Published in final edited form as:

J Struct Biol. 2018 April ; 202(1): 100–104. doi:10.1016/j.jsb.2017.11.004.

New crystal forms of the integral membrane *Escherichia coli* quinol:fumarate reductase suggest that ligands control domain movement

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Abstract

Quinol:fumarate reductase (QFR) is an integral membrane protein and a member of the respiratory Complex II superfamily. Although the structure of *Escherichia coli* QFR was first reported almost twenty years ago, many open questions of catalysis remain. Here we report two new crystal forms of QFR, one grown from the lipidic cubic phase and one grown from dodecyl maltoside micelles. QFR crystals grown from the lipid cubic phase processed as P1, merged to 7.5 Å resolution, and exhibited crystal packing similar to previous crystal forms. Crystals grown from dodecyl maltoside micelles processed as P2₁, merged to 3.35 Å resolution, and displayed a unique crystal packing. This latter crystal form provides the first view of the *E. coli* QFR active site without a dicarboxylate ligand. Instead, an unidentified anion binds at a shifted position. In one of the molecules in the asymmetric unit, this is accompanied by rotation of the capping domain of the catalytic subunit. In the other molecule, this is associated with loss of interpretable electron density for this same capping domain. Analysis of the structure suggests that the ligand adjusts the position of the capping domain.

Graphical Abstract

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1. Introduction

The integral-membrane *Escherichia coli* quinol:fumarate reductase (QFR) is a bioenergetic protein and a member of the respiratory Complex II superfamily. It is composed of four polypeptide chains, two of which are soluble (flavoprotein, FrdA; iron-sulfur protein, FrdB) and two of which are membrane-spanning (FrdC and FrdD). The *E. coli* QFR was among the first membrane proteins of known structure (Iverson et al., 1999), with crystals grown from protein solubilized in the polyoxyethylene detergent C₁₂E₉. Since the initial report of the *E. coli* QFR structure, structures of five additional integralmembrane Complex II superfamily members have been reported (Huang et al., 2006; Lancaster et al., 1999; Shimizu et al., 2012; Sun et al., 2005; Yankovskaya et al., 2003). This makes the Complex II superfamily one of the better structurally characterized membrane protein families.

Nevertheless, questions remain about catalysis in Complex II. One outstanding question relates to interdomain motions during fumarate/succinate interconversion. The active site for this transformation is located within the flavoprotein (FrdA) subunit and contains a catalytic FAD at the interface of two domains, termed a flavin-binding domain (residues 1–232 and 353–602) and a capping domain (residues 233–352) (Iverson et al., 1999). Substrate interacts with residues of both domains, and interdomain movement may stabilize the transition state (Tomasiak et al., 2008). However, the positions of the flavin-binding and capping domains are influenced by crystal contacts in reported crystal forms of the *E. coli* QFR (Iverson et al., 1999; Starbird et al., 2017). Thus, it is difficult to evaluate the interplay between domain motion and catalysis without a new crystal form. Here, we report two new crystal forms of *E. coli* QFR grown from the lipid cubic phase (LCP) or dodecyl maltoside (DDM) micelles. We determined each structure and analyzed the structure of the *E. coli* QFR grown from DDM micelles.

2. Protein purification and detergent stability

In addition to its role in bioenergetics, QFR binds to the FliG subunit of the switch-motor of the bacterial flagellar rotor and promotes clockwise rotation of the flagellum (Cohen-Ben-Lulu et al., 2008), which is essential for chemotaxis (Eisenbach, 2004). Our study crystallized both isolated *E. coli* QFR and the *E. coli* QFR-FliG complex. Crystallization of QFR-FliG used wild-type and the PAA variant of the *E. coli* FliG subunit, which biases flagellar rotation clockwise (Togashi et al., 1997).

QFR was expressed and purified as described (Maklashina et al., 2006) with the final size exclusion column equilibrated in 20 mM Tris pH 7.4, 0.1 mM EDTA and the appropriate

detergent. Wild-type or PAA *E. coli* FliG was expressed in *E. coli* BL21 pLysS and expression induced with 1 mM IPTG. Cells were lysed by sonication in 50 mM NaH₂PO₄, 300 mM NaCl, and 10 mM imidazole. FliG was purified by Ni affinity.

QFR and FliG (1:9 ratio) was purified by size exclusion chromatography in 20 mM Tris pH 7.4, 0.1 mM EDTA and detergent. C₁₂E₉ (0.05%) resulted in one major peak (Fig. 1A); SDS-PAGE analysis revealed that this peak contained bands corresponding to all four subunits of QFR plus the FliG subunit (Fig. 1A, **inset**). DDM (0.02%) resulted in a doublet; one peak contained QFR-FliG, and the second contained QFR (Fig. 1B).

