An *Escherichia coli* Mutant Quinol:Fumarate Reductase Contains an EPR-detectable Semiquinone Stabilized at the Proximal Quinone-binding Site*

(Received for publication, February 19, 1999, and in revised form, June 17, 1999)

Cecilia Hägerhäll‡‡¶¶, Sergey Magnitsky‡¶, Vladimir D. Sled‡¶, Imke Schröder‡¶, Robert P. Gunsalus‡¶, Gary Cecchini**††‡‡‡, and Tomoko Ohnishi¶¶¶

From the ‡Department of Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, Pennsylvania 19104, the ¶Department of Microbiology and Molecular Genetics, University of California, Los Angeles, California 90095, the **Molecular Biology Division, Veterans Affairs Medical Center, San Francisco, California 94121, and the ‡‡‡Department of Biochemistry and Biophysics, University of California, San Francisco, California 94143

The EPR and thermodynamic properties of semiquinone (SQ) species stabilized by mammalian succinate:quinone reductase (SQR) in situ in the mitochondrial membrane and in the isolated enzyme have been well documented. The equivalent semiquinones in bacterial membranes have not yet been characterized, either in SQR or quinol:fumarate reductase (QFR) in situ. In this work, we describe an EPR-detectable QFR semiquinone using *Escherichia coli* mutant QFR (FrdC E29L) and the wild-type enzyme. The SQ exhibits a g = 2.005 signal with a peak-to-peak line width of ~1.1 milliteslas at 150 K, has a midpoint potential (Em(pH 7.2)) of ~56.6 mV, and has a stability constant of ~1.2 x 10^-2 at pH 7.2. It shows extremely fast spin relaxation behavior with a P1/2 value of >500 milliwatts at 150 K, which closely resembles the previously described SQ species (SQ1) in mitochondrial SQR. This SQ species seems to be present also in wild-type QFR, but its stability constant is much lower, and its signal intensity is near the EPR detection limit around neutral pH. In contrast to mammalian SQR, the membrane anchor of *E. coli* QFR lacks heme; thus, this prosthetic group can be excluded as a spin relaxation enhancer. The trinuclear iron-sulfur cluster FR3 in the [3Fe-4S]1+ state is suggested as the dominant spin relaxation enhancer of the SQ1 in this enzyme. *E. coli* QFR activity and the fast relaxing SQ species observed in the mutant enzyme are sensitive to the inhibitor 2-n-heptyl-4-hydroxyquinoline N-oxide (HQNO). In wild-type *E. coli* QFR, HQNO causes EPR spectral line shape perturbations of the iron-sulfur cluster FR3. Similar spectral line shape changes of FR3 are caused by the FrdC E29L mutation, without addition of HQNO. This indicates that the SQ and the inhibitor-binding sites are located in close proximity to the trinuclear iron-sulfur cluster FR3. The data further suggest that this site corresponds to the proximal quinone-binding site in *E. coli* QFR.

Succinate:quinone reductase (SQR) and quinol:fumarate reductase (QFR) are structurally and functionally similar enzymes with an interesting evolution (1–3). They consist of two well conserved subunits protruding from the membrane. A larger flavoprotein subunit (denoted Fp) harbors the dicarboxylate-binding site and a covalently bound FAD cofactor; a smaller iron-sulfur protein subunit (denoted Ip) contains three distinct iron-sulfur clusters. The [2Fe-2S]1+, [4Fe-4S]1+, and [3Fe-4S]1− clusters are called S1 or FR1, S2 or FR2, and S3 or FR3 in SQR and QFR, respectively. The membrane anchor domain of the enzyme is more variable and may consist of one or two hydrophobic polypeptides (SdhC/FrdC and SdhD/FrdD) and contain zero, one, or two b hemes depending on the enzyme species. When two hemes are present, they are denoted heme b₄₃ and heme b₄. The primary sequence similarity is also much lower in this part of the enzyme. Nevertheless, accumulated evidence indicates that the membrane anchors have a conserved general structure (3, 4). One exception is a group of SQRs lacking the membrane domain and instead containing two different, more or less hydrophilic subunits (5).

