Fumarate Reductase and Succinate Oxidase Activity of Escherichia coli Complex II Homologs Are Perturbed Differently by Mutation of the Flavin Binding Domain

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The Escherichia coli complex II homologues succinate:ubiquinone oxidoreductase (SQR, SdhCDAB) and menaquinol:fumarate oxidoreductase (QFR, FrdABCD) have remarkable structural homology at their dicarboxylate binding sites. Although both SQR and QFR can catalyze the interconversion of fumarate and succinate, QFR is a much better fumarate reductase, and SQR is a better succinate oxidase. An exception to the conservation of amino acids near the dicarboxylate binding sites of the two enzymes is that there is a Glu (FrdA Glu-49) near the covalently bound FAD cofactor in most QFRs, which is replaced with a Gln (SdhA Gln-50) in SQRs. The role of the amino acid side chain in enzymes with Glu/Gln/Ala substitutions at FrdA Glu-49 and SdhA Gln-50 has been investigated in this study. The data demonstrate that the mutant enzymes with Ala substitutions in either QFR or SQR remain functionally similar to their wild type counterparts. There were, however, dramatic changes in the catalytic properties when Glu and Gln were exchanged for each other in QFR and SQR. The data show that QFR and SQR enzymes are more efficient succinate oxidases when Gln is in the target position and a better fumarate reductase when Glu is present. Overall, structural and catalytic analyses of the FrdA E49Q and SdhA Q50E mutants suggest that coulombic effects and the electronic state of the FAD are critical in dictating the preferred directionality of the succinate/fumarate interconversions catalyzed by the complex II superfamily.

Succinate dehydrogenase (succinate:ubiquinone oxidoreductase (SQR)), complex II and fumarate reductase (menaquinol:fumarate oxidoreductase (QFR)) couple the interconversion of succinate and fumarate with quinone and quinol. Succinate dehydrogenase is part of the aerobic respiratory chain and citric acid cycle of most organisms, whereas fumarate reductase is found in anaerobic or facultative bacteria and lower eukaryotes that live a portion of their life cycle in a reduced oxygen environment. Based upon amino acid sequence analysis, biochemical studies, and their overall structures, it has been proposed that both enzymes arose from a common evolutionary ancestor (2–4). Succinate dehydrogenase and fumarate reductase from Escherichia coli are composed of four nonidentical subunits organized into two domains. A membrane-extrinsic domain comprises two polypeptide chains: a 64–66-kDa flavoprotein subunit, containing a covalently bound FAD cofactor and the substrate binding site, and a 27-kDa iron-sulfur subunit containing three iron-sulfur clusters ([2Fe-2S]2+, [4Fe-4S]3+, and [3Fe-4S]4+). The membrane-extrinsic domain is bound to the membrane through interactions with the hydrophobic subunits of the complex. These subunits comprise two membrane anchor polypeptides, each containing three transmembrane helices and providing a binding site(s) for quinone (for reviews, see Refs. 5–8). In addition, the E. coli SQR hydrophobic peptides bind one b-type heme, whereas the E. coli QFR lacks heme.

Comparison of the structures of complex II is possible due to the availability of x-ray crystallographic structures for both SQR and QFR of E. coli (9, 10), the porcine SQR (11), the QFR from Wolinella succinogenes (12), and soluble homologs of the flavoprotein subunit that function as periplasmically localized fumarate reductases (13–16). The flavoprotein subunits from the E. coli SQR and QFR are highly homologous, with 64% similarity and 44% identity of amino acid residues (3). The sequence similarity within the SQR/QFR superfamily is reflected in structural alignments of members whose structures are known (17–19). Within this group, the backbone Cα atoms can be superimposed with a maximal root mean square deviation of 1.5 Å (10). A detailed hydride transfer mechanism for fumarate reduction has been proposed based on structural data and enzyme assays of wild type and mutant enzymes (15, 18–20). QFRs and SQRs all contain covalently bound FAD and are bidirectional (i.e. they will catalyze both succinate oxidation and fumarate reduction). There are, however, significant differences for the kinetics of fumarate reduction by fumarate reductase and succinate dehydrogenase. In addition to conventional steady-state solution kinetics, these differences have been measured by electro-
Succinate Dehydrogenase and Fumarate Reductase

chemical experiments (protein film voltammetry) in which the enzymes exhibit high electrocatalytic activity when adsorbed on a graphite electrode. In these studies, SQR is proficient at reducing fumarate above an electrode potential of approximately -60 mV (pH 7.0), whereas catalysis is severely constrained at potentials below this value (21, 22). This electrochemical property of succinate dehydrogenase has been described as being analogous to that of a tunnel diode, which is a device displaying negative resistance in a certain potential region (21, 22). Native QFRs by contrast, exhibit normal positive order kinetics (i.e. the rate of fumarate reduction increases with increasing thermodynamic driving force). The reasons for the differences in catalytic activity between the enzyme complexes remain unclear but are likely to relate to differences between the FAD and/or dicarboxylic acid binding sites of SQR and QFR (23, 24).

The remarkable degree of conservation of amino acid residues involved in substrate binding and interaction with the flavin in SQR and QFR has a notable exception. In E. coli FrdA, Glu-49 is located within 5 Å of the FAD cofactor, whereas in E. coli SdhA, a Gin residue (Gln-50) is found at this position. The majority of enzymes classified as QFRs contain a Glu at this position, whereas all SQRs contain a Gin. The soluble fumarate reductase homologs are exceptions, since they contain an Ala at this position. However, their structures reveal that this location is filled by the propionate group of a heme moiety, not present in the QFR/SQR family, which conserves the negative charge found in the QFRs. It should also be noted that the soluble flavoprotein homologs contain a noncovalently bound FAD cofactor and are essentially unable to oxidize succinate (25).

