Plasticity of the Quinone-binding Site of the Complex II Homolog Quinol:Fumarate Reductase*

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Background: Different quinone substrates are used by complex II.

Results: Structural and kinetic analyses show that two arginine residues modulate the enzyme interaction with different quinones.

Conclusion: Specific arginines compensate for each other in proton transfer during quinone oxidoreduction in the complex II homolog fumarate reductase.

Significance: Plasticity in quinone binding may be important for bioenergetic transformations.

Respiratory processes often use quinone oxidoreduction to generate a transmembrane proton gradient, making the 2H+/2e− quinone chemistry important for ATP synthesis. There are a variety of quinones used as electron carriers between bioenergetic proteins, and some respiratory proteins can functionally interact with more than one quinone type. In the case of complex II homologs, which couple quinone chemistry to the interconversion of succinate and fumarate, the redox potentials of the biologically available ubiquinone and menaquinone aid in driving the chemical reaction in one direction. In the complex II homolog quinol:fumarate reductase, it has been demonstrated that menaquinol oxidation requires at least one proton shuttle, but many of the remaining mechanistic details of menaquinol oxidation are not fully understood, and little is known about ubiquinone reduction. In the current study, structural and computational studies suggest that the sequential removal of the two menaquinol protons may be accompanied by a rotation of the naphthoquinone ring to optimize the interaction with a second proton shuttling pathway. However, kinetic measurements of site-specific mutations of quinol:fumarate reductase variants show that ubiquinone reduction does not use the same pathway. Computational docking of ubiquinone followed by mutagenesis instead suggested redundant proton shuttles lining the ubiquinone-binding site or from direct transfer from solvent. These data show that the quinone-binding site provides an environment that allows multiple amino acid residues to participate in quinone oxidoreduction. This suggests that the quinone-binding site in complex II is inherently plastic and can robustly interact with different types of quinones.

Living cells adapt to their environment by a variety of complex regulatory processes. In many bacteria and lower eukaryotes, major changes in cellular protein composition are found when there is a transition from aerobic to anaerobic respiration (1–3). In addition to changes in protein composition, a number of organisms including Escherichia coli can produce a variety of different redox components to accommodate the physiological niche to which the cell must adapt. Quinones are small, membrane-bound, freely diffusible lipophilic redox components that can vary in identity depending upon environmental conditions. In E. coli, three types of quinones are synthesized: the benzoquinone ubiquinone (UQ), and two naphthoquinones, demethylmenaquinone (DMQ) and menaquinone (MQ) (4). The composition of the membrane-bound quinone pool varies depending upon the aerobic-anaerobic environment of the cell. Ubiquinone (UQ) and DMQ are most prevalent under aerobic conditions, comprising ∼65 and 32% of the quinone pool, with the remaining ∼3% being MQ (5). This ratio changes, however, upon the switch to a more anoxic environment with about a 20-fold increase in MQ levels, a slight 1.5–2-fold increase in DMQ, and an 8-fold reduction in UQ (5). The quinones vary in redox potential with MQ (Em,7 = −80 mV) having the lowest potential and DMQ (Em,7 = +40 mV) and UQ Em,7 = +110 mV) having higher poten-

The abbreviations used are: UQ, ubiquinone, where subscript numbers indicate the number of isoprene repeats in the quinone tail group (n in Fig. 1); QFR, quinol:fumarate reductase; SQR, succinate:quinone reductase; MQH2, reduced MQ; MQH1, menaquinone; DMQ, demethylmenaquinone; Em,7, midpoint redox potential at pH 7; Qp, quinone-catalytic site; C,D, nonoxyl-ethylene n-dodecyl ether; HQNO, 2-heptyl-4-hydroxyquinoline N-oxide; Bistris propane, 1,3-bis[tris(hydroxymethyl)methylamino]propane.

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2 The atomic coordinates and structure factors (code 4KX6) have been deposited in the Protein Data Bank (http://wwpdb.org/).
3 Background: Different quinone substrates are used by complex II.

Results: Structural and kinetic analyses show that two arginine residues modulate the enzyme interaction with different quinones.

Conclusion: Specific arginines compensate for each other in proton transfer during quinone oxidoreduction in the complex II homolog fumarate reductase.