The membrane-bound enzymes catalyze the oxidation of succinate or the reduction of fumarate in the bacterial cytoplasm or mitochondrial matrix and the reduction or oxidation of quinone/quinol in the membrane. It should be emphasized that when provided with suitable substrates in vitro, SQRs and QFRs generally can carry out both reactions; however, in vivo, they serve separate physiological functions. Thus, organisms capable of both aerobic and anaerobic life contain genes encoding both enzymes that are expressed during different growth conditions. There are three functionally distinct classes of SQR/QFR defined by the type of quinones that they use as electron acceptors/donors. Class 1 SQRs donate electrons to a quinone with a higher redox midpoint potential (Em) such as ubiquinone, whereas Class 2 QFRs and Class 3 SQRs use a quinone with a lower Em such as menaquinone (3). How the directionality of the enzyme reaction is achieved in vivo is not well understood, particularly for the Class 3 enzymes, but it is clear that the Em values of the iron-sulfur clusters are differently tuned in the enzymes of a different functional class.

The presence of two quinone-binding sites on the membrane anchor, located toward opposite sides of the membrane, has been demonstrated in SQR/QFR enzymes by various methods.

---

*The abbreviations used are: SQR, succinate:quinone reductase; QFR, quinol:fumarate reductase; Q, ubiquinone; TTF-A, 2-thienyltrifluoroacetone; HQNO, 2-n-heptyl-4-hydroxyquinoline N-oxide; SQ, semiquinone; QH₂, quinol; BisTris, 2-[lsqb][bis(2-hydroxyethyl)-amino]-2-(hydroxymethyl)-propane-1,3-diol; mW, milliwatt(s); mT, millitesla(s).*
Both membrane anchor polypeptides of *Bos taurus* SQR were photolabeled with [3H]arylazoquinone derivatives (6). In subsequent labeling studies using the same enzyme, two peptide regions, one in SdhC and the other in SdhD, were assigned as quinone-binding sites (7, 8). Recently, the N-terminal part of SdhC from *Escherichia coli* SQR was photoaffinity-labeled with a [3H]arylazoquinone analogue (9). Mutagenesis studies of the *E. coli* QFR membrane anchor polypeptides also outlined two quinone-binding regions (10) that overlap both with peptide stretches indicated in the bovine enzyme and with the stretch implied in bacterial SQR (see Fig. 1). This corroborates the structural similarity between the heme-less and the heme-containing membrane anchors. Apparently, extensive sequence variability is tolerated at the quinone-binding sites, but their location in the protein is nevertheless conserved. There is a quinone-binding region formed by amino acid residues from SdhCD/FrdCD located near the bacterial cytoplasmic or mitochondrial matrix side of the membrane. This region is referred to as Q-proximal (previously denoted Qp), whereas an additional quinone-binding area located farther from the Fp and Ip subunits and near the other side of the membrane is termed Q-distal (or Qd).

There are a number of inhibitors that interfere with the interaction of SQR/QFR with quinones. The most well known are 2-thienylfluorouacetonate (TTFA), 3-methylcarboxin, and 2-n-heptyl-4-hydroxyquinoline N-oxide (HQNO). Sensitivity to these inhibitors varies among species and SQR/QFR enzyme types. The two former compounds exhibit some structural similarity and are specific inhibitors (11, 12), whereas HQNO also inhibits various other enzymes interacting with quinones (13). Studies with resistant mutants of *Ustilago maydis* (14) and *Paracoccus denitrificans* (15) indicate that the carboxin-binding site overlaps with Q-proximal and that amino acids from both Ip (in fact, a residue within the cluster S3 ligation motif) and SdhD contribute to carboxin binding. Close proximity of S3 and the inhibitor-binding site is also apparent from the *E. coli* shift of cluster S3 in pigeon heart submitochondrial particles (16) and bovine heart submitochondrial particles (17) caused by TTFA.