3. LCP crystallization

Protein (QFR or QFR-FliG, 25 mg/ml) was mixed in a 40:60 ratio with monoolein (1-(9Z-octadecenoyl)-rac-glycerol, 9.9 MAG) in a gas-tight syringe. Crystals were grown using 50 nl mesophase and 800 nl precipitant (200 mM NH₄F, 100 mM Bis-Tris pH 7.5, 22% PEG 400 and 5% pentaerythritol propoxylate) (Fig. 2A). Crystals of QFR grew using the same conditions as crystals of QFR-FliG, with the crystals from the QFR-FliG mixture being better suited to diffraction analysis.

Data were collected at the Advanced Photon Source (APS) beamline 23-ID-D at -173 °C using a wavelength of 1.03 Å and a Pilatus3 6M detector. Data were processed in HKL2000 (Otwinowski and Minor, 1997) in space group P1 with unit cell dimensions $a = 131.5 \text{ Å}$ $b = 133.9 \text{ Å}$ $c = 259.6 \text{ Å}$ $\alpha = 90.8^\circ$ $\beta = 90.1^\circ$ $\gamma = 91.6^\circ$ and merged to 7.5 Å resolution (Table 1). Molecular replacement used QFR lacking the capping domain of the FrdA subunit (PDB entry 1KF6 (Iverson et al., 2002)) in Phaser (McCoy et al., 2007). The crystals had modified type I crystal packing (Fig. 3A) similar to that reported for QFR grown from C₁₂E₉ micelles (Iverson et al., 1999) (Fig. 3B). Consistent with a correct solution, omitting the Fe:S clusters resulted in the appearance of difference electron density for these cofactors (Fig. 3A). Given the resolution, refinement is not warranted; the capping domain was not placed into the model and is omitted from Fig. 3A.

4. Micellar crystallization

Micellar crystallization of QFR-FliG in 20 mM Tris pH 7.4, 0.02% DDM (Fig. 1B) used the sitting drop vapor diffusion method with 200 nl of protein (25 mg/ml) and 200 nl of reservoir; crystals appeared in a range of reservoir solutions (10–20% PEG (400 – 900), 15–50 mM divalent cation (CaCl₂, Ca(CH₃COO)₂, MgCl₂), and 50 mM Bis-tris, pH 6.5) (Fig. 2B). Data were collected at APS beamline 21-ID-D at a wavelength of 1.13 Å, and merged to 3.35 Å resolution in HKL2000 (Otwinowski and Minor, 1997) (Table 1). Crystals belonged to space group P2₁ with unit cell dimensions $a=113.3 \text{ Å}$, $b=118.0 \text{ Å}$, $c=125.3 \text{ Å}$, $\beta=98.8^\circ$. The structure was determined by molecular replacement in Phaser (McCoy et al., 2007) using PDB entry 1KF6 (Iverson et al., 2002) as the search model. The structure was improved using Coot (Emsley and Cowtan, 2004) and Phenix (Adams et al., 2010) to an R_{free} of 0.285/0.316.

This crystal form lacks dicarboxylate ligand and exhibits unique crystal packing (Fig. 3C) that does not influence capping domain position; this impacts each of the two molecules in

the asymmetric unit differently. In the first molecule (chains ABCD) comparisons to the structure of fumarate-bound *E. coli* QFR (PDB entry 3P4P (Tomasiak et al., 2008)) using DynDom (Hayward and Berendsen, 1998) show that the capping domain rotates 7° (Fig. 4), which results in a maximal displacement of Ca atoms of 3.9 Å and large changes at the active site. However, the structure of fumarate-bound *E. coli* QFR (PDB entry 3P4P (Tomasiak et al., 2008)) has a capping domain position intermediate between the open and closed states; comparison to the structure of the *W. succinogenes* QFR in the fully closed state (PDB entry 2B52 (Lancaster et al., 2001)) shows a rotation of 15° and a maximal displacement of 5.4 Å. This domain rotation is within the context of unchanged folds, with an RMS deviation of the flavin-binding domain (residues 1–232 and 353–602) of 0.44 Å and an RMS deviation of the capping domain (residues 233–352) of 0.34 Å. Two notable catalytic side chains lack interpretable electron density: the catalytic proton delivery residue FrdA^{R287} (Doherty et al., 2000; Tedeschi et al., 2001), and a glutamate important for covalent flavinylation, FrdA^{E245} (Starbird et al., 2017) (Fig. 5A).

In the second molecule (chains EFGH), a near absence of interpretable electron density is associated with the capping domain, suggesting mobility. Indeed, only three short regions of sequence (residues 232–244, 287–293, 332–340) within this 120 amino acid domain are associated with clear electron density. These visible regions are those that are nearest the flavin-binding domain and contain active site residues. As a result, despite the loss of capping domain electron density the positions of all catalytic residues could be determined. The exclusion of coordinates for much of capping domain of the E chain may be the underlying physical basis for the high final R-factors.