Significance: Plasticity in quinone binding may be important for bioenergetic transformations.
Using E. coli mutant strains with compromised ability to synthesize UQ, DMQ, and MQ, it has been shown that quinol oxidases such as fumarate reductase do not only interact with their normal substrates UQ and MQ (Fig. 1) but can function with reduced DMQ (DMQH₂) and that DMQ can also act as an electron acceptor during succinate oxidation (4, 7).

Quinol:fumarate reductase (QFR; FrdABCD; Fig. 2) is a terminal electron acceptor during anaerobic respiration with fumarate and is also a member of the complex II (succinate dehydrogenase, succinate:quinone reductase (SQR; SdhABCD)) family of respiratory proteins (8–10). Complex II provides electrons to the quinone pool during aerobic respiration in both bacteria and the mitochondrion of eukaryotes and is also the only membrane-bound component of the citric acid cycle. QFR is a structural and functional homolog of complex II, although it is physiologically poised to function in anaerobic respiration where it oxidizes reduced MQ (MQH₂) with the subsequent reduction of fumarate to succinate (8–10). QFR, however, can physiologically substitute for complex II by working in reverse, i.e. oxidizing succinate to fumarate and reducing UQ to ubiquinol (UQH₂) (10, 11).

E. coli QFR and SQR have been structurally characterized by x-ray crystallography (12–14). Both are heterotetramers composed of a soluble domain comprising a flavoprotein subunit (~66 kDa; SdhA/FrdA) and an iron-sulfur protein subunit (~27 kDa; SdhB/FrdB). The soluble domain is attached to the membrane through interaction with two subunits, each containing three membrane-spanning helices (12–15). In QFR, the quinone-binding domain comprises the membrane-bound subunits FrdC (~15 kDa) and FrdD (~13 kDa) as well as amino acid residues from the iron-sulfur subunit FrdB. Structural and biochemical analyses have shown that QFR can accommodate both MQ and UQ at a single quinone-catalytic site termed Qp (12, 13, 15, 16).

Structural analyses, however, have only investigated the binding of MQ and MQ inhibitors within this pocket, and the binding mode of UQ has instead been inferred from examination of the amino acid residues surrounding the pocket. Interestingly, biochemical studies highlight differences in potential pathways by which MQ and UQ are deprotonated and protonated, respectively, in QFR (16). This suggests that there may be differences in amino acid residues that participate in the different activities with quinones. Two amino acid residues were shown to be essential for proficient menaquinol-fumarate reductase activity (12, 16, 17). Lys-B228, where the “B” indicates that this residue is part of the FrdB subunit, was shown to form a strong hydrogen bond to the C1 carbonyl oxygen of MQ, and Glu-C29 forms a hydrogen bond to the C4 carbonyl oxygen of MQ (12, 16, 18) (Fig. 2). Kinetic and spectroscopic investigations of these two amino acids showed that Lys-B228 was essential for both menaquinol-fumarate reductase activity as well as succinate-UQ reductase activity. Eliminating the negative charge of Glu-C29, although having a profound effect on menaquinol-fumarate reductase activity, had only negligible effects on succinate-ubiquinone reductase activity (16). This suggested that although MQ and UQ analogs bind in the same quinone-binding pocket (12, 13) other amino acid residues besides Glu-C29 are required for succinate-UQ reductase activity.

In the present study, MQ and UQ binding to wild-type E. coli QFR and the FrdC E29L variant were investigated by x-ray crystallographic analysis and molecular docking, respectively, to...
query whether additional amino acids are important for quinone binding and catalysis. This information provided insight into two structurally adjacent amino acids that could be involved in protonation reactions with quinones. Therefore, a series of site-directed substitutions of Arg-C28 and Arg-D81 were constructed to evaluate the nature of these residues in UQ reduction and MQH$_2$ oxidation as discussed below.

**EXPERIMENTAL PROCEDURES**

**QFR Expression and Purification**—Both the wild-type and the FrdC E29L variant of the *E. coli* QFR were produced in *E. coli* strain DW35 (ΔfrdABCD, sdhC::kan) (17) containing the pBR3 plasmid and grown under microaerophilic conditions in terrific broth as described previously (20). Following cell disruption, membranes were isolated by centrifugation at 120,000 × g and washed three separate times as described (20). The isolated cell membrane pellets were stored at −80 °C.