**E. coli** QFR and *Bacillus subtilis* SQR, both of which use menaquinone as an electron donor/acceptor, are not sensitive to carboxin and TTFA, but are sensitive to HQNO (with apparent *K* values of 0.2 and 0.4 μM, respectively (18); the former at pH 7.8 and the latter at pH 7.5). Both studies show that the apparent *K* values increase with increasing pH. In *B. subtilis* SQR as well as in isolated SdhC polypeptide, HQNO binding causes a shift in the EPR spectrum line shape and induces a shift in the *E* m of heme b₃ by about −60 mV, without affecting the heme b₃ properties (19). This demonstrates that the HQNO-binding site in *B. subtilis* SQR is located in the vicinity of heme b₃, i.e. that it corresponds to Q-distal. It has also been shown that HQNO elicits a significant change in the EPR line shape of *E. coli* Fr3, indicating the close proximity of the quinone-binding site and iron-sulfur cluster (20). Such EPR line shape changes were not detected in the *B. subtilis* enzyme (21, 22) in the presence of HQNO.

In mammalian mitochondria, multiple EPR signals at *g* = 2.04, 1.99, and 1.96 arising from spin-spin interactions were observed at temperatures <15 K (23). These spin-coupled spin signals were absent in ubiquinone-depleted membranes and reappeared in ubiquinone-replenished membranes, indicating that one of the interacting partners was a semiquinone (SQ). Spectral simulations suggested a semiquinone (either semiquinone or flavosemiquinone) spin-spin interaction overlapped with the cluster S3 signal (23). If the spin-coupled split signals were assumed to arise only from dipole-dipole interaction, the distance between the interacting spins was estimated to <7.7 Å (23).

### Table I

<table>
<thead>
<tr>
<th>SQ species</th>
<th><em>K</em></th>
<th>pH</th>
<th>mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. taurus SMP</td>
<td>SQR</td>
<td>10</td>
<td>110</td>
</tr>
<tr>
<td>B. taurus SMP</td>
<td>SQR</td>
<td>12</td>
<td>102</td>
</tr>
<tr>
<td>B. taurus SMP</td>
<td>SQR</td>
<td>5</td>
<td>102</td>
</tr>
<tr>
<td>B. taurus SMP</td>
<td>SQR</td>
<td>4</td>
<td>102</td>
</tr>
<tr>
<td>B. taurus SMP</td>
<td>SQR</td>
<td>1</td>
<td>102</td>
</tr>
<tr>
<td>R. capsulatus chromophore</td>
<td>SQR</td>
<td>10-10</td>
<td>90</td>
</tr>
<tr>
<td>R. capsulatus chromophore</td>
<td>SQR</td>
<td>10-14</td>
<td>80</td>
</tr>
<tr>
<td>R. capsulatus chromophore</td>
<td>SQR</td>
<td>10-11</td>
<td>80</td>
</tr>
<tr>
<td>Q pool</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Note: SMP, submitochondrial particles.*

In this work, we describe an EPR-detectable thermodynamically stabilized semiquinone in *E. coli* QFR using an FrdC E29L mutant (10). The semiquinone is sensitive to HQNO and demonstrates extremely fast spin relaxation behavior, similar to the previously described *Q*⁺-*Q*⁻ pair of mitochondrial SQR. Furthermore, we demonstrate that in contrast to *B. subtilis* SQR, in *E. coli* QFR, HQNO interacts with the proximal quinone-binding site. The SQ species is also found in wild-type QFR, but has a much lower *K*ₚ and is detectable only in the higher pH range.

### MATERIALS AND METHODS

The *E. coli* strains, plasmids, and phage used in this study have been previously described (10). To obtain higher expression levels of the mutant forms of QFR discussed in this work, it was necessary to reconstruct the *frdC* mutations that had been previously made using a low copy number, *i.e.* two-plasmid expression system (10). Thus, site-directed mutagenesis was performed using the *in vitro* mutagenesis system from Bio-Rad based on the method developed by Kunkel (27) and Kunkel et al. (28) using single-stranded M13 DNA containing the *frdC* genes as template. Oligonucleotides were designed and synthesized on a Biosearch Model 8700 nucleic acid synthesizer to change the nucleotides in *frdC* encoding Gnu-29, His-82, and Trp-86 to codons for the selected amino acid substitutions. The mutations were confirmed by DNA sequence analysis using the dideoxy termination procedure (29) and a Ladderman DNA sequencing kit (Panvera Corp., Madison, WI).