Potentially contributing to the capping domain mobility is the lack of a bound substrate mimetic. Without dicarboxylate, electron density for a likely anionic molecule is observed at a position shifted from the position of bound substrate (Fig. 5A–C). This molecule is of similar size as a sulfate or phosphate, molecules that have been shown to activate the enzyme (Kearney et al., 1974; Robinson and Weiner, 1981); it is tentatively assigned as a phosphate. Binding of this anion subtly shifts the positions of several side chain residues normally involved in catalysis (Fig. 5A–C).

5. Discussion

Previously reported structures of QFR were grown from C₁₂E₉ micelles, where crystals diffracted at resolutions between 2.7 Å – 4.3 Å (Iverson et al., 1999; Starbird et al., 2017). Here, we screened for new crystallization conditions. These crystals formed from mixtures of QFR and FliG, but electron density was only observed for QFR in each case. Although it is not clear why the presence of FliG promotes these new crystal forms, one possibility is that FliG shielded surfaces of QFR during the initial stages of crystallization, which affected crystal growth. Crystals grown from the LCP (Fig. 2A) exhibited low resolution diffraction, yet the structure could be determined with reasonable confidence (Fig. 3A). Crystals grown from DDM micelles (Fig. 2B) diffracted to 3.35 Å resolution, and the structure could be determined and refined, although a 120-residue domain lacked interpretable electron density in one of the two molecules of the asymmetric unit.

Notably, this structure provides the first view of Complex II lacking dicarboxylate. Instead, another anion may neutralize the positive charges of the active site (Fig. 5). Tentatively assigned as phosphate, the possibility that an anion was retained through the purification process suggests that Complex II active sites are unstable without ligand. Each copy in the asymmetric unit is associated with a slightly different position of this anion, however, both differ from the position of true substrate (Fig. 5).

Analysis of the structure suggests new principles of Complex II catalysis as relates to interdomain mobility. Studies over 40 years ago suggested activation-associated conformation changes (Kearney et al., 1974). That these changes involve the angles between two domains was first shown in structures of *Shewanella putrefaciens* flavocytochrome *c* (Leys et al., 1999), a single-subunit homolog of the FrdA subunit of QFR. Soon thereafter, in a second soluble homolog of the FrdA subunit, the *E. coli* L-Aspartate oxidase, a structure lacking FAD had a large rotation of the capping domain as compared to a structure with FAD incorporated (Bossi et al., 2002; Mattevi et al., 1999). This is complemented by structural comparisons of integral-membrane Complex II from different organisms bound to different ligands, which showed differences in position of the capping domain (Huang et al., 2006; Iverson et al., 2000; Lancaster et al., 2001). Finally, mutagenesis of FrdA^{T234}, a residue located at the hinge between domains in the *E. coli* QFR, promoted domain opening (Tomasiak et al., 2008). Contradicting this evidence for interdomain motion was the finding that engineered disulfide bond designed to restrain interdomain motions in the soluble flavocytochrome *c* fumarate reductase from *Shewanella frigidimarina* retained catalytic turnover, albeit at a reduced level (Rothery et al., 2004).

In the present study, the finding that the capping domain either rotates or becomes disordered without dicarboxylate supports a model of ligand-dependent interdomain rotation (Fig. 4), where anion binding to the active site controls the capping domain position. Given that substrate-binding residues are found on the flavin-binding domain (FrdA^{H355}, FrdA^{R390}), the capping domain (FrdA^{T244}, FrdA^{R287}) and the hinge (FrdA^{H232}), substrate-based control of the capping domain seems plausible. One could envision a mechanism where a true substrate correctly orients the capping domain and catalytic residues for catalysis. The catalytic proton donor/acceptor FrdA^{R287} appears to undergo the largest change upon substitution of dicarboxylate for other anions at the active site and may be particularly important for responding to the ligand (Fig. 5). As FrdA^{R287} is essential for catalytic protonation/deprotonation of fumarate/succinate, an intriguing mechanism for substrate control of capping domain position is via the charge on the dicarboxylate affecting the pKa of FrdA^{R287}. Ligand control of domain position also suggests a mechanism for ingress and egress of substrate facilitated by the changes in the locations of active site residues.

4. Conclusions

The structure of *E. coli* QFR in new crystallization conditions suggests that the ligand at the active site controls the domain position during catalysis. Because the capping domain is not influenced by crystal contacts, this crystal form may be particularly useful for examining catalysis in *E. coli* QFR.