QFR was extracted from membranes in buffer containing 20 mM Tris-HCl and 0.1 mM EDTA, pH 7.4 (Buffer A) with complete protease inhibitor mixture (Roche Applied Science) and 2% C$_{12}$E$_9$ (Sigma). Detergent extraction proceeded for 2 h at 4 °C. The extract was then centrifuged at 22,000 × g, and the supernatant containing solubilized QFR was subjected to further purification.

QFR was purified at 4 °C using a three-step chromatographic procedure similar to that described (20). The solubilized extract was first applied to a 100-ml Q-Sepharose fast flow column equilibrated in Buffer A containing 0.05% C$_{12}$E$_9$ and then with 3 column volumes of Buffer A containing 0.05% C$_{12}$E$_9$ and 100 mM NaCl. The protein was eluted using a gradient of 100–250 mM NaCl over ~8 column volumes.

The dark brown QFR protein fraction was concentrated using an Amicon centrifugal concentrator at 4,500 × g. The dark brownish supernatant was then diluted into Buffer A containing 0.05% C$_{12}$E$_9$ and loaded onto a 10-ml Poros column. The column was washed with 4 column volumes of Buffer A containing 0.05% C$_{12}$E$_9$ and then with 3 column volumes of Buffer A containing 0.05% C$_{12}$E$_9$ and 100 mM NaCl. The protein was eluted using a gradient of 100–250 mM NaCl over ~8 column volumes.

**Crystallization**—Prior to crystallization, purified QFR was diluted to 30 mg of protein/ml in buffer containing 20 mM Tris-HCl, 0.1 mM EDTA, and 0.05% Anagrade C$_{12}$E$_9$. Crystals were grown at 22 °C using the hanging drop vapor diffusion method with 0.8 μl of protein (30 mg of protein/ml) and 1 μl of reservoir solution. Crystals were grown using reservoir solutions of 13–15% polyethylene glycol (PEG) 5000 monomethyl ether, 85–115 mM MgCl$_2$, 100 mM sodium citrate, 0.01 mM DTT, and 0.1 mM EDTA, pH 5.8. Crystals formed within 24 h and grew to maximal size within 3–5 days. Crystals were cryoprotected in a solution containing all crystallization reagents and 30% ethylene glycol.

**Crystallographic Data Collection and Refinement**—Diffraction data were collected at the Advanced Photon Source Life Sciences Collaborative Access Team (LS-CAT) beamline ID-G at a temperature of 100 K using a MARCCD detector and the wavelengths listed in Table 1. Data were processed using HKL2000 (21). Crystals formed in the orthorhombic space group P2$_1$2$_1$2$_1$ with unit cell dimensions listed in Table 1. Structure Determination and Refinement—Crystals were isomorphous with crystals of previously determined structures of QFR and were determined by rigid body refinement in CNS using the structure of QFR bound to fumarate (Protein Data Bank code 3P4P) (22) as the starting model. Model building was performed in Coot (23), and refinement was performed using CNS (24) and Refmac (25) with translation-liberation-screw (TLS) parameters determined with the TLSMD server (26). The statistics for the refined model are listed in Table 1. Three complete data sets with a maximal resolution better than 3.2 Å were collected from three independent crystals of the FrdC E29L variant of QFR. During model building, the electron density was averaged in Coot (23) to improve ligand placement. However, the final model is of refinement against the highest resolution data set, and only those statistics are listed in Table 1. Similarly, an additional data set of wild-type QFR diffracting to 2.9-Å resolution was collected and used for map averaging, but refinement against these data was not performed.

**In Silico Modeling of Quinone Binding**—Wild-type and FrdC E29L variant structures were prepared for modeling with Maestro (Schrödinger LLC) (27) by creating a file with only the iron-sulfur protein subunit FrdB and the membrane anchor subunits FrdC and FrdD. Protons were added to all structures and then minimized using the molecular mechanics force field OPLS_2005. A membrane system was introduced into the structures using the Orientations of Proteins in Membrane (OPM) database (28) and the Desmond System Builder component of Maestro. Docking grids were created for both wild-type QFR and the FrdC E29L variant with Glide Receptor Grid Generation. Ligands (MQH$_2$, HQNO, and UQ) were prepared for docking using the Ligprep module by adding explicit hydrogen
Protein Control of Quinone Binding and Catalysis

atoms. These ligands were then inserted into the Qp site of the minimized protein. The Glide XP subroutine in Maestro was used for ligand docking. The relative free energy of binding was estimated with the extended precision scoring function (Glide-XP) (27). During docking and calculation, flexibility of the ligand was allowed, whereas the protein was held rigid.

