 units of glucose oxidase/catalase were added to ensure a totally anaerobic solution. The absorbance of the heme at 560 nm was monitored using a three-point drop method with the data points at 500 and 575 nm. A base-line absorbance reading was established for 30 s prior to the addition of succinate into the cuvette to a final concentration of 8.7 mM. All readings were performed with a Hewlett Packard 8453 UV-VIS diode array spectrophotometer.

EPR Spectroscopy—In preliminary studies of “as is” air-oxidized membranes, samples containing 180 μg of membrane vesicles in 100 mM MOPS/5 mM EDTA, pH 7, of roughly 30 mg ml^{-1} protein were prepared. To simulate turnover conditions, 30 μl of 100 mM succinate was added to 150 μl of the same membranes and incubated at 23 °C for 30 s prior to freezing. For potentiometric titrations, membrane vesicles were prepared in 100 mM Tricine/5 mM EDTA buffer, pH 8, at a protein concentration of ∼20 mg ml^{-1}. Titrations were carried out in an anaerobic vessel at 25 °C as previously described (25). The following redox dyes were added to a final concentration of 25 μM: dichlorophenolindophenol, 1,2-naphthoquinone, toluylen blue, phenazine methosulfate, thionine, methylene blue, resurufin, indigotrisulfonate, indigocarmine, anthroquinone-2-sulfonic acid, and neutral red. EPR spectra were recorded using a Bruker ESP300E spectrometer equipped with a Bruker liquid nitrogen-evaporating cryostat at 150 K, 20-milliWatt microwave power at 9.43 GHz, and a modulation amplitude of 2G_{pp}. All spectra reported are the average of five scans.

RESULTS

Mutagenesis of Q-site Residues—Site-directed mutants of several conserved residues within the membrane intrinsic domain of E. coli SQR were generated. Based upon the x-ray structure, the chosen residues are all located around the Qp site. Ser-C27 was mutated to an Ala residue; Arg-C31 and Asp-D82 were both mutated to Leu. Tyr-D83 was mutated to Phe, Ala, and Lys. All mutants and wild-type enzymes were expressed in E. coli DW35, which contains a deletion in the frdABCD operon and an inactivated sdhCDAB operon to eliminate any chromosomally encoded enzyme contributing to the activity. The chosen residues are located directly within hydrogen bonding distance of the bound quinone molecule. Ser-C27 is a candidate for hydrogen bonding to the O4 atom of ubiquinone, while Tyr-D83 lies on the opposite side, acts as a ligand for the O1 atom, and has been postulated to act as a proton donor during quinone reduction. Asp-D82 lies in close proximity to ubiquinone as well and could potentially interact directly with the quinone or act through its interaction with Tyr-D83. Arg-C31 is also a major structural component of the quinone binding site as it lies equidistant between the heme and the ubiquinone; additionally, this residue has been proposed to act as a modulator of the pK_a of the side chain OH of Tyr-D83 (2).