In this paper, we compare the catalytic and dicarboxylate binding effects of Glu, Gin, and Ala residues at position 49 in E. coli FrdA and position 50 in E. coli SdhA. The results show that Ala substitutions do not significantly alter the catalytic properties of QFR or SQR. Substitution at this position with the residue found in the complementary enzyme has profound effects on catalytic activity, with the SdhA-Q50E SQR mutant becoming a more efficient fumarate reductase, whereas the FrdA-E49Q QFR becomes a better succinate dehydrogenase. These substitutions, however, give no detectable alteration of the kinetic properties related to the “tunnel diode” effect. The results are discussed in the context of altered substrate binding, supported by an x-ray structure of the FrdA E49Q mutant QFR, and effects on the electronic status of the FAD moiety.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

E. coli strain DW35 (ΔfrdABCD, sdhC::kan), which was used as the host for expression of wild type and mutant forms of SQR and QFR, has been previously described (26). Plasmid pH3 (frdA+ B+ C+ D+) was used for expression of wild type QFR (26), and plasmid pFAS (Pfrd, sdhC+ D+ A+ B+) was used for expression of wild type QFR (27). Plasmid pFAB-HT (frdA+ B+) containing a C-terminal His tag has been previously described (24) and was used for expression of the two-subunit soluble fumarate reductase (FrdAB).

Mutagenesis

Plasmids pH3 and pFAS were used as the templates for mutagenesis of the frdA and sdhA genes in QFR and SQR. Plasmid pFAB-HT was used as template for mutagenesis of frdA in the soluble FrdAB enzyme. Site-directed mutagenesis was performed utilizing the QuikChange site-directed mutagenesis kit from Stratagene (La Jolla, CA). Primers for mutagenesis were obtained from Qiagen (Valencia, CA). Nucleotide changes to construct the mutants are underlined. To construct the SdhA Q50A mutant, primer 5′-ACCGTTTCTGCGGAGGCG- GATTACCG-3′ and its reverse complement were designed. The SdhA Q50E mutant was constructed using the primer 5′-CCGTTTCTGCGGAGGCGATTACC-3′ and its reverse complement. Following mutagenesis, the 1390-base pair HindIII-BstXI fragment containing either the SdhA Q50E or Q50A mutation was cloned into pFAS for expression of the mutant enzyme.

For the QFR FrdA E49Q mutation, the primer 5′-CATACCGTT- GCTGCAAGGGGGTCC-3′ and its reverse complement were designed. The 1277-base pair BstEI-Apal fragment containing the FrdA E49Q mutation was cloned into pH3 for the expression of the four subunit QFR and into pFAB-HT for the expression of the two-subunit His-tagged fumarate reductase. The QFR FrdA Q49A mutant was constructed using primer 5′-CCGTTGCTGAGCGAGGGC- GCCGCTCGGC-3′ and its reverse complement. Cloning of this mutant into plasmid pH3 used a similar strategy to that used for the FrdA E49Q mutant. All mutations were confirmed by DNA sequencing using the University of California San Francisco biomolecular core facility. Plasmids encoding the mutations were subsequently transformed into E. coli DW35 for expression of mutant enzyme.

Growth Conditions

QFR, SQR, and FrdAB were expressed in DW35 cells harboring the appropriate plasmid and grown under microaerophilic conditions. Starter cultures (25 ml) in Luria-Bertani (LB) medium with 150 μg/ml ampicillin were grown overnight at normal aeration. These cells were then used to inoculate 2-liter flasks filled with 1.4 liters of Terrific Broth medium containing ampicillin (150 μg/ml), and the cells were grown overnight at 37 °C with moderate aeration (150 rpm) on a gyratory shaker. E. coli used to express soluble FrdAB was grown using an identical protocol for 16 h. Cells were collected by centrifugation (10 min at 5000 × g) and stored at -80 °C.

Enzyme Purification

Isolation of the membrane fractions enriched with wild type and mutant SQR and QFR enzymes was as previously described (28, 29). Protein extraction with the detergent Thesit (Anapoe C12E9 (polyoxyethylene 9-dodecyl ether; Anatrace Inc., Maumee, OH) and purification on Q-Sepharose fast flow chromatography were carried out as previously described for wild type and mutant QFR and SQR (28, 29). Purified enzyme fractions were pooled, concentrated under nitrogen using an Amicon cell and YM30 membrane, and stored at -80 °C. Isolation of the soluble His-tagged FrdAB suitable for protein film voltammetry was performed as previously described (24) using nickel affinity resin (Qiagen). Soluble FrdA E49QB mutant enzyme was purified by a protocol identical to that used for wild type FrdAB.

Activation of the Enzymes

FrdAB as isolated exhibits high initial rates of succinate oxidation and does not require preactivation. SQR and QFR as isolated, however, are only partially active due to the presence of tightly bound oxaloacetate at their active sites. To activate the enzymes, SQR and QFR were diluted to 1–2 mg of protein/ml in 30 mM bis-Tris-propane buffer, pH 7.0, 0.1 mM EDTA, 0.05% Thesit, 3 mM malonate and incubated for 20 min at 30 °C. Activated enzymes were stored on ice for the duration of the experiment.