Mutagenesis—Plasmid pH3 (frdA* B* C* D*) was used as the template for mutagenesis as described previously (17). The QuikChange XL-1 site-directed mutagenesis kit from Agilent Technologies (Santa Clara, CA) was used to construct FrdC R28L, FrdC R28N, FrdD R81K, FrdD R81A, and FrdD R81E mutants using appropriate polymerase chain reaction primers. A series of a combination of the above mutants was also constructed including FrdC R28L/E29L, FrdC R28E/E29L, and FrdC R28L/FrdD R81A. All mutant enzymes assembled at typical levels in the E. coli inner membrane (data not shown).

Measurement of Enzyme Activity—Membranes containing wild-type and mutant QFR enzymes were activated to remove inhibitory oxaloacetate using malonate as described previously (29). The standard assay medium contained 30 mM Bistris propane, pH 8.0, 0.1 mM EDTA, and 3 mM potassium cyanide at an oxygen-free medium as described previously (29). Succinate-ubiquinone reductase activity was determined in a reaction mixture including FrdC R28L/E29L, FrdC R28E/E29L, and FrdC R28L/FrdD R81A. The affinity of wild-type and QFR mutant enzymes for quinones was determined using fluorescence quench titrations of HQNO binding by methods similar to those described previously (12, 29). The MQ to FrdC E29L variant. The docking calculations (Fig. 3D, black bonds) preserved the hydrogen bonding interactions (Fig. 3D, pink bonds) but suggested that the naphthoquinone ring rotated away from the hydrophobic leucine side chain and toward a more favorable, polar environment.

Because of the weak electron density associated with MQ at the Qp site, the placement of the ligand was verified using docking calculations with the program Glide. Docking studies were first performed with MQ and wild-type QFR where it was found that naphthoquinone docked into the Qp site of the wild-type enzyme (Fig. 3C, black bonds) into a position almost identical to the best fit for the electron density (Fig. 3C, pink bonds). In this docking model, the hydrogen bonds between the protein and both the C1 carbonyl and the C4 carbonyl observed in the experimental structure were replicated, suggesting that computational docking is a valid method to support the experimental placement. Similar docking studies were then performed for MQ to the FrdC E29L variant. The docking calculations (Fig. 3D, black bonds) preserved the hydrogen bonding interactions between the protein and the C1 carbonyl that had been modeled into the electron density (Fig. 3D, pink bonds) but suggested that the naphthoquinone ring rotated in the opposite direction. This slight difference in best binding location as calculated by crystallography and docking suggests that the limited quality of the electron density reflects some rotational disorder of MQ within the Qp site in the FrdC E29L variant (Fig. 4).

RESULTS

Structure of the FrdC E29L Variant—The FrdC E29L variant has been shown previously to stabilize the menasemiquinone (MQ7) intermediate at the Qp-binding site (19). This implies that the Glu-C29 is a necessary part of the proton shuttle during menaquinol oxidation. To better define the role of Glu-C29 in quinone chemistry, we determined the x-ray structure of the FrdC E29L variant. At a global level, each subunit of the wild-type and the FrdC E29L variant enzymes was similar with a root mean square deviation of less than 0.32 Å. In addition, introduction of the mutation did not significantly shift the position of the main chain surrounding the Qp-binding pocket. Indeed, the only detectable significant difference was the identity of the side chain.

Binding of MQH2 at the Qp Site—In previous structures (12, 22, 32), diffuse electron density for MQ was present at the Qp site even in the absence of exogenous addition of this substrate (Fig. 3A), suggesting that MQ co-purifies with QFR. Because the affinity between the FrdC E29L variant and MQ is similar to that between wild-type enzyme and MQ, we anticipated that crystallization of the FrdC E29L variant without added ligand would result in electron density being observed for menaquinone.