Characterization of Enzyme Activities Is Shown in Table 1—To ensure that loss of activity was not a result of breakdown of the enzyme, SDS-PAGE was performed on all membrane preparations (data not shown). This indicated that the wild-type as well as all the mutant enzymes were equally overexpressed to very high levels, whereas assembly and membrane targeting were unaffected. These results were verified by use of a covalently bound flavin assay to determine the exact concentration of SQR within the bacterial membrane. PMS-MTT (phenazine methosulfate-3-(4,5-dimethylthiazolyl-2-)-2,5,di-phenyltetrazolium bromide) assay results, which measure the functionality of the SdhAB dimer, were unchanged between the wild-type and mutant enzymes (data not shown). The succinate/Q0 reductase assay monitors the physiological activity of SQR, and all of the mutants showed drastically reduced activity compared with the wild type. As mutants of residues Ser-C27, Arg-C31, and Asp-D82 were unable to reduce the ubiquinone analog Q0, measurement of the enzyme K_m for Q0 was not possible. Mutants of Tyr-D83 were also defective in their ability to reduce Q0, although there was retention of 15–28% of wild-type activity. When Tyr-D83 was mutated to an Ala or Lys, the K_m for Q0 was elevated as well, whereas a Phe substitution showed a K_m similar to wild type. SQR is capable of operating in the reverse direction as a fumarate reductase, and this can be monitored by the plumbagin/fumarate assay. These mutations had different effects on the ability of the enzyme to oxidize reduced plumbagin, a menaquinol analog. The Ser-C27 mutant only reduced plumbagin oxidation by 33% in contrast to its severe effect on Q0, reduction (Table 1). Mutation of Tyr-D83 resulted in an elevated K_m for plumbagin, and the loss of the ability to oxidize reduced plumbagin was similar to the effect seen on Q0 reduction. The Arg-C31 and Asp-D82 mutants were severely affected in their ability to oxidize reduced plumbagin, similar to the effect seen with succinate-Q0 activity. These results con-
firm that Arg-C31 and Asp-D82 are integral to the quinone chemistry of the enzyme in both the forward and reverse direction, whereas Ser-C27 is more essential for quinone reduction than for menaquinol oxidation and Tyr-D83 probably is more essential for quinone binding rather than being a direct participant in the chemistry of the QP site.

The ability to complement growth of *E. coli* DW35 on aerobic minimal medium supplemented with succinate as the sole carbon source is a simple measure of *in vivo* functionality of the enzyme. Fig. 1 shows growth curves for cultures complemented with plasmids expressing each of the mutants. Cells harboring the wild-type enzyme on pFAS plasmid grew with a doubling time of 2.0 h. Mirroring the physiological activity of succinate oxidation, cells harboring plasmids expressing SdhCDAB, SdhC611DAB, and SdhCD823LAB failed to grow. Despite the significant loss of physiological activity *in vitro*, the mutants SdhCD33FAB, SdhCD33AAB, and SdhCD33KAB were still able to grow on succinate-supplemented minimal medium with doubling times of 2.7, 2.0, and 2.2 h, respectively. That we did not observe a more significant difference in growth rates given the wide range of enzymatic activities can be attributed to the high degree of enzyme expression from the pFAS plasmid.

The ability of the mutant SQR to complement growth in *E. coli* DW35 in anaerobic glycerol-fumarate minimal medium was also examined. None of the mutants was able to support growth, whereas *E. coli* DW35 harboring the wild-type plasmid could (data not shown). *E. coli* SQR is a very poor fumarate reductase, and when this activity is impaired the enzyme can no longer support growth on glycerol-fumarate medium despite the high expression levels.

### TABLE 1

Characterization of SdhCDAB activities in the different mutant SQR

| Mutant         | Succinate/Q<sub>0</sub> turnover x10<sup>7</sup> min<sup>-1</sup> | K<sub>M</sub> Q<sub>0</sub> µM | Plumbagin/fumarate turnover x10<sup>7</sup> min<sup>-1</sup> | K<sub>M</sub> plumbagin µM | SQ signal |
|----------------|---------------------------------------------------------------|---------------------------|---------------------------------------------------------------|---------------------------|-----------|
| DW35           | 0                                                             | ND                        | 0                                                             | ND                        | No        |
| SdhCDAB        | 26 ± 2                                                        | 0.24 ± 0.01               | 16 ± 1                                                         | 80 ± 1                    | Yes       |
| SdhC511DAB     | 1.1 ± 0.1                                                     | ND                        | 10.6 ± 0.8                                                    | 80 ± 2                    | No        |
| SdhC611DAB     | 0.6 ± 0.1                                                     | ND                        | 1.0 ± 0.1                                                     | 53 ± 2                    | No        |
| SdhC823LAB     | 0.5 ± 0.1                                                     | ND                        | 2.3 ± 0.3                                                     | 81 ± 1                    | No        |
| SdhC33FAB      | 3.9 ± 0.3                                                     | 0.28 ± 0.01               | 4.9 ± 0.6                                                     | 260 ± 6                   | No        |
| SdhC33AAB      | 7.2 ± 0.6                                                     | 0.93 ± 0.03               | 8.8 ± 0.7                                                     | 283 ± 4                   | No        |
| SdhC33KAB      | 6.7 ± 0.5                                                     | 0.57 ± 0.04               | 6.8 ± 0.7                                                     | 134 ± 2                   | No        |