Measurement of Enzyme Activity

The standard assay medium contained 30 mM bis-Tris-propane buffer (pH range 6.0–8.5), 0.1 mM EDTA, 0.006% Thesit, and 3 mM potassium cyanide at 30 °C.
**Succinate Oxidation**—The succinate oxidase reaction of SQR was monitored by the decrease of the absorbance at 600 nm in the presence of succinate, 1.5 mM phenazine ethosulfate, and 50 μM 2,6-dichlorophenol (ε\textsubscript{600} = 21.8 mM\textsuperscript{-1} cm\textsuperscript{-1} (pH 7.8)). The succinate-ferricyanide reductase activity of QFR was determined at 420 nm with 0.5 mM potassium ferrocyanide (ε\textsubscript{420} = 1 mM\textsuperscript{-1} cm\textsuperscript{-1}) and 20 mM succinate.

**Fumarate Reduction**—Fumarate reduction activities of QFR and SQR with quinol analogues were determined in a reaction coupled to DT diaphorase (NADH:quinate reductase) as previously described (30) with menaquinone (MQ\textsubscript{1}) and ubiquinone (UQ\textsubscript{1}) for QFR and SQR, respectively (MQ\textsubscript{1} and UQ\textsubscript{1} were kindly provided by Eisai Co. Ltd., Tokyo, Japan). Fumarate reduction with a 0.2 mM concentration of the low potential electron donors benzylioviolin (BV) or methylviologen was performed in 3-ml screw top cuvettes under a continuous flow of argon. Prior to initiation of the reaction, 10 mM glucose, glucose oxidase, and catalase were added to the assay medium to maintain anaerobiosis. A stoichiometric amount of sodium dithionite was added to reduce the viologens (starting absorbance ~1.8), and the reaction was initiated by the addition of either enzyme or fumarate. The progress of the reaction was monitored by the decrease of viologen absorbance at 602 nm (9.6 mM, pH 7.0), \( k_{\text{red}} \) was calculated using covalently bound FAD content.

**Electrochemistry**

The soluble FrdA domain containing the FrdA E49Q mutation was used for voltammetry experiments using a mixed buffer system at 25 °C as previously described (24, 31). A pyrolytic graphite edge rotating disk working electrode was used in conjunction with an EG&G model electrode rotator.

**Crystallization of FrdA E49Q**

Crystallization of the FrdA E49Q variant of *E. coli* QFR was performed by the hanging drop vapor diffusion method using 0.7 μl of protein solution (20 mg/ml, 20 mM Tris, pH 7.4, 0.7% Thesit) and 0.7 μl of mother liquor (13.5% polyethylene glycol 5000 monomethyl ether, 200 mM magnesium acetate, 100 mM sodium citrate, pH 5.6, 100 μM ethylene diamine tetraacetic acid, and 0.001% dithiothreitol) at a temperature of 20 °C. Protein crystals grew after 1–2 days and were cryo-cooled after 3–5 days. Prior to flash cooling in liquid nitrogen, crystals were quickly dipped into cryosolution containing mother liquor and 30% ethylene glycol.

**Diffraction Data Collection and Processing**

Diffraction data were collected at the European Synchrotron Radiation Facility (ESRF), beamline ID-13, using a wavelength of 0.9537 Å. Radiation damage was minimized by translating the crystals after every three degrees of data collection. Crystals belonged to the orthorhombic space group P2\textsubscript{1}2\textsubscript{1}2\textsubscript{1} with unit cell dimensions \( a = 96.80 \text{ Å}, b = 139.53 \text{ Å}, c = 273.97 \text{ Å} \) (Table 1). Data were processed using DENZO and SCALEPACK (32) and the CCP4 suite of programs (33). In order to improve the completeness, data from two crystals were merged (Table 1).

**Structure Solution and Refinement**

Since the crystals were isomorphous with previously determined structures (9, 34), the structure of QFR (Protein Data Bank code 1KF6), with the Frd Glu-49 side chain truncated to Ala, was subjected to rigid body refinement, geometric minimization, and simulated annealing using CNS (35) and then used as a starting model. Density of the side chain of Gln-49 was observed after this procedure, and the identity was changed to Gln. Model building was performed using the program O (36), and refinement was performed using both CNS (35) and REFMAC (37), whereas the quality of the stereochemical parameters was evaluated with the program PROCHECK (38). The final model of FrdA E49Q has an \( R_{\text{free}} \) of 0.246 and an \( R_{\text{cryst}} \) of 0.285. Geometric indicators of model quality are reasonable with root mean square deviation in bond lengths of 0.024, angles of 2.60°, and 74% of residues in the most favored region of the Ramachandran diagram (Table 1). Figs. 1 and 7 and supplemental Fig. S1 were made with MOLSCRIPT (39) BOBSCRIPT (40), and RASTER3D (41). The mutant structure was deposited in the Protein Data Bank with code 2B76.
**Succinate Dehydrogenase and Fumarate Reductase**

**TABLE 2**
Comparison of catalytic parameters for succinate-oxidase (Succ-ox) and fumarate-reductase (Fum-red) reactions catalyzed by *E. coli* wild type (WT) and mutant SQR enzymes and QFR enzymes

Enzymatic activities were assayed as described under “Materials and Methods” at 30 °C, pH 8.0.