Following mutagenesis, the 1070-base pair *DraIII*-XhoI fragment con-

---


*3* V. Borisov, I. Smirnova, C. Hägerhäll, A. Konstantinov, and L. Hederstedt, manuscript in preparation.
taining the desired mutation was cloned into plasmid pH3 (30) to restore the complete frdABCD operon with the desired mutation. Plasmids containing the mutations were then transformed into strain DW35 (ΔfrdABCD sdhCΔkan) (10) to give high level expression of membrane-bound QFR. E. coli strain DW35 cells containing pH3 plasmids encoding wild-type or mutant forms of QFR were grown anaerobically on glucose/fumarate medium as described previously (31). Cells were harvested in the early stationary phase of growth, and the membrane fraction was prepared from a French pressure cell lysate as described previously (32).

Redox titrations were performed at room temperature (25 °C) in an air-tight vessel flushed with oxygen-free argon and equipped with a magnetic stirring device, an Ag/AgCl-platinum combination electrode, and a pH electrode essentially as described (33). The redox potential of the reference electrode was calibrated versus a saturated solution of quininhydron (285 mV versus normal hydrogen electrode at pH 7). The following redox mediators, dissolved in H2O (indigo dyes) or Me2SO, were used at 25–50 μM final concentrations: 5,5'-indigodisulfonate (−125 mV), 5,5',7',8'-indotetrasulfonate (−46 mV), 2-hydroxy-1,4-naphthoquinone (−152 mV), 1,4-naphthoquinone-2-sulfonate (+113 mV), 1,4-naphthoquinone (+50 mV), and duroquinone (+7 mV). In total, <0.15% Me2SO was added. Reductive titrations were carried out by stepwise addition of an anaerobic sodium dithionite solution, and samples were transferred anaerobically to EPR tubes, then in a cold mixture of isopentane/methylcyclohexane (5:1) at about −80 °C, and stored in liquid nitrogen until EPR analyses. The relative concentration of the intermediate SQ form (y) as a function of the ambient redox potential (Ed) is described by Equation 1,

\[
y = \frac{E_{y,1}^{1/2}}{1 + 10^{(y - E_{y,2}^{1/2})/\Delta y_{1/2}}} + 10^{(y - E_{y,2}^{1/2})/\Delta y_{1/2}}\frac{E_{y,2}^{1/2}}{1 + 10^{(y - E_{y,2}^{1/2})/\Delta y_{1/2}}}\]  

(1)

where \(E_{y,1}^{1/2}\) and \(E_{y,2}^{1/2}\) are \(E_n\) values of two consecutive 1-electron transfer steps. Alternatively, membranes were poised at different ambient \(E_n\) values using the substrate couple succinate/fumarate (1:1) at a 20 mM total concentration under argon by changing the ambient pH in the absence of redox mediator dyes. The same titration vessel and setup as for the potentiometric titration were used. The pH, initially 4.0, was gradually altered by small additions of 5 M NaOH and, when approaching a pH of −9.5, was followed by similar additions of 5 M HCl. EPR samples were taken 5 min after each addition to ensure equilibrium and were frozen and stored as before. In both cases, the E. coli membranes were suspended in 50 mM BisTris and 3 mM EDTA (pH as indicated) at 25 mg/ml membrane protein. At this protein concentration, the membranes routinely contained 32 ± 5 μM QFR, based on spin quantitation of the iron-sulfur cluster FR1. H2ONO, when present, was added at −5:1 stoichiometry (150 μM). Protein was determined as described (34). Spin quantitation under non-power-saturated conditions was performed as described (35) using 0.5 mM CuEDTA as a standard. EPR spectra were recorded using an X-band Bruker ESP-300E EPR spectrometer equipped with an Oxford Instruments ESR-9 helium flow cryostat. Other EPR conditions were as indicated in the figure legends. The power saturation data were analyzed by computer fitting to Equation 2,

\[
A = \sum_{i=1}^{n} n C \left( \frac{P}{TP(1/2)^{1/2}} \right)^{n/2} 
\]

(2)

where \(A\) is the amplitude of the ith-type free radical, \(C\) is a coefficient for the actual content of the ith-type free radical in the sample, \(P_{frd}\) is the half-saturation microwave power, \(b_i\) is the “inhomogeneity parameter,” and \(n\) is numbers of components (36, 37). Simulation of the power saturation and redox titration curves was performed using the software Origin (MicroCal Software, Inc.) using the Marquardt-Leneberg algorithm and simplex method for nonlinear least-square fitting.