**FIGURE 1.** Growth of *E. coli* DW35 cells harboring plasmids expressing wild-type and mutant SQR on minimal medium supplemented with succinate (A) glycerol-fumarate (B). ●, DW35 host; ○, SdhCDAB; ■, SdhC511DAB; ●, SdhC611DAB; ▲, SdhC823LAB; ◆, SdhC33FAB; ○, SdhC33AAB; ✡, SdhC33KAB.
Impairment of the Q Binding Site Disrupts Heme Reduction

The redox state of the heme can be measured spectrophotometrically at 560 nm. In this way, it is possible to observe whether the heme sandwiched between the membrane subunits participates in the electron transfer relay. Heme reduction was monitored following the addition of 10 mM succinate. The rate of reduced heme appearance as well as the extent of heme reduction once equilibrium was reached. These data suggest that impairment of the Q-site is directly responsible for the impediment to heme reduction.

**EPR Spectroscopy of Mutant SQR**—To determine whether the effects on heme reduction could be related to defective quinone reduction, we examined membranes enriched for wild-type or mutant enzyme using EPR and focused on the SQ radical intermediate. An SQ radical signal has been previously identified in *E. coli* fumarate reductase as well as preparations of submitochondrial particles (33). An SQ intermediate has yet to be confirmed in *E. coli* SQR, although an SQ has been detected by EPR in eukaryotic membrane preparations (34) as well as in *Paracoccus denitrificans* (35).

We were able to visualize a radical signal at $g = 2.005$ in the membranes expressing wild-type SdhCDAB in both "as is" air-oxidized membranes as well as a significantly larger signal under turnover conditions (Fig. 3). Neither signal was present in the background *E. coli* DW35 membranes. Under turnover conditions, the EPR line shape is slightly altered with wings characteristic of a FAD radical (7). A conventional potentiometric redox titration on *E. coli* DW35 membranes expressing wild-type SdhCDAB identifies two radical species within the sample (Fig. 4). These species can be effectively resolved along the potential domain into a high potential species, which we identify as the SQ signal, and a low potential species, due to a radical at the FAD cofactor in SdhA. The low potential signal was highly attenuated in membranes not activated with malonate or membranes containing oxaloacetate, which when bound to the active site can interact directly with the FAD (data not shown). Curve fitting of this FAD signal reveals a midpoint potential of $-148 \text{ mV}$ at pH 8.0 for the two-electron reduction of FAD to $\text{FADH}_2$. This is in reasonable agreement with the parameters previously reported by us (7).

The higher potential SQ signal has a midpoint potential of 60 mV at pH 8.0. Both the $E_{\text{m1}}(\text{SQ/Q})$ and $E_{\text{m2}}(\text{QH}_2/\text{SQ})$ transitions had equivalent values, which equates to 33% of the quinone being EPR visible ($K_{\text{STAB}} \sim 1.0$). That the SQ signal has a smaller amplitude than the flavin signal is likely due to a lower occupancy at the $Q_p$ site (ubiquinone concentration depend-
ent) compared with the FAD site (covalently bound flavin). It is likely that the growth conditions used resulted in a large concentration of menaquinone compared with ubiquinone in the membrane preparations used.

In contrast to the wild-type enzyme, when redox titrations were performed on the Q-site mutants by EPR, each of these mutants retained the FAD radical signal whereas they all lacked the SQ signal (Table 1). The FAD signal appears at the same midpoint potential as that seen in the wild type, yet no other signal is observed at any other potential. Representative redox titrations for the mutants SdhCS27ADAB and SdhCDY83FAB are shown in Fig. 5.