|          | Succinate oxidation | | | | | Fumarate reduction | | | | | Catalytic efficiency ($k_{cat}/K_m$) | | | |
|----------|---------------------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
|          | $k_{cat}$ | $K_{m}$ | $K_{m}$ | $K_{m}$ | $K_{m}$ | $K_{m}$ | $K_{m}$ | $K_{m}$ | $K_{m}$ | $K_{m}$ | $K_{m}$ | $K_{m}$ | $K_{m}$ | $K_{m}$ | $K_{m}$ | $K_{m}$ |
|          | succinate/s⁻¹ | μM | mM | μM | mM | μM | mM | μM | mM | μM | mM | μM | mM | μM | mM | μM |
| WT SQR | 110.0 ± 4 | 110 ± 10 | 2 ± 0.2 | 0.07 ± 0.01 | 20.0 ± 0.2 | 100 ± 12 | 1000 | 20 |
| SdhA Q50A | 27.3 ± 0.5 | 60 ± 10 | 4 ± 0.5 | 0.10 ± 0.01 | 0.9 ± 0.1 | 90 ± 7 | 450 | 10 |
| SdhA Q50E | 1.0 ± 0.3 | 460 ± 30 | 750 ± 50 | 8.0 ± 0.5 | 0.5 ± 0.1 | 50 ± 5 | 2 | 10 |
| WT QFR | 30.0 ± 0.5 | 550 ± 38 | 25 ± 3 | 0.3 ± 0.03 | 250 ± 10 | 20 ± 2 | 54 | 222 |
| FrdA E49A | 2.4 ± 0.1 | 220 ± 15 | 10 ± 1 | 0.1 ± 0.02 | 32 ± 2 | 30 ± 0.5 | 11 | 97 |
| FrdA E49Q | 4.0 ± 0.2 | 110 ± 12 | 5 ± 1 | 0.06 ± 0.02 | 1 ± 0.4 | NA | 36 | NA |

*Activity for the FrdA E49Q enzyme was estimated by extrapolation to low fumarate concentration.*

*NA, value for $K_m$ for fumarate with the FrdA E49Q enzyme was difficult to determine due to strong fumarate inhibition.

**FIGURE 2.** Menaquinol-fumarate reductase reaction catalyzed by QFR and FrdA E49Q. The reaction was assayed as described under “Materials and Methods” under anaerobic conditions in the presence of NADH and DT diaphorase to continuously regenerate MQ$_2$. A, reduction of 15 μM fumarate by wild type QFR and FrdA E49Q by MQ$_2$H$_2$ regenerated in the presence of NADH and DT diaphorase. WT, wild type.

**Analytical Methods**

FAD content was determined as previously described (42). Protein concentration was determined by the BCA method (Pierce) with bovine serum albumin as a standard in the presence of 1% (w/v) SDS.

**Potentiometric Titrations and EPR Spectroscopy**

In order to obtain data as representative as possible of the characteristics for fumarate reduction and succinate oxidation of the mutants compared and are summarized in Table 2.

**SQR Mutants**—Alanine substitution of the target residue in SdhA (Gln-50) causes a minimal effect on the catalytic properties of the mutant enzyme. It retains high succinate oxidation and fumarate reductase activity and has no significant alteration of the kinetic parameters for inhibition by malonate or oxaloacetate. Glutamate substitution at the SdhA Gln-50 position, however, severely impairs succinate dehydrogenase activity, with the maximal observed $k_{cat}$ turnover number only 1% of wild type. Succinate oxidation catalyzed by this mutant still retains sensitivity to malonate and oxaloacetate inhibition (Table 2) but with $K_i$ values increased at least 2 orders of magnitude compared with wild type SQR. These data are consistent with a major effect on the binding of dicarboxylate substrates being introduced into the SQR substrate-binding site by substituting a charged residue (Glu) for the neutral Gln at SdhA position 50.

**QFR Mutants**—Elimination of the negatively charged side chain by introduction of Ala at FrdA Glu-49 results in enzyme that catalyzes succinate oxidation and fumarate reduction with a turnover number between 8 and 13% of wild type QFR. The $K_m$ values for succinate and fumarate and $K_i$ values for malonate and oxaloacetate are similar to wild type in the FrdA E49A mutant.
Glutamine substitution at FrdA Glu-49 results in mutant enzyme that demonstrates typical Michaelis-Menten kinetics in the succinate oxidase reaction and retains 13% of wild type QFR activity. The $K_{m}$ value for succinate and the $K_{i}$ values for malonate and oxaloacetate decrease 5-fold in this mutant. Analysis of the quinol-fumarate reductase reaction, however, reveals a new kinetic property of the mutant enzyme (i.e. strong substrate inhibition by fumarate) (Fig. 2A). In contrast to wild type QFR, the initial rate of the reaction catalyzed by the FrdA E49Q mutant is inhibited dramatically by increasing the fumarate concentration. Maximum activity can only be estimated at low fumarate concentrations (15 μM) and is 0.25% of wild type QFR activity (Table 2). The $K_{m}$ for wild type QFR is 20 μM (Table 2), but this concentration of the substrate strongly inhibits FrdA E49Q activity (Fig. 2A). The strong substrate inhibition exhibited by this mutant precludes any simple calculation of its kinetic parameters. Fig. 2B shows traces of the catalytic activity of wild type QFR and FrdA E49Q in response to low fumarate concentrations (15 μM) in an assay for fumarate reduction using DT diaphorase to continually recycle the quinol (30). With wild type QFR, the reaction is first-order with respect to fumarate concentration. By contrast, menaquinol-fumarate reductase activity of the FrdA E49Q mutant is initially inhibited by fumarate but increases as the substrate is utilized during the course of the assay (Fig. 2B) in the conditions when the stable reducing potential of MQ$_{2}$H$_{2}$ is supported by continuous regeneration of menaquinol by DT diaphorase in the presence of NADH.