Acknowledgments

We thank Q Chen for assistance with LCP crystallization and P Sharma for making Figure 3. This work was supported by the Department of Veterans Affairs (BX001077 to GC), and the National Institutes of Health (NIH) (GM061606 to GC/TMI). CAS was supported by NIH T32GM008320 and National Science Foundation DGE: 0909667. TMT was funded by NIH T32 GM65086. This research used resources of the Advanced Photon Source, a U.S. Department of Energy (DOE) Office of Science User Facility operated for the DOE Office of Science by Argonne National Laboratory under Contract No. DE-AC02-06CH11357. Use of the LS-CAT Sector 21 was supported by the Michigan Economic Development Corporation and the Michigan Technology Tri-Corridor (Grant 085P1000817). GM/CA@APS has been funded in whole or in part with Federal funds from the National Cancer Institute (ACB-12002) and the National Institute of General Medical Sciences (AGM-12006).

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Figure 1. Detergent screening of QFR-FliG

A. Size exclusion chromatogram of QFR (green) superimposed with a chromatogram of QFR-FliG (black) in buffer containing 50 mM KPi , pH 7.4, and 0.05% C_{12}E_9 . Units for each run are on different y-axes. The inset shows an SDS-PAGE gel of the central peak from the QFR-FliG purification. B. Size exclusion chromatogram of isolated QFR (green/magenta) superimposed with a chromatogram of QFR-FliG (black/red) performed in PBS and 0.02% DDM. A doublet is observed for the QFR-FliG mixture, suggesting heterogeneity.

Figure 2. New crystal forms of QFR

A. Crystals grown from LCP using a precipitant solution of 200 mM ammonium fluoride, 100 mM Bis-TRIS pH 7.5, 22% PEG 400 and 5% pentaerythritol propoxylate. B. Crystals grown from DDM micelles using a reservoir solution of 15% PEG 550 MME, 25 mM CaCl_2 , 50 mM Bis-tris, pH 6.5. In each panel, the scale bar represents 40 μm .

Figure 3. Crystal packing

In all panels, the FAD is shown as red sticks and Fe:S clusters are shown as orange and yellow cubes. A. Crystal packing of QFR grown in the LCP. In the inset, F_0-F_c maps (*green*) were calculated after the omission of the Fe:S clusters and are contoured at 4σ . B. Crystal packing of QFR grown from $C_{12}E_9$ micelles, as previously reported (PDB entry 1KF6 (Iverson et al., 2002)). C. Crystal packing of QFR grown from DDM micelles.

Figure 4. Capping domain position with and without dicarboxylate

The *E. coli* QFR is shown as an inset on the left. The FrdA subunit is *cyan* (flavin binding domain) and *blue* (capping domain), the FrdB subunit is *red*, the FrdC subunit is *green*, and the FrdD subunit is *magenta*. On the right, the FrdA subunit is rotated 90° so that the view is from the membrane and looking through the top of the complex. A 7° rotation of the capping domain is observed in the absence of dicarboxylate (*blue*) as compared to the structure with fumarate (*grey*, PDB entry 3P4P (Tomasiak et al., 2011)).

Figure 5. Active site of dicarboxylate-free QFR

In all panels, FAD is *yellow*, side chains from the flavin-binding domain are *cyan*, side chains from the capping domain are *blue*, and ligand is *green*. A. Active site of the A chain FrdA subunit. The putative ligand closely approaches FrdA^{H232} and FrdA^{R390}, likely making hydrogen-bonding interactions. B. Active site of the E chain FrdA subunit. The putative ligand is shifted in location with respect to its position in the A chain and is in close proximity to FrdA^{H232}, FrdA^{H355}, and FrdA^{R390}. C. Active site of the fumarate-bound FrdA subunit of QFR grown from C₁₂E₉ micelles (PDB entry 3P4P (Tomasiak et al., 2011)).

Table 1

Data collection and refinement statistics.

Data Collection	LCP	DDM Micelles
Resolution	7.5 Å	3.35 Å
Space group	P1	P2 ₁
R _{sym}	0.095 (0.425)	0.172 (1.20) *
R _{pim}	n/a	0.120 (0.872)
I/σ	5.4 (1.1) &	7.61 (3.54)
Completeness (%)	72.6 (72.8)	89.8 (82.6)
Redundancy	1.7 (1.6)	2.7 (2.6)
CC _{1/2}	0.652	0.986
<u>Refinement Statistics</u>		
PDB entry		6AWF
R _{cryst}		0.285
R _{free}		0.318
RMSD bond lengths		0.011
RMSD bond angles		1.37°
Ramachandran		
Favored		91.4%
Allowed		7.6%
Disallowed		1.0%

Values in parentheses correspond to the highest resolution shell.

* For the crystals grown from DDM micelles, radiation damage impacted the R_{sym} and R_{pim} values; R_{sym} in the shell containing data from 3.97-3.77 Å resolution was 0.447.

& For the crystals grown from LCP, I/σ was > 2 at 8.4 Å resolution.