DISCUSSION

E. coli SQR shares significant sequence and structural similarity with its mitochondrial ortholog. Although most of the similarity is found within the two membrane-extrinsic subunits, there are significant portions within the membrane domain that contain a number of very highly conserved residues, mainly surrounding the heme moiety and the Qp-site. In this study, we mutated several conserved residues within the Q-site that are poised in prime positions for a direct interaction with the bound substrate according to the high resolution crystal structure (2). The locations of the chosen residues are shown in Fig. 6.

We investigated the possible roles of residues Ser-C27, Arg-C31, Asp-D82, and Tyr-D83. We found that each mutant, although expressed to equivalent levels compared with wild-type enzyme, had a serious defect in the ability to reduce ubiquinone. Mutations in the three former residues resulted in enzymes inactive in quinone reduction, whereas mutations in Tyr-D83 led to active enzymes although with significantly reduced physiological activities. This highlights the critical importance of these residues in defining the binding pocket and/or the mechanism of quinone reduction. Ser-C27, Arg-C31, and Asp-D82 have structural significance within the Qp-site as their side chains are in positions able to hydrogen bond to the ubiquinone. The loss of physiological activity associated with these mutants may be caused by loss of quinone binding.

Ser-C27 and Arg-C31 have previously been identified as key residues within the Q-site (36), and Tyr-D83 has been suggested to play an active role in reduction of the quinone as a possible proton donor due to its close proximity to the O1 oxygen of ubiquinone (2). The results in this communication, however, suggest that Tyr-D83 plays a more important role in binding of the quinone and is not essential for donating a proton to the quinone. Removal of the OH side chain in the Y83F mutant resulted in an 85% loss in activity but the $K_m$ of Q0 was not altered. Mutation to either Ala or Lys reduced activity 75% and additionally had an effect on quinone binding as evidenced by the increase in $K_m$ for Q0. This would suggest that Tyr-D83 plays a role in defining the proper architecture and orientation of the quinone in the binding site but is not essential for protonation of the quinone. Rather, another amino acid residue and/or water molecule may be the direct proton donor (37).

The mutations in these residues also had an effect on activity when the enzyme acted as a fumarate reductase. Mutants defective in Q0 reduction also had corresponding decreases in their plumbagin/fumarate activities, suggesting that both quinone reduction and oxidation occur at the same site within the
enzyme. Ser-C27 did not have as great an effect on plumbagin oxidation as was seen on \( Q_0 \) reduction. The differences in structure between ubiquinone and menaquinol may cause the molecules to bind and interact with different residues within the Q-site, so differences in activity in either direction can be expected. This result is consistent with recent observations with *E. coli* fumarate reductase where a slightly different orientation for menaquinone and ubiquinone have been shown for the \( Q_p \) binding site in that SQR paralog (38).

It was not anticipated that mutations made to the Q-site would have effects on electron transfer to the heme. Electrons at the [3Fe-4S] cluster face a bifurcating pathway to either the heme or the Q-site and would be expected to tunnel to both sites given the distances involved. Indeed, it has been proposed that the heme serves as an electron sink such that electrons are donated to the heme first, and when both the [3Fe-4S] and the heme are reduced, this would allow for the concomitant two-electron reduction of the bound quinone (37). In this mechanism, the heme would hold an electron until the [3Fe-4S] cluster was reduced, thus preventing formation of an SQ radical that could interact with oxidants in the native, aerobic environment in which the enzyme operates in vivo, in such a way as to prevent production of high amounts of reactive oxygen species resulting in oxidative stress that has been shown to be harmful to cells (39). Although such a hypothesis may be appealing, the results here do not indicate that this is the preferred mechanism.