**pH Dependence of the Succinate Oxidase Reaction**—The succinate dehydrogenase reactions catalyzed by QFR and SQR are strongly pH-dependent with pH profiles similar to those of a theoretical titration curve for deprotonation of a monobasic acid. E. coli SQR and QFR exhibit simple sigmoidal dependences with estimated $pK_{a}$ values of 7.3 and 7.5, respectively (Fig. 3). Both QFR mutants demonstrate an acidic shift in the $pK_{a}$ values to 6.9 for FrdA E49A and 6.6 for FrdA E49Q. The substitution of SdhA Q50A has no detectable effect on the pH profile of the reaction, whereas introducing a negative charge in SdhA Q50E results in a bell-shaped pH profile for succinate oxidation with maximum activity observed at pH 7.0.

**Protein Film Voltammetry Studies**—In light of the solution assays of the mutant FrdA E49Q, it was desirable to determine if the FrdA E49Q mutant would behave like wild type SdhAB and demonstrate tunnel diode-like behavior (21, 23) in protein film voltammetry (PFV) studies. To undertake the PFV studies, the two-subunit (FrdAB) soluble form of the mutant was constructed. Nonturnover voltammograms show sharp, prominent signals dominated by the cooperative two-electron oxidation and reduction of the FAD. At pH 7.0, the FAD reduction potential in FrdA E49Q mutant is $-115$ mV, whereas the value for wild type enzyme is $-88$ mV. The pH dependence of the FAD signal from the voltammogram was determined over the range 5.0–8.5 (Fig. 4A) and produced a linear gradient of $-38$ mV/pH unit for FrdA E49Q mutant with a range of fumarate concentrations. Increasing quantities of fumarate were added to the cell up to a concentration of 1 mM. After this, the cell was washed, and the enzyme-coated electrode was placed back into the cell with fresh, fumarate-free buffer. The resulting CV is demonstrated as a typical Michaelis-Menten reaction (24), although the data obtained by wild type FrdAB over this pH range reveal curvature suggesting that the “intermediate” gradient actually hides transitions between two-electron/two-proton and two-electron/one-proton reactions. Consistent
with the solution assays, which show strong fumarate inhibition, only a
small catalytic wave is observed in a voltammogram of the FrdA<sup>E49Q</sup>B
variant in the presence of fumarate (Fig. 4B). As the fumarate concen-
tration is increased to 10 mM, an increase in current (activity) is seen;
however, at higher substrate concentrations, the current decreases,
indicating substrate inhibition. After quickly washing the voltammetry
cell with buffer, the trace amounts of fumarate remaining are sufficient
to restore the higher catalytic activity of FrdAE49QB. Thus, the substrate
inhibition is reversible, and the decrease in current is not due to protein
film loss or denaturation effects. Although the FrdA<sup>E49Q</sup>B mutant dem-
strates low activities in both solution assays and PFV studies, the catalytic
current is a function of electrode potential as it is for FrdAB.
Typical SdhAB tunnel diode behavior, characterized by low activity at
low potentials is not observed in the FrdA<sup>E49Q</sup>B mutant.

In agreement with PFV studies, the solution experiments using the
benzyl viologen-fumarate reductase assay revealed that the substitution of
FrdA Glu-49 by Ala and Gln does not change the reaction profile; the
decrease in activity correlates with a decrease of the driving force (ox-
idation of reduced BV). Additionally, alteration of SdhA Gln-50 to either
Glu or Ala does not eliminate the tunnel diode behavior, suggesting that
other residues contribute to mediating this phenomenon (data not shown).

**EPR Study of Flavin Semiquinone**—In bovine SQR, the FAD semiqui-
none is clearly detectable by EPR at neutral or basic pH values (45).
Spectra of the flavin semiquinone have not been reported for either SQR
or QFR from *E. coli*. Fig. 5A shows representative spectra of redox-
poised EPR samples of membranes enriched in wild type and SdhA Q50E SQR.
The spectra are very similar to those reported by Ohnishi
*et al.* (45), exhibiting a line width (peak to trough) of 9 and 10 G for the
wild type and SdhA-Q50E mutant, respectively (Fig. 5A). Fig. 5B shows

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wild type and SdhA-Q50E mutant, respectively (Fig. 5A). Fig. 5B shows

the results of potentiometric titrations of the FAD semiquinone of the
wild type and mutant enzyme, generating estimates for the *E<sub>mo</sub>* of the
FAD/FADH<sub>2</sub> couple of −138 mV (E<sub>e</sub><sub>1</sub> − E<sub>e</sub><sub>2</sub> = 0 mV; K<sub>stab</sub> = 1.0) and
−190 mV (E<sub>e</sub><sub>1</sub> − E<sub>e</sub><sub>2</sub> = 75 mV; K<sub>stab</sub> = 18), respectively (Fig. 5B). Thus,
the SdhA-Q50E mutation elicits a change in potential (∆*E<sub>mo</sub>*) of −52 mV
for the FAD/FADH<sub>2</sub> couple and increases the amount of FAD semiqui-
none observable from 33 to ~68% of its total concentration.

