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)

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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 × 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 (SQs) 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 SQFR spins 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.

* This work was supported in part by National Science Foundation Grants MCB-9418694 (to T. O.) and MCB-9728778 (to G. C.), the Department of Veterans Affairs (to G. C.), and National Institutes of Health Grant HL-16251 (to G. C. and I. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This work is dedicated to the memory of Vladimir D. Sled.

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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.

Succinate:quinone reductase (SQR)1 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 helices depending on the enzyme species. When two helices are present, they are denoted heme b5 and heme b6. 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 *vivo*, 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 differentially tuned in the enzymes of a different functional class.

1 The abbreviations used are: SQR, succinate:quinone reductase; QFR, quinol:fumarate reductase; Q, ubiquinone; TTFA, 2-thienyl-trifluoroacetone; HQNO, 2-n-heptyl-4-hydroxyquinoline N-oxide; SQ, semiquinone; QH2, quinol; BisTris, 2-[isobutyl]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 Q₃), 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 Q₄).

There are a number of inhibitors that interfere with the interaction of SQR/QFR with quinones. The most well known are 2-thienylfluorouracetone (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 *Eₚₗ₃ 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 the respiratory chain compared with species functioning solely as n = 2 components.

| Table I |
| Stability constants of different semiquinone species functioning as a converter in n = 1 ↔ n = 2 electron transfer processes in the respiratory chain compared with species functioning solely as n = 2 components |

| SQ species | K₉ | Eₚₗ₃ | pH |
|-----------|----|-----|----|
| Converters of n = 1 ↔ 2 e⁻ transfer processes |
| B. taurus SMP⁵ | SQ₉ | 10 | +110 | 7.4 |
| B. *coli* Frd C E29L membrane | SQ₉⁰ | 1.2 x 10⁻² | -57 | 7.2 |
| B. taurus SMP | SQ₉ | 5 x 10⁻² | +80 | 7.0 |
| R. *spheroides* chromatophore | SQ₉ | 4 x 10⁻² | +150 | 8.0 |
| B. *taurus* bc₁ complex | SQ₉ | 1 x 10⁻² | +90 | 7.0 |
| R. *capsulatus* chromatophore | SQ₉ | 1 x 10⁻¹ | +150 | 7.0 |
| Q pool | SQ₉ | 10⁻¹⁰ | +90 | 7.0 |
| R. *capsulatus* chromatophore | SQ₉ | 10⁻₁⁴ | +80 | 7.0 |
| R. *capsulatus* chromatophore | SQ₉ | 10⁻¹¹ | +80 | 7.0 |

² BMP, submitochondrial particles.
³ none or flavosemiquinone (spin-off) 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). Subsequently, Ingledew and Ohnishi (16) and Salerno and Ohnishi (17) showed that these split signals arise from a semiquinone anion (Q⁻Q⁺) pair, based on detailed EPR and thermodynamic analysis of the rapidly relaxing Q⁻ g = 2.00 signal and the spin-coupled split signals. Both Q⁻ and the Q⁺ signals are sensitive to the SQR-specific inhibitors carboxin and TTFa. The spin-coupled Q⁺ pair have similar Eₚₗ₃ values: Eₚₗ₃(Q⁻/Q) and Eₚₗ₃(QH₂/Q⁻) = 140 and 80 mV, respectively, which correspond to a stability constant (K₉) of 10. This is many orders of magnitude greater than the *K₉* of the quinone pool, indicating a preferential binding of *Q*⁻ relative to Q and QH₂ (Table I). Similar spin-spin interaction signals have been observed in mitochondria from various plants (24) and the fungus *Neurospora crassa* (25). Neither semiquinone nor the spin-coupled split EPR signals have previously been directly demonstrated in bacterial SQR and QFR enzymes, although recent results based on spectral simulations suggest the presence of a similar semiquinone pair in *P. denitrificans* SQR (26).

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⁻ g = 2.00 signal 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 *K₉* 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 frdCD genes as template. Oligonucleotides were designed and synthesized on a Biosearch Model 8700 nucleic acid synthesizer to change the nucleotides in frdC encoding Gln-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 Ladderless DNA sequencing kit (Panvera Corp., Madison, WI). Following mutagenesis, the 1070-base pair DraIII-XhoI fragment con-
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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 carbinox 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 W68R (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 HQNO.