RESULTS

The previous EPR studies using mammalian SQR showed that semiquinone signals were more readily detected in submitochondrial particles compared with more resolved preparations or the isolated enzyme (38–40). In mitochondrial and bacterial inner membranes, other free radical species are also present. SQR-specific inhibitors such as carboxin and TTFA can be used in the mammalian experimental system, but for E. coli QFR, we have no specific inhibitors available. Thus, we compared redox titrations of membranes from E. coli strain DW35, deleted of both the QFR- and SQR-encoding operons (10), with membranes from DW35 expressing wild-type or mutant QFR. The overexpression of QFR also facilitated detection of QFR-bound semiquinone versus other unrelated free radicals in the system. In this study, attention was focused on the proximal quinone-binding site in QFR; and thus, we selected three of the most promising of the previously generated membrane anchor mutants predicted to reside in this area, i.e. FrdC E29L, H82R, and W86R (10) (Fig. 1).

EPR analyses of cytoplasmic membranes of E. coli, poised by conventional potentiometric redox titrations, showed that a weak SQ free radical g = 2.00 signal was present in both DW35 (QFR- and SQR-deleted) membranes and DW35 membranes containing wild-type QFR. The SQ species showed \(E_m\) values of approximately −30 and −50 mV, respectively, with about the same spin concentration/mg of membrane protein (data not shown). Both semiquinone signals were very slow relaxing; and in addition, neither signal was affected by H2ONO.

In contrast, membranes from FrdC E29L mutant QFR exhibited another SQ free radical species with much faster spin relaxation behavior (\(P_{frd} \gg 500\) mW) at 123 K similar to the SQ species of bovine heart SQR, in addition to the slow relaxing SQ species. Fig. 2 shows a potentiometric titration curve of the semiquinone g = 2.005 signal in the cytoplasmic membrane of the FrdC E29L mutant. SQ spectra were recorded at 5-mW microwave power to minimize the overlapping slow relaxing SQ signals. Curve-fitting computer analysis provided \(E_m\) values such as −112 mV and \(E_{m/2}\) values (98% of \(SQ\)) = +1.2 mV, which correspond to \(E_m/QH_2\) = −5.66 mV and a SQ stability constant (\(K_s\)) of \(1.2 \times 10^{-2}\). Both first and second electron transfer steps were assumed as \(n = 1\) steps. The \(E_m\) value corresponds to the peak redox potential of the bell-shaped titration curve, which equals the average of \(E_m\) and \(E_{m/2}\). This SQ signal was quenched by H2ONO. The SQ g = 2.005 spectra of the sample poised near the titration peak is shown below in Fig. 4.

The amplitude of the SQ signal in FrdC E29L mutant membranes at a sample temperature of 150 K was plotted as a function of microwave power in Fig. 5A. The observed biphasic saturation curve was resolved into two distinct components.

---

4 Commercial X-band EPR spectrometers can measure to a maximum of only a 200-mW level. Although we obtained very high \(P_{frd}\) values such as >500 mW by computer fitting, it means that the sample has extremely fast spin relaxation from a practical point of view.
with $P_{1/2}$ values of 0.095 and 788 mW, respectively. At 1- and 10-mW microwave power levels, ~90 and ~95% of the spectral contribution arise from signals of the extremely rapidly relaxing SQ component, respectively. Shown in Fig. 3B are the power saturation profiles of the wild-type enzyme compared with the two remaining mutant QFR species, FrdC H82R and W86R. These samples show $P_{1/2}$ values of 0.095, 0.12, and 0.13 mW, respectively. Only the fast relaxing ($P_{1/2} = 788$ mW component) SQ species of FrdC E29L is sensitive to low concentrations of HQNO (HQNO/QFR = 5:1). The EPR signal of the low $P_{1/2}$ SQ species of the wild-type enzyme and the FrdC H82R and W86R QFR mutants is insensitive to HQNO at this concentration range. The slow relaxing SQ signal is almost completely power-saturated under the EPR condition used in Fig. 2.