The FAD semiquinone of *E. coli* QFR has not been thoroughly inves-
tigated by EPR spectroscopy. It was not detected by this technique in
studies of a menasemiquinone radical localized at the quinone-binding
site (Q<sub>p</sub>) (46). The menasemiquinone radical anion is sensitive to the
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**TABLE 3** Comparison of two-electron reduction potentials of covalently
bound FAD obtained by EPR and PFV (pH 8.0, 25°C)

| Enzyme | E<sub>mo</sub><sub>PFV</sub> (mV) | E<sub>mo</sub><sub>PFV</sub> (mV) |
|--------|-----------------|-----------------|
| SQR    | −138 ± 10       |                 |
| SdhA Q50E | −190 ± 10       |                 |
| QFR    | −185 ± 10       | −122 ± 5        |
| FrdA E49Q | −185 ± 10       | −153 ± 5        |

FIGURE 5. EPR and potentiometric characterization of SQR and SdhAQ50E mutant.
A, EPR spectra of wild type SQR- and SdhA Q50E-enriched membranes poised at E<sub>e</sub><sub>1</sub> values of approximately −140 and −190 mV, respectively. EPR spectra were recorded under
the following conditions: temperature, 150 K; microwave power, 20 milliwatts at 9.437
GHz; modulation amplitude, 2 Gpp at 100 KHz. B, plots of semiquinone signal intensity
versus E<sub>e</sub><sub>1</sub> for wild type SQR (triangles) and SdhA Q50E mutant (squares). Data were fit to
E<sub>mo</sub> and E<sub>e</sub><sub>1</sub> − E<sub>e</sub><sub>2</sub> values as described under “Results.” 100% of the signal intensity corre-
sponds to 33 and 68% as a percentage of total FAD concentration for SQR and SdhA
Q50E, respectively, WT, wild type.

FIGURE 6. EPR and potentiometric characterization of the QFR FrdAE49Q mutant. A,
EPR spectra of wild type QFR and FrdAE49Q poised at E<sub>e</sub><sub>1</sub> values of approximately −171
and −195 mV, respectively. EPR spectra were recorded under the following conditions:
temperature, 150 K; microwave power, 2 milliwatts at 9.437 GHz; modulation amplitude,
2 Gpp at 100 KHz. B, plots of semiquinone signal intensity versus E<sub>e</sub><sub>1</sub> for the wild type
(triangles) and the FrdA E49Q mutant (squares). For the FrdA E49Q mutant, data were fit
to an E<sub>mo</sub> of −185 mV and an E<sub>e</sub><sub>1</sub> − E<sub>e</sub><sub>2</sub> value of −80 mV (K<sub>stab</sub> = 2.15) as described under
“Results.” 100% of the signal intensity corresponds to 70% of total FAD concentration for
FrdAE49Q. WT, wild type.
The FAD semiquinone titrates with an $E_{\text{m}}$ of $-185 \pm 10$ mV at pH 8 (Fig. 6B), comparable with the value $-153 \pm 5$ mV obtained by voltammetry under similar conditions (Table 3), and shows increased stability ($E_1 - E_2 = 80$ mV; thus, $K_{\text{stab}} = 21.5$) in the FrdA E49Q variant. Interestingly, the FAD semiquinones in the resulting FrdA E49Q and SdhA Q50E proteins show strong similarities in their EPR properties (Figs. 5 and 6). We have not analyzed in detailed the pH dependence of the FAD radical; however, at pH 7.0, the intensity of the radical in the wild type SQR remained at about 70% of that determined at pH 8.0, whereas in the SdhA Q50E variant, the FAD radical concentration is negligible (data not shown), indicating that stabilization of the radical in the mutant involves a change in protonation state of the FAD environment.

**Succinate Dehydrogenase and Fumarate Reductase**

**FIGURE 7. Comparison of the active site structures in wild type (Protein Data Bank code 1KF6) and FrdA E49Q QFR (Protein Data Bank code 2B76).** The wild type (teal) and FrdA E49Q variant (yellow) are superimposed in the same view as Fig. 1. Only the main chains from the E49Q variant are shown for clarity. Oxygen atoms are in red, nitrogen atoms are in blue, and carbon atoms follow the color of the molecule. Little structural perturbation is observed, with a slight movement of the side chain functional group of Arg-287. However, an altered hydrogen-bonding pattern is observed, with a new hydrogen bonding interaction to the side chain of Glu-245, altered directionality of the hydrogen bond to Arg-287, and a putative hydrogen-bonding interaction with the N-5 of the FAD. The latter two have been shown to directly participate in the fumarate reduction reaction.

The mutations did, however, dramatically impair the catalytic activity for each enzyme in their respective physiological direction. As a result, the FrdA E49Q mutant becomes a better succinate dehydrogenase than fumarate reductase, and SdhA Q50E becomes more efficient in catalyzing fumarate reduction relative to succinate oxidation. The negative charge within close proximity to FAD and the dicarboxylate binding site may cause significant long range coulombic effects. This may result in changes in enzyme conformation, substrate binding parameters, and altered flavin electronic properties, including changes in reduction potential and stability of the flavinsemiquinone.