Indeed, the structure of the FrdC E29L variant is associated with electron density at the Qp site. However, it is noted that although this is consistent with MQ the electron density is weaker than was observed for MQ bound to wild-type QFR (12), suggesting either decreased occupancy or increased mobility (Fig. 3B). It is difficult to place ligands accurately with the quality of this electron density; however, in our best assignment, the naphthoquinone ring is rotated away from Leu-C29 as compared with the deposited coordinates of the wild-type structure (Protein Data Bank code 1L0V) (Fig. 4). The molecular origin of this rotation likely comes from both the loss of the stabilizing hydrogen bonding interaction between the side chain of Glu-C29 and the (presumably protonated) C4 carbonyl of MQ and the movement of the C4 carbonyl away from the hydrophobic leucine side chain and toward a more favorable, polar environment.
The UQ-binding site in QFR has not been experimentally structurally elucidated. To propose how the binding differs from MQ, we used computational methods to dock UQ into both wild-type and FrdC E29L enzymes. In both enzymes, UQ docked similarly with the C1 carbonyl of UQ calculated as making hydrogen bonding contacts with Lys-B228, Gln-B225, and Trp-D14 that resembled those experimentally identified between QFR and the C1 of MQ (Fig. 3, E and F). Interestingly, however, docking of UQ to wild-type QFR (Fig. 3 E) suggested that the C4 carbonyl formed a hydrogen bond to both Arg-C28 and Arg-D81 in addition to Glu-C29. In comparison, docking of UQ to the FrdC E29L variant of QFR only suggested a hydrogen bond to Arg-C28 (Fig. 3 F). As a part of this predicted alteration in position of UQ compared with that of MQ, the benzoquinone ring is shifted toward the [3Fe-4S] cluster by 0.6 Å.

Evaluation of the Role of FrdC Arg-28, FrdC Glu-29, and FrdD Arg-81 in MQ Oxidation and UQ Reduction—Given (i) the alteration of hydrogen bonding between MQ and the FrdC E29L variant, (ii) the computational analysis suggesting that hydrogen bonds could form between UQ and either Arg-C28 or Arg-D81, and (iii) previous observations that mutation of Glu-C29 does not influence succinate-UQ reductase activity in QFR (16), we assessed whether Arg-C28 and/or Arg-D81 is important for MQ oxidation or UQ reduction by QFR. A series of
mutations involving these amino acid residues was constructed, and the resulting mutant proteins were expressed in E. coli strain DW35 (ΔfrdABCD, sdhC::kan), which lacks any succinate-quinone reductase or menaquinol-fumarate reductase activity (17). It was found that all mutant enzymes assembled normally and were expressed to levels similar to that of wild-type QFR (data not shown).

The mutations included substitutions at Arg-C28 and Arg-D81 designed to neutralize the positive charge or introduce a potential negative charge at these positions. The substitutions would also remove the potential hydrogen bond to the C4 carboxyl group of UQ from Arg-C28. In addition, a double substitution of FrdC R28L/E29L eliminates two of three charged residues, and R28E/E29I moves the location of the negatively charged residue. In another construct, both Arg-C28 and Arg-D81 were substituted with amino acids that would neutralize the charge. As shown in Table 2, kinetic analysis of succinate oxidase activity by measuring succinate-ferricyanide reductase activity of the isolated membranes enriched with QFR confirms that all mutant enzymes retain near normal levels of activity at the dicarboxylate active site. Even the mutant enzyme from the double Arg substitution (Arg-C28/Arg-D81) retained up to 70% of wild-type activity. It was found that extraction of some of the mutant enzymes rendered them less stable than wild-type protein; therefore, the kinetic analysis was done using enriched membrane fractions. The succinate-UQ reductase activity predominantly reflects the ability of the enzyme to catalyze quinone chemistry. This activity in the membrane-bound enzyme was not severely impaired in the majority of the mutant enzymes except for the double substitution FrdC R28E/E29L, which retained only 7% of wild-type activity, and the double substitution FrdC-R28L/FrdD-R81A, which had no measurable UQ reductase activity (Table 2). By contrast, there was a much greater effect on menaquinol-fumarate reductase activity in the majority of mutant enzymes (Table 3).