The maximal amplitudes of both the fast and slow relaxing SQ signals were increased by changing the ambient pH of the potentiometric titrations from 7 to 9, suggesting that these semiquinone species are mostly in an anionic form ($Q^-$) within this pH range. The semiquinone EPR spectra of E29L mutant QFR poised potentiometrically near the titration peak at pH 7.2 and 9.0 are shown in Fig. 4. The signal amplitude of SQ at pH 9.0 is higher than at pH 7.2, indicating that SQ is in an anionic form ($Q^-$) in this pH range. Both spectra exhibit an ~1.1-mT peak-to-peak line width with a gaussian-type EPR line shape. Around pH 7, the SQ signal was almost completely quenched by HQNO at a concentration ratio of 5:1 molar excess to QFR, whereas at pH 9, only ~80% of the signal was quenched (HQNO is known to be a less effective inhibitor at higher pH). We concluded that the fast relaxing SQ radical in the FrdC E29L mutant is QFR-associated and that the SQ state is more strongly bound to QFR than the oxidized or fully reduced states. In contrast to mitochondrial SQR in situ, no spin-coupled split signals indicative of a spin-coupled SQ pair were detected in *E. coli* QFR over a wide range of low temperatures.

Glu-29 in FrdC was among the first residues in the *E. coli* QFR membrane anchor to be implicated in interactions with quinones. This residue was proposed to facilitate protonation/deprotonation of the quinone (10), in analogy with a glutamate residue in the photosynthetic reaction center QB (41). It should also be noted that Glu-29 from FrdC is located in the vicinity of a glutamate residue in the Q-proximal and thus near to the iron-sulfur cluster FR3 (Fig. 1). In the structural model of the membrane anchor, Glu-29 is predicted to be located at or close to Q-proximal and thus near to the iron-sulfur cluster FR3 (Fig. 1). Fig. 5A shows the EPR spectrum of the FR3 $[3Fe-4S]^{1+}(1+0)$ cluster in wild-type membranes in the oxidized state. Addition of the inhibitor HQNO to wild-type membranes altered the EPR spectral line shape in the central region of the FR3 spec-
trum (Fig. 5B) as described (20). EPR line shape perturbation of the FR3 spectrum in the E29L mutant is similar to that seen when the wild-type QFR FR3 spectrum is perturbed by HQNO (Fig. 5C). No further FR3 line shape changes occurred after HQNO addition to FrdC E29L mutant QFR (data not shown). These observations provide evidence that FR3 is in close proximity to the Q-proximal binding site and agree with the observations of Rothery and Weiner (20). Furthermore, the location of the observed semiquinone in the vicinity of FR3 is consistent with the position of Glu-29 of FrdC in the structural model (Fig. 1) (3, 4) of the QFR membrane anchor.

To circumvent the interference with intensified g = 2.00 signals from the redox mediator dyes in the high pH range, we poised QFR under anaerobic conditions using the substrate couple succinate/fumarate at a 1:1 ratio at a total concentration of 20 mM, which is 3 orders of magnitude higher than the QFR concentration (Fig. 6). The ambient redox potential ($E_a$) was altered by gradually changing the pH from 6.5 to 9.5 by addition of small aliquots of alkali or acid, using the pH dependence (~60 mV/pH unit) of the succinate/fumarate redox couple. These experimental conditions were non-deleterious to QFR since the sequential oxidative and reductive titrations could be performed with reasonable reproducibility. As presented in Fig. 6, SQ peak-to-peak signal amplitude as a function of the ambient pH showed a biphasic curve, increasing SQ signal amplitude with increasing pH. Unfortunately, DW35 (QFR- and SQR-deleted) membranes cannot be used as a control in this system. Nevertheless, we observed biphasic power saturation profiles of the SQ species with extremely high (..500 mW) and low (0.05, P1/2, 0.3 mW) P1/2 values in the E29L mutant membranes, similar to those observed during potentiometric titration. The inhibitory effect of HQNO decreases with increasing pH in the range above pH 8.5. Although the SQ signal intensity is much lower in the wild-type membranes, the presence of an HQNO-sensitive SQ signal is clearly discernible at a pH range higher than 9.0.