**DISCUSSION**

**Change in Catalytic Efficiency of the SQR and QFR Mutant Enzymes—** This study was conducted in order to investigate the role of the highly conserved Glu-49/Gln-50 residue found near the dicarboxylate binding site of the flavoprotein subunit of \textit{E. coli} QFR and SQR. Comparison of available structures of complex II type enzymes (9 –16) does not provide an obvious suggestion for the importance of a negative charge or polar group at this position. One of the prominent differences between SQR and QFR, known as a tunnel diode effect, was observed in several SQR but not QFR enzymes and has been associated with the FAD site of the proteins (21 –24). We anticipated that the Glu/Gln residue $\sim 5$ Å from the FAD redox center may contribute to the tunnel diode phenomenology. In the present work, this hypothesis was examined by constructing mutants where the Glu/Gln residues were exchanged in the FrdA Glu-49 and SdhA Gln-50 positions. Both kinetic and protein film voltammetry analysis of the mutants exclude an important role of a charge versus polar residue in the target position for the tunnel diode effect. The mutations did, however, dramatically impair the catalytic activity for each enzyme in their respective physiological direction. As a result, the FrdA E49Q mutant becomes a better succinate dehydrogenase than fumarate reductase, and SdhA Q50E becomes more efficient in catalyzing fumarate reduction relative to succinate oxidation. The negative charge within close proximity to FAD and the dicarboxylate binding site may cause significant long range coulombic effects. This may result in changes in enzyme conformation, substrate binding parameters, and altered flavin electronic properties, including changes in reduction potential and stability of the flavinsemiquinone.

The direct comparison of catalytic efficiency of wild type and mutant \textit{E. coli} SQR and QFR (Table 2) provides an opportunity to understand catalytic differences between members of the SQR/QFR superfamily. In studies carried out using conventional steady-state solution kinetics, SQR was 50-fold more proficient in oxidizing succinate than reducing acetate. Previous structure determinations of \textit{E. coli} QFR are likely to have citrate bound at the active site, but the density was misinterpreted as cis-oxaloacetate. This can be explained, because QFR as isolated is a mixture of enzyme containing oxaloacetate at the dicarboxylate binding site and free (uninhibited) enzyme (\textit{i.e.} only some of the enzyme molecules have oxaloacetate bound) (see supplementary Fig. S1). The additional electron density in the FrdA E49Q variant also indicates that citrate is bound at the active site in this variant, and several new hydrocarbon binding interactions are formed, one to the side chain of FrdA Glu-245 and one to the N-5 of the FAD (Fig. 7). In addition, a significant alteration of hydrogen bonding at the active site is caused by the side chain guanidine of Arg-287, which is the proposed proton shuttle during fumarate reduction. Both Arg-287 and the N-5 of the FAD are directly involved in catalysis. Taken together, the altered protonation pattern at the active site in the E49Q variant highlights the importance of this side chain.

More substantial are the structural alterations in the second copy of QFR in the asymmetric unit (chains M–P). The density for the soluble domain of this second copy of QFR is of significantly poorer quality than that of the first copy (chains A–D). Despite this, it is clear that the citrate bound at the active site occupies a position distinct from that observed in molecule 1 (chains A–D) and all other structures of complex II homologs determined to date. Shifts in the polypeptide backbone of the soluble subunits are large and are observed over $20 \text{ Å}$ away from the active site, resulting in a new conformation of the soluble domain of the enzyme. Further study is needed to resolve the physiological relevance of the conformational differences in the second copy of the FrdA E49Q QFR.
fumarate (although note that voltammetry on the soluble enzyme suggests that fumarate reduction is effective only when the driving force is very small), whereas QFR was 230-fold more proficient as a menaquinol-fumarate reductase. Kinetic analysis of the alanine variants in both enzymes reveals that these substitutions decrease the catalytic efficiency in both succino-oxidase and fumarate reductase directions, but both enzymes continue to function as a succinate dehydrogenase and fumarate reductase, respectively. The FrdA E49Q and SdhA Q50E substitutions, however, cause dramatic changes in the catalytic properties of the mutant enzymes. The SdhA Q50E substitution results in a 500-fold reduction in the ability of the enzyme to oxidize succinate, but this is accompanied by only a 2-fold reduction in the ability to reduce fumarate. The $k_{cat}/K_m$ ratio of succinate oxidation catalyzed by the FrdA E49Q variant remained at 65% of that of the wild type. The major kinetic effect observed in the mutant is strong substrate inhibition by fumarate, attributed only to the fumarate reductase direction, whereas the succinate oxidation reaction retained normal Michaelis-Menten parameters. The change in catalytic efficiency reflects both a decrease of $k_{cat}$ and changes in the ligand binding properties. In the wild type enzymes, dicarboxylate site inhibitors generally have higher affinities for SQR than QFR. In the mutants, there is a change in the relative ligand affinities, as evidenced by changes in the $K_i$ for the inhibitors malonate and oxaloacetate (Table 2). The SdhA Q50E mutant exhibits binding parameters that are remarkably similar to wild type QFR (Table 2). The converse is observed for the FrdA E49Q enzyme, which has increased affinity for the dicarboxylates such that the $K_i$ values are similar to wild type SQR. The differences in affinity of the dicarboxylate site inhibitors for oxidized (succinate oxidase direction) and reduced enzymes (fumarate reductase direction) observed for enzymes from the complex II superfamily suggest structural changes at the substrate binding site upon enzyme reduction; however, no x-ray structures of the reduced form of SQR/QFR homologs are available. Fig. 7 demonstrates that there are some alternations in hydrogen bonding between the citrate dicarboxylate carboxyl group and residues thought to be involved in the proton transfer pathway (FrdA Arg-287 and Glu-245). It is possible that structural changes resulting from the FrdA E49Q substitution do not significantly affect the substrate binding site; however, they may make it somehow more compact, as reflected by decreased binding parameters for the inhibitors. More dramatic changes in the active site structure are expected upon reduction of the variant enzyme, and the second fumarate molecule could be hydrogen-bonded to FrdA Arg-287/Glu-245, thus preventing catalytic turnover and explaining the fumarate inhibition.