The results presented here indicate that the quinone, not the heme, likely receives the first electron from the [3Fe-4S] cluster, although rapid electron equilibration between the heme and quinone cannot be ruled out (15). When we disrupt the Q-site through mutation, we observe that the rate of electron transfer to the heme is decreased. We conclude that a functional Q-site is required for optimal heme reduction. Electron tunneling rates can be affected by distances between, as well as midpoint potentials of, each cofactor, although the effect of the latter may be minimal (13). In the case of *E. coli* SQR, the heme is only slightly closer, edge-to-edge, to the [3Fe-4S] compared with the bound quinone. However, the midpoint potential of the ubiquinone pair is significantly higher than the midpoint potential of the heme \( (E_{m,c} = -15 \text{ mV}) \), suggesting that the quinone may be the preferred receptor of the electron from the [3Fe-4S]. Our results show that in the absence of a functional Q-site, there is still a slow leakage of electrons to the heme but compared with the wild-type enzyme the rate is radically slower. This low rate of electron transfer is surprising considering the close distance between these two centers. This distance between the [3Fe-4S] and the heme should lend itself to fast redox equilibrium regardless of the state of the Q-site, but given that the opposite is observed, perhaps a gating mechanism may be in place to prevent the electrons from tunneling directly to the heme from the [3Fe-4S] cluster. A potential candidate here is residue His-B207, which lies directly between the cluster and the heme. Its electron density in the crystal structure suggests a mobile side chain that could modulate electron flow between these redox centers (37). His-B207 is in direct proximity to the [3Fe-4S] cluster, the bound ubiquinone, and a heme \( b \) propionate. Any hydrogen-bonding network in this vicinity would likely override the heme, and an empty Q-site may disrupt this network.

Using potentiometric methods in combination with EPR spectroscopy, we have demonstrated for the first time the presence of a ubisemiquinone intermediate bound to the \( Q_p \) site of *E. coli* SQR. This SQ signal can be convincingly separated from any interfering FAD radical along the potential domain. Given the stability of the SQ, it is likely that regular turnover of the enzyme occurs through this radical intermediate. A ubisemiquinone has also been demonstrated in *E. coli* menaquinol:fumarate oxidoreductase, but only in a site-specific mutant (FrdC-E29L) (38). It is perhaps notable that it is the wild-type form of *E. coli* SQR in which the SQ is observed.

Moreover, all the mutants studied here lacked the SQ signal as only the FAD radical was observed in the EPR experiments. There is a correlation between loss of enzyme activity and lack of a ubisemiquinone intermediate. This effect may be due to either the inability to bind quinone or a decrease in the stability of the radical. The latter may be the case in the Tyr-D83 mutants that knockout the radical signal but retain some activity. In this instance, the stability of the quinone may be so reduced that little or no EPR signal may be detected.

We propose a mechanism in which electrons derived from succinate oxidation at the FAD tunnel along the [Fe-S] relay until the [3Fe-4S] cluster. These electrons are subsequently transferred to an awaiting ubiquinone molecule nested within the Q-site, although there is a small possibility that this electron may tunnel to the heme instead. Following the first single electron reduction step, a semiquinone radical species is formed that may be stabilized by any number of the residues that were
discussed in this report. The free electron of the SQ may then tunnel back and forth between the heme and the quinone in equilibrium prior to a second electron arriving from the [3Fe-4S] cluster to provide full reduction of the ubiquinone to ubiquinol. In this way, the heme cofactor may still behave as an electron sink, but its role is to prevent the interaction of the SQ with molecular oxygen by providing a more suitable oxidizing agent. Mutations that create anomalies in the Q-site or around the heme will surely impair these processes and are likely to result in an increase in the levels of reactive oxygen species generated by the enzyme as well as have functional consequences on enzyme activity. Although the root cause of many of the Complex II-associated diseases remains a mystery, the fact that many of these mutations are found in the same areas as described in this study may again suggest that disruption of enzymatic activity and possible reactive oxygen species production could be the key to understanding these disease states.

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