In Fig. 7, the EPR spectra of FR3 poised at redox potentials of +15.6, −40.2, and −94.8 mV are presented, which correspond to pH values of 7.2, 8.2, and 9.1, respectively. Since the $E_m$ value of the trinuclear iron-sulfur cluster FR3 in both wild-type and E29L mutant QFR is in the range of approximately −70 to −50 mV and is pH-independent, the relative concentration of the oxidized [3Fe-4S]$^{1+}$ cluster FR3 is decreased when the ambient pH of the succinate/fumarate redox couple is increased (Table II). Resolution of the biphasic power saturation curves showed that the ratio of high P1/2 SQ versus...
low $P_{1/2}$ SQ varied as 2.8, 2.3, and 1.2 in parallel with pH changes of 7.2, 8.2, and 9.1, respectively. Concomitantly, the concentration of the oxidized FR3 amplitude decreased as 3.9, 2.4, and 1.0, respectively. This strongly suggests that the trinuclear cluster FR3 in the oxidized state ([3Fe-4S]$^{1-}$ spin 1/2 ground state) is more stable than the wild-type enzyme. The EPR-detectable semiquinone thermal stability of QFR in the oxidized state versus in the reduced state was clearly detectable in the pH range of 9.0–9.5, although its signal intensity was equivalent to only 15–20% of the counter-

![Figure 7](image)

**Fig. 7.** EPR spectra of the FR3 cluster in E29L mutant QFR poised with 20 mM succinate/fumarate buffer at three different pH values. EPR conditions were as described in the legend to Fig. 5.

have previously been directly demonstrated only in eukaryotic organisms.\(^5\)

The SQ spectra of E. coli FrdC E29L membranes at three different pH values are presented in Fig. 8. Notably, the SQ spectral line shape of the succinate/fumarate poised system is more a Lorentzian-type than that obtained by potentiometric titration, although the peak-to-peak width is the same. The SQ signal intensity increased with increasing pH in the same manner as the potentiometrically poised system (Fig. 4). This indicates that the SQ species in E. coli QFR is in the anionic form ($Q^{-}$) in the pH 7–9 range.

**DISCUSSION**

In this work, we have shown that an E. coli mutant (FrdC E29L) QFR contains an EPR-detectable semiquinone thermodynamically more stable than the wild-type enzyme. Semiquinone species associated with succinate:quinone oxidoreductase have been previously detected in bacterial QFR. In both the previously described mammalian SQR and E. coli QFR, the observed semiquinone is apparently stabilized at the proximal quinone-binding site. The effect of quinone-binding site inhibitors on the semiquinone is apparently stabilized at the proximal quinone-binding site. The effect of quinone-binding site inhibitors on SQR (both Class 1 SQRs donating electrons to ubiquinone), the semiquinone is stabilized in the wild-type enzyme, whereas in E. coli QFR (oxidizing menaquinol), the semiquinone at the proximal site is not detected in the wild-type enzyme. However, an HQNO-sensitive SQ species was clearly detectable in the pH range of 9.0–9.5, although its signal intensity was equivalent to only 15–20% of the counter-

**Table II**

| pH | $K_{s}$ | $P_{SO}$ | Relative conc of SQ-fast | Relative conc of [FR3]$^{1-}$
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>9.1</td>
<td>94.8</td>
<td>813</td>
<td>1.2</td>
<td>1.0</td>
</tr>
<tr>
<td>8.2</td>
<td>40.2</td>
<td>982</td>
<td>2.3</td>
<td>2.4</td>
</tr>
<tr>
<td>7.2</td>
<td>15.6</td>
<td>813</td>
<td>2.8</td>
<td>3.9</td>
</tr>
</tbody>
</table>