Based upon these data, we again performed docking studies of oxidized and reduced MQ and UQ into models of each site-specific QFR variant (not shown). Interestingly, the total number of predicted hydrogen bonds between the protein and the quinone correlated with succinate-UQ reductase activity. This suggests that any hydrogen bond donor to the bound quinone could promote proton transfer to ubiquinone bound at Qp.

It was shown previously that substitution of the Glu-C29 residue severely impairs MQH2-fumarate activity (16) in agreement with the data in Table 3; however, it is interesting to note that substitution of Arg-C28 to neutral residues only reduced activity by about 70%. Also as seen in Table 3, substitution of Arg-D81 to a neutral or negatively charged amino acid severely reduced fumarate reductase activity, whereas a Lys substitution, which is expected to maintain the positive charge, retained about 55–65% of wild-type activity at pH 7 and 8, respectively, suggesting a role in pKa modulation of Glu-C29. Importantly, only a trace of HQNO-sensitive menaquinol-fumarate reductase activity is retained when both Arg-C28 and Arg-D81 are substituted by a neutral amino acid residue. To assess the changes in binding properties of the modified Qp site, we used fluorescence quench titrations of HQNO upon binding to the enzyme (30, 31). Fluorescence quench titration of HQNO shows that substitution of either Arg-C28 or Arg-D81 significantly decreases the affinity of HQNO. There was no binding observed when both arginines were substituted (Fig. 5). These data are consistent with both Arg-C28 and Arg-D81 having an important role in the substrate binding at the Qp site of QFR.

**DISCUSSION**

**Movement of the MQ Naphthoquinone during Catalytic Oxidation**—We first probed the mechanisms of MQ oxidation using the FrdC E29L variant as a tool (16, 17). In QFR, the mutation of Glu-C29 to a hydrophobic residue has long been

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**TABLE 2**

Comparison of kinetic parameters of succinate oxidase reactions catalyzed by membranes enriched with wild-type and mutant QFR enzymes

| Membranes | Succinate-K$_4$Fe(CN)$_6$ | Succinate-UQ$_2$ |
|-----------|--------------------------|------------------|
| Wild-type QFR | $k_{cat}$ | $K_{cat}$ | $K_{app(UQ)}$ |
| FrdC R28N | 24.6 | 19.0 | 1.5 |
| R28L | 27.1 | 19.3 | 1.5 |
| R28L/E29L | 28.9 | 13.9 | 2.0 |
| R28E/E29I | 26.4 | 1.7 | 3.8 |
| FrdD R81K | 28.1 | 17.4 | 0.6 |
| R81E | 30.4 | 32.2 | 1.3 |
| R81E | 27.3 | 10.7 | 0.5 |
| FrdC R28L/FrdD R81A | 20.1 | 0 |

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**TABLE 3**

Comparison of kinetic parameters of MQH$_2$-fumarate reductase reaction of membrane bound QFR enzymes

| Membranes | pH 7 | pH 8 |
|-----------|------|------|
| Membranes | $k_{cat}$ | $K_{app(MQH2)}$ | $K_{cat}$ |
| FrdC R28N | 222.2 | 5.4 | 211.1 |
| R28L | 68.6 | 3.1 | 65.4 |
| R28L/E29L | 71.1 | 3.0 | 67.8 |
| R28E/E29I | 12.2 | 3.9 | 11.1 |
| FrdD R81K | 10.6 | 0.9 | 6.1 |
| R81E | 123.7 | 2.7 | 138.9 |
| R81E | 14.0 | 1.3 | 12.8 |
| FrdC R28L/FrdD R81A | <1 |

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known to stabilize the menasemiquinone intermediate (19), suggesting that this side chain is required for shuttling of one of the two protons during quinol oxidation. Later studies demonstrated substantial changes in the reaction kinetics of MQH₂-coupled fumarate reduction, consistent with the idea that Glu-C29 acts as a proton acceptor during MQH₂ oxidation (16). The current study suggests that the QFR FrdC E29L variant binds MQ with an ensemble of slight rotations of the substrate. This binding may mimic the semiquinone-bound state of the protein. Previous Fourier transform infrared studies of MQ bound to the QFR FrdC E29L variant (16) have identified that the introduction of the hydroscopic side chain results in a tightening of the hydrogen bonding interactions on the other side of the molecule with Lys-B228. This is not observed in the structures here. The difference may reflect limitations in the x-ray data or may result from a different quinone oxidation state being bound.