In contrast, membranes from FrdC E29L mutant QFR exhibited another SQ free radical species with much faster spin relaxation behavior (P_{1/2} > 500 mW)^4 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 of approximately –112 mV and E_n2 (SQ/Q) = +1.2 mV, which corresponds to E_m (TH) = –56.6 mV and a SQ stability constant (K_s) of 1.2 × 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_m1 and E_n2. This SQ signal was quenched by HQNO. 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

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4 Commercial X-band EPR spectrometers can measure to a maximum of only a 200-mW level. Although we obtained very high P_{1/2} 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 the peptide stretch recently labeled with [3H]azidoquinone in E. coli SQR (9) (see 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]$^{1+} \rightarrow [2Fe-2S]$ 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 P 1/2, 0.3 mW) P 1/2 values in the E29L mutant membrane, 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 P 1/2 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) seems to be a more effective spin relaxant of the spin 2 ground state. It should be pointed out that we estimated the relative concentrations of the FR3 cluster ([3Fe-4S]$^{1+}$ spin $1^2$ ground state) based on the amplitude of the 2.02 $g_z$ peak, which is consistent with the calculated redox change in the $E_m$ and $E_h$ values of the three selected EPR samples of the titration in Fig. 6. However, it is discernible that the central EPR spectral line shape of the FR3 cluster could be significantly altered during the titration from curve A to C (Fig. 7). Detailed computer simulations are needed for more rigorous analysis of the correlation suggested above.

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 previously been directly demonstrated only in eukaryotic organisms.$^5$

Our results represent the first direct observation of a stabilized semiquinone 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 cluster S3 in mitochondria (16, 17) in combination with the location of mutations in other species giving resistance to the same inhibitors (14, 15) demonstrates this fact. In E. coli QFR, the effect of the E29L mutation on the EPR line shape of cluster FR3, similar to that observed in the wild-type enzyme in the presence of HQNO, in combination with the effect of HQNO on the E29L stabilized semiquinone is consistent with a closeness between the proximal quinone-binding site and the iron-sulfur cluster FR3. In addition, these data are consistent with the position of Glu-29 in the current structural model (3, 4) of the QFR membrane anchor. However, in bovine SQR and seemingly in P. denitrificans 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-

$^5$ 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 SQRs/QFRs, it is known that semiquinones are necessary for the transition of the \( n = 1 \leftrightarrow n = 2 \) 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 \textit{E. coli} QFR is meant to produce a thermodynamically relatively unstable semiquinone for its physiological \( n = 1 \leftrightarrow n = 2 \) 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 \textit{Rhodobacter capsulatus} chromatophore \( bc_1 \) complex (42, 43).

In a recent study by Ishii \it{et al}. (44), it was demonstrated that a glycine-to-glutamate mutation in the \textit{Caenorhabditis elegans} SdhC subunit resulted in oxidative stress and premature aging in the nematode. Alignment of SdhC/FrdC subunits from various species places this glycine residue in the vicinity of Glu-29 in FrdC (4). As shown in this work, mutation of Glu-29 in \textit{E. coli} QFR results in the stabilization and easier detection of SQ. Long-lived semiquinones are prone to react with oxygen. Thus, similar perturbation of the quinone-binding environment in \textit{C. elegans} mutant SQFR could be responsible for the increased oxidative stress and premature aging.