**Properties of FAD**—Both EPR titrations and PFV were used to study the effect of the mutations on the properties of FAD. PFV is very useful for investigating the properties of the redox centers of soluble FrdAB; however, these centers are not clearly resolved in soluble SdhAB. Conversely, stabilized EPR-detectable FAD radicals are observed in wild type SQR but not in QFR. As seen in Figs. 5 and 6, the reciprocal Glu/Gln mutations in SQR and QFR resulted in increased stabilization of the FAD radical compared with their wild type counterparts. The correlation between EPR line width and optical data on neutral and anionic semiquinone has been previously established (47). The differences in line width of the flavin radicals appear to be a reflection of the fact that these two species of flavin have significantly different hyperfine interaction constants with the various magnetic nuclei of the isoalloxazine ring. Thus, all three EPR-detecatable FAD radicals detected here for SQR, SdhA Q50E, and FrdA E49Q appear to be anionic at pH 8.0 based on the narrow EPR signal line width of 9–10 G. The $pK_a$ value of N-5 of the FAD semiquinone in bovine SQR remains controversial. The pH analysis of the line width of the FAD radical clearly suggests that the narrow line width of 12 G observed at both pH 6.1 and 9.1 is indicative of an anionic flavosemiquinone (48). However, in a different EPR study (45), the FAD radical is suggested to be neutral below pH 8.0 and anionic above. It should be noted that the $pK_a$ value for N-5 of the FAD semiquinone determined for E. coli QFR by PFV was also suggested to be 8.15 (24).

The reduction potential for the FAD in E. coli SQR has not been previously reported. Table 3 summarizes data on $E_{m\text{r}}$ for FAD in the SQR/QFR enzymes used in this study. $E_{m\text{r}}$ values for the FAD in E. coli SQR and QFR are found to be −138 and −122 mV, respectively, at pH 8.0. Interestingly, both variants demonstrate a moderate decrease in FAD potential, −52 mV in SdhA Q50E and −31 to −62 mV in FrdA E49Q. The $E_{m\text{r}}$ values for FAD in FrdA E49Q determined by PFV and EPR methods are in good agreement, and the −30-mV lower value determined by EPR (within experimental error) may reflect the presence of malonate, as has been previously shown for QFR and SQR (24, 49). The decrease in $E_{m\text{r}}$ for FAD in both FrdA E49Q and SdhA Q50E variants may partly explain the decrease in succinate oxidase activity for both mutant enzymes.

The two-electron reduction potential of FAD in bovine SQR shows a −60 mV/pH dependence below pH 7.7 and −30 mV/pH above (45). The observed $pK_a$ of 7.7 can be assigned to protonation of N-1 in the isoalloxazine ring in the reduced flavin (50). The mechanism of fumarate reduction/succinate oxidation involves hydride transfer from the N-5 position of the isoalloxazine ring of the FAD, implying that N-5 must be protonated in QFR upon flavin reduction to be catalytically competent (18). Cyclic voltammetry experiments with SdhAB from bovine and E. coli SQR suggest a straight line in pH dependence of the FAD midpoint potential with a slope of −60 mV (2H$^+$/2e$^-$) in the pH 6–8 interval, indicating that the $pK_a$ of N-1 for reduced FAD is expected to be close to pH 8.0 (24). E. coli FrdAB, by contrast, showed a linear dependence in the pH range of 5–8 that is close to −30 mV, corresponding to a 1H$^+$/2e$^-$ ratio and suggesting that N-1 in reduced FAD should be deprotonated above pH 5.0 (24). Thus, changes in the protein environment around the isoalloxazine moiety of FAD affect the properties of the molecule that result in changes in the reduction potential and increased stabilization of the FAD radical.

Hydride transfer between N-5 of FAD and substrate is a key step in catalysis. Based on structural and mutagenesis studies, FrdA Arg-287 is in a suitable position for direct proton donation to C-5 of fumarate (9, 12, 13, 18). The putative proton pathway to FrdA Arg-287 involves FrdA Glu-245 and FrdA Arg-248; however, the proton pathway to reproto- nate N-5 of FAD is not obvious. In the W. succinogenes QFR structure determined in the presence of fumarate (12), the residue (FrdA Gln-48) equivalent to FrdA Glu-49 is hydrogen-bonded through its amide nitrogen to a water molecule, which further forms a hydrogen bond to N-5 of the flavin. This would be one potential pathway for reproto- nation of the N-5 of the FAD. There is no water molecule found near the flavin N-5 position included in the models of either the E. coli QFR or SQR structures (10, 34). Since these two structures were both determined in the presence of dicarboxylate-binding inhibitors and at a resolution where water molecules were added conservatively, it is difficult to evaluate if an ordered water molecule would be located in that position when substrate is bound. Replacement of FrdA Glu-49 with a Gln residue may perturb the binding of a catalytically relevant water molecule and thus affect the reproto- nation of N-5. Significant stabilization of the FAD radical in SdhA Q50E at pH 8.0 may indicate altered proton uptake/release from the reduced FAD upon ionization of Glu-50 that results in an inhibition of the succinate oxidase reaction.
Conclusions—The data presented here show that mutation of FrdA Glu-49 or SdhA Gln-50 has effects on the electronic properties of the covalently bound flavin moiety. Additionally, substrate binding is significantly changed by alteration of these amino acids such that fumarate reductase becomes more like succinate dehydrogenase with glutamine in the FrdA 49-position, and SQR with glutamate in the SdhA 50-position is more efficient as a fumarate reductase. Although redox potentials are known to control the direction of electron transfer, we believe this study also demonstrates that other factors, including coulombic effects investigated here, may be of importance in controlling the direction of the reaction.

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