Both the movement of quinones and the protein backbone and the alteration of the hydrogen bonding environment have been reported in many quinone-coupled oxidoreduction reactions (33–35). These changes in the quinone environment may increase the catalytic reactivity of the quinone, help stabilize the semiquinone, or help prevent back-reaction during an energy harvesting process. Interestingly, the observed movement of the MQ naphthoquinone ring in the FrdC E29L structure is rather slight as compared with some of these other systems but still alters the hydrogen bonding pattern. It is possible that the architecture of the Q₆ site in E. coli QFR is optimized such that it does not require as large of a movement of the quinone to prevent a back-reaction. It is also possible, however, that the use of the site-specific mutation does not allow the full range of motion that is normally associated with the wild-type enzyme.

**Differences in Proton Shuttling Pathway(s) for the UQ and MQ Substrates of QFR—**QFR and SQR are homologs that are catalytically poised to be more proficient in one direction of catalysis as assessed by a comparison of the activity in their respective forward and reverse directions (9, 10, 16, 29). Previous analyses have suggested a role of the reduction potentials of the Fe–S clusters, the properties of the covalently bound FAD cofactor, and quinone substrates in catalytic proficiency (10, 14, 18, 32). Because electrons are substrates and products, the tuning of the potentials can control substrate availability at each active site. However, the reactions catalyzed by QFR and SQR are somewhat unusual in that each direction of catalysis has (in fact) a different reaction pathway at both active sites. For example, it has previously been suggested that the pKₐ near the N1 of the isovalloxazine ring of the covalent FAD cofactor is different in QFR and SQR, altering the stability of the flavin semiquinone radical and the directionality of the enzymes (36).

Previous studies of QFR similarly suggest that a different proton shuttling pathway is used for UQ reduction and MQH₂ oxidation within the Q₆-binding site (16). The essential nature of Glu-C29 for MQH₂ oxidation is clear (16, 17); however, it is not required for UQ reduction. To investigate aspects of oxidoreduction that differ for the MQ and UQ substrates, the current computational and biochemical studies were performed. The in silico docking studies are consistent with the previous findings that imply that UQ and MQH₂ occupy a single functional quinone-binding pocket. However, as suggested by the docking calculations described here (Fig. 3), the orientation of the headgroups of each quinone species is slightly shifted when compared with each other such that the hydrogen bonding appears to differ. Although the difference is only slight, it strongly suggests that each quinone substrate interacts with possible proton shuttling amino acid residues differently.

The mutagenesis and catalytic data shown in Tables 2 and 3 support the idea that two neighboring arginine residues in addition to Glu-C29 play important roles in the protonation/deprotonation reactions of UQ/MQ in QFR. Surprisingly the data

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**TABLE 4**

Calculated pKₐ values of selected amino acids at Q₆ site

The PROPKA3.1 module was used to calculate all pKₐ values. QFR-MQ refers to the wild-type QFR structure (Protein Data Bank code 1LOV (12)) with MQ present used for the calculation. E29L-MQ refers to the x-ray structure of the FrdC E29L variant enzyme described. QFR-UQ used coordinates from the wild-type QFR (Protein Data Bank code 1LOV (12)) with UQ₄ docked into the Q₆ site. E29L-UQ used coordinates for the FrdC E29L mutant enzyme with UQ₄ docked into the Q₆ site. n/a indicates not applicable.