During the preparation of this manuscript, we learned that X. Yang and L. Yu have detected semiquinone signals from wild-type E. coli SQR in situ (L. Yu, personal communication).
part signal detectable in the E29L mutant (Fig. 6). In the catalytic reactions of SQR/QFR, it is known that semiquinones are necessary for the transition of the n = 1 ↔ n = 2 electron transfer steps. However, for the same functional role, a wide range of stability constants for semiquinones can be found in the literature with differences of several orders of magnitude depending on the preparation. Even larger differences are seen depending on the physiological function of certain quinone-binding site(s) (2, 3) (see Table I). The E29L mutant is in fact severely defective in both quinol oxidase and quinone reductase activities, and one may speculate that the proximal-Q site in *E. coli* QFR is meant to produce a thermodynamically relatively unstable semiquinone for its physiological n = 1 ↔ n = 2 converter function. Analogously, a decrease in enzyme activity was reported upon the stabilization of SQ, in the case of an H271R mutant of cytochrome *b* in the *Rhodobacter capsulatus* chromatophore (24) and in mitochondria from *E. coli* (25). Although HQNO is a potent inhibitor of QFR, it also inhibits other components in the respiratory chain. The *Q* site of the cytochrome *bc* complex interacts with HQNO, and formate dehydrogenase and a number of quinol-oxidizing enzymes are HQNO-sensitive, including *QH*-nitrate reductase, the *o*- and *d*-type ubiquinol oxidases (47), and Me2SO reductase. A recent paper describes the interaction of an engineered [3Fe-4S] cluster in *Me2SO* reductase with HQNO (48), indicating the presence of a proximal HQNO-binding site also in this quinol-oxidizing enzyme, analogous to that in *E. coli* QFR. The structure of HQNO resembles a seminaphthoquinone. The apparent *K* values for HQNO of *B. subtilis* SQR and *E. coli* QFR increase with increasing pH, indicating that the deproto- nated inhibitor is less efficient (18). Thus, one may speculate that HQNO binds to the opposite quinone-binding site in *E. coli* QFR versus that in *B. subtilis* SQR. This is particularly interesting in light of the reverse function and different directionality of these two enzymes, which both use menaquinone as the electron donor/acceptor.

Although HQNO is a potent inhibitor of QFR, it also inhibits other components in the respiratory chain. The *Q* site of the cytochrome *b* complex interacts with HQNO, and formate dehydrogenase and a number of quinol-oxidizing enzymes are HQNO-sensitive, including *QH*-nitrate reductase, the *o*- and *d*-type ubiquinol oxidases (47), and Me2SO reductase. A recent paper describes the interaction of an engineered [3Fe-4S] cluster in *Me2SO* reductase with HQNO (48), indicating the presence of a proximal HQNO-binding site also in this quinol-oxidizing enzyme, analogous to that in *E. coli* QFR. The structure of HQNO resembles a seminaphthoquinone. The apparent *K* values for HQNO of *B. subtilis* SQR and *E. coli* QFR increase with increasing pH, indicating that the deproto- nated inhibitor is less efficient (18). Thus, one may speculate that HQNO binds to the opposite quinone-binding site in *E. coli* QFR versus that in *B. subtilis* SQR. This is particularly interesting in light of the reverse function and different directionality of these two enzymes, which both use menaquinone as the electron donor/acceptor.

Acknowledgment—T. O. thanks R. Lin for excellent general assistance in preparing this manuscript.

REFERENCES


*After the original submission of this manuscript, the x-ray crystallographic structure of *E. coli* quinol:formate reductase at 3.3 Å resolution was completed (49). Our proposed proximity of FrdC Glu-29 to the Q-proximal site and detection of the spin-spin interaction between SQ-proximal and [3Fe-4S]FR3 are consistent with the determined center-to-center distances of 4–5 and 9–11 Å (49), respectively.*
26164  

Semiquinone in Escherichia coli Fumarate Reductase