The semiquinone detected in E29L mutant QFR demonstrates extremely fast spin relaxation behavior, similar to that found for the SQ semiquinone from \textit{B. taureus} mitochondria. In the latter case, it has been suggested that spin relaxation of the SQ semiquinone is enhanced by the very fast relaxing S3 spins in the oxidized \((S = 1/2)\) spin state and/or by the heme spins of the membrane anchor cytochrome \( b \) (17, 39). Since QFR lacks heme, the spin interacting partner of the E29L semiquinone has to be the \([3Fe-4S]\) cluster FR3. The \( E_m \) of the succinate/fumarate couple is pH-dependent (\(-60 \text{ mV/pH unit} \)), whereas the \( E_m \) of the FR3 cluster is not. Thus, during the succinate/fumarate pH titrations shown, the iron-sulfur cluster FR3 is changed from oxidized at low pH to more reduced states at higher pH (Fig. 7). In the oxidized \([3Fe-4S]^{1+}\) state of the iron-sulfur cluster FR3, three high spin \((S = 5/2)\) Fe\(^{3+}\) atoms are anti-ferromagnetically coupled to give an \( S = 1/2 \) ground state in the low temperature range; in this case, one unpaired valence electron is delocalized among three Fe\(^{3+}\) atoms. In the reduced \([3Fe-4S]^{0}\) state of the FR3 cluster, the overall system is in the \( S = 2 \) spin state, arising from antiferromagnetic interaction between a valence-delocalized Fe\(^{3+}\)/Fe\(^{2+}\) pair \((S = 9/2)\) and a valence-localized Fe\(^{3+}\) site \((S = 5/2)\) (45). Although the FR3 cluster is paramagnetic in both the oxidized and reduced states, it is more efficient in relaxing the spin of the reduced cluster FR3 in the oxidized \( S = 1/2 \) form than in its reduced \( S = 2 \) spin state (1). As shown in Fig. 6 and Table I, spin relaxation of SQ in FrdC E29L mutant QFR is also more effectively enhanced by the oxidized \( S = 1/2 \) state FR3 than by the reduced \( S = 2 \) state FR3, as in the case of the \( Q^*Q^* \) pair in mitochondrial SQ and the \([2Fe-2S]_{21}\) cluster in \textit{Micrococcus luteus} SQR (46). The spin-coupled \( Q^*Q^* \) split signals were not detected in \textit{E. coli} E29L mutant QFR. Notably, in addition to mammalian mitochondria SQR, the spin-coupled \( Q \) semiquinone pair has been observed only in some green plant mitochondria (24) and in mitochondria from \textit{N. crassa} (25). This may be due to the fact that EPR signals from an interacting semiquinone pair are much more sensitive to perturbations than a single semiquinone. If the two interacting semiquinones function as independent electron (or proton) transfer components, the concentration of the interaction signals would be the square function of the individual semiquinone concentrations. A modest shift of \(-40 \text{ mV} \) in the \( E_m \) of one bound quinone relative to the other would cause an almost complete lack of signal (17). We cannot, however, exclude the possibility that in \textit{E. coli} QFR, only a single quinone molecule exists at the Q-proximal domain rather than the interacting pair.\(^6\)

In a previous study by Westenberg \it{et al}. (10), Glu-29 of FrdC was replaced by aspartate, lysine, or phenylalanine, in addition to the replacement by leucine (10). To understand more about the stabilization of this semiquinone in \textit{E. coli} QFR, we will perform EPR and thermodynamic analysis of membranes from QFR FrdC mutants such as E29D, E29F, and E29R.

The location of the HQNO-binding site is also of interest. As mentioned in the Introduction, in \textit{B. subtilis} SQR, HQNO binding induces a shift in the \( E_m \) of heme \( b_1 \) of about \(-60 \text{ mV} \), but has no effect on the \( E_m \) of heme \( b_2 \) (19), indicating that the HQNO-binding site in \textit{B. subtilis} SQR corresponds to Q-distal. Furthermore, in \textit{B. subtilis}, no effect of HQNO on the EPR properties or thermodynamic behavior of S3 was detected (21, 22), although a Q-proximal site is seemingly present in \textit{B. subtilis} SQR (21, 22). In this study, it is clear that in \textit{E. coli} QFR, the inhibitor HQNO interacts with the proximal quinone-binding site. We can thus conclude that HQNO binds to the opposite quinone-binding site in \textit{E. coli} QFR versus that in \textit{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 \( bc_1 \) complex interacts with HQNO, and formate dehydrogenase and a number of quinol-oxidizing enzymes are HQNO-sensitive, including \( QH_2 \)-nitrate reductase, the \( o- \) and \( d \)-type ubiquinol oxidases (47), and \( MeS \) oxidase. A recent paper describes the interaction of an engineered \([3Fe-4S]\) cluster in \( MeS \) oxidase with HQNO (48), indicating the presence of a proximal HQNO-binding site also in this quinol-oxidizing enzyme, analogous to that in \textit{E. coli} QFR. The structure of HQNO resembles a semi(naphtho)quinone. The apparent \( K \) values for HQNO of \textit{B. subtilis} SQR and \textit{E. coli} QFR increase with increasing pH, indicating that the deprotected inhibitor is less efficient (18). Thus, one may speculate that HQNO binds to topographically different, but perhaps functionally similar sites in \textit{E. coli} QFR and \textit{B. subtilis} SQR.

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

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\(^6\) After the original submission of this manuscript, the x-ray crystallographic structure of \textit{E. coli} quinol:fumarate reductase at 3.3Å 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 \([3Fe-4S]_{21}\) are consistent with the determined center-to-center distances of \(-4–5 \) and \(-9–11 \) Å (49), respectively.
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