| Residue | pKₐ of free amino acid in solution | Predicted pKₐ in QFR |
|---------|----------------------------------|---------------------|
|         |                                  | QFR-MQ              | E29L-MQ | QFR-UQ | E29L-UQ |
| Arg-A287| 12.5                             | 7.96                | 7.63    | 7.95   | 7.31    |
| (control)|                                 |                     |         |        |         |
| Lys-B228| 10.5                             | 7.41                | 7.76    | 7.82   | 7.67    |
| Arg-C28 | 12.5                             | 11.51               | 9.69    | 11.66  | 9.78    |
| Glu-C29 | 4.5                              | 3.45                | n/a     | 3.89   | n/a     |
| Arg-D81 | 12.5                             | 13.04               | 11.07   | 12.93  | 11.1    |
show that site-directed mutations of either Arg-C28 or Arg-D81 to a neutral amino acid residue resulted in only minor effects on UQ reduction (Table 2) and moderate effects on MQH₂ oxidation (Table 3). One possible explanation is that either Arg-C28 or Arg-D81 can act as a proton shuttle during UQ reduction such that each can functionally compensate for each other. Thus, the double mutation where both amino acids were replaced with neutral residues has the potential to be quite informative. Although no UQ reduction is observed, and there is only very minimal MQH₂ oxidation (Tables 2 and 3), each of the Arg substitutions disturbs the H-bonding pattern of the bound quinones. This might be expected to be reflected as a decrease of quinone substrate binding. In agreement with this suggestion, the increase in $K_a$ values for HQNO in Arg-C28 and Arg-D-81 mutant enzymes and the loss of HQNO binding in the double mutant as assessed by fluorescence quench titrations (Fig. 5) indicates that quinone binding has been perturbed. The $K_{app}$ shown in Tables 2 and 3, however, shows smaller changes than observed for the $K_a$ values, which may reflect the endogenous quinones present in the membrane fractions used for kinetic analysis.

The results shown in Tables 2 and 3 parallel previous findings with E. coli SQR where site-directed substitutions near the UQ-binding site did not identify any single amino acid side chain that was essential for proton shuttling to UQ (37–39). Studies on SQR have included the mutation of side chains that stabilize ordered water molecules leading into the UQ-binding site, and the substitution of these has measurable but limited effects on quinone reduction (38).

An altered reaction pathway between the forward and reverse directions of quinone oxidation/reduction may be key for enzyme regulation and imperfect reversibility. It will be interesting to identify whether similar modification of the reaction pathway is an evolutionarily conserved mechanism to tune the kinetics of bidirectional catalysis in parallel systems.

A Dual Role for the $q_p$ Arginines in $pK_a$ Modulation—in the case of MQH₂ oxidation, the two arginine residues may play an additional role in modulating the $pK_a$ of Glu-C29. Charge reversal of Arg-C28 via mutation to glutamic acid significantly reduces both MQH₂ oxidation (Table 3) and UQ reduction as evidenced by changes in $k_{cat}$ (Table 2). This strongly suggests that the $pK_a$ values of neighboring side chains and water molecules are influenced by the charges. This is supported by analysis using the PROPKA3.1 module indicating that the protein environment and presence of quinone influence the $pK_a$ of Arg-C28 and Arg-D81 (Table 4) in the FrdC E29L mutant enzyme. Thus, both Arg-C28 and Arg-D81 in wild-type QFR may modulate the $pK_a$ of Glu-C29 to assist in MQH₂ oxidation. In addition, during UQ reduction, it is possible that these amino acid residues may substitute for one another to assist in protonation of UQ and/or to modulate the $pK_a$ of a water molecule that could act as the direct proton donor.

Implications for Plasticity in Quinone Binding—in QFR and SQR, the different redox potentials of the MQ and UQ and the redox centers in the proteins likely provide the driving force to alter the direction of the reaction. Indeed the succinate/fumarate couple (+30 mV) lies between the couple of MQ (−74 mV) and UQ (+110 mV). It is not uncommon, however, for bioenergetic proteins to catalyze the oxidation or reduction of more than one quinone type (40–43), and depending upon the redox potential of the coupled reaction, this does not always change the reaction direction.

No matter whether the driving force is changed, multiple quinone types universally bind within the same pocket. Physiologically, this could allow different chemical reactions to proceed within the same enzyme during sudden changes in respiratory conditions. For example, membrane-bound glucose dehydrogenase can couple oxidation of glucose to reduction of either MQ or UQ with similar catalytic efficiency (44). Similarly the rescue of the pink1 Parkinson disease-associated mutation with the MQ analog vitamin K₂ (45) suggests that the naphthoquinone headgroup may interact functionally with proteins involved in eukaryotic respiration. This suggests that the plasticity of quinone binding is an evolutionarily conserved mechanism for maximizing or regulating respiratory function.

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