JOURNAL OF BACTERIOLOGY, Aug. 1995, p. 4587–4592 Vol. 177, No. 16
0021-9193/95/$04.00+0

Aerobic Inactivation of Fumarate Reductase from *Escherichia coli* by Mutation of the [3Fe-4S]-Quinone Binding Domain

GARY CECCHINI,[1]* HARRY SICES,[1] IMKE SCHRÖDER,[2] AND ROBERT P. GUNSALUS[2]

Molecular Biology Division, Veterans Administration Medical Center, San Francisco, California 94121, and Department of Biochemistry and Biophysics, University of California, San Francisco, California 94143,[1] and Department of Microbiology and Molecular Genetics and Molecular Biology Institute, University of California, Los Angeles, California 90024[2]

Received 26 January 1995/Accepted 6 June 1995

Fumarate reductase from *Escherichia coli* functions both as an anaerobic fumarate reductase and as an aerobic succinate dehydrogenase. A site-directed mutation of *E. coli* fumarate reductase in which FrdB Pro-159 was replaced with a glutamine or histidine residue was constructed and overexpressed in a strain of *E. coli* lacking a functional copy of the fumarate reductase or succinate dehydrogenase complex. The consequences of these mutations on bacterial growth, assembly of the enzyme complex, and enzymatic activity were investigated. Both mutations were found to have no effect on anaerobic bacterial growth or on the ability of the enzyme to reduce fumarate compared with the wild-type enzyme. The FrdB Pro-159-to-histidine substitution was normal in its ability to oxidize succinate. In contrast, however, the FrdB Pro-159-to-Gln substitution was found to inhibit aerobic growth of *E. coli* under conditions requiring a functional succinate dehydrogenase, and furthermore, the aerobic activity of the enzyme was severely inhibited upon incubation in the presence of its substrate, succinate. This inactivation could be prevented by incubating the mutant enzyme complex in an anaerobic environment, separating the catalytic subunits of the fumarate reductase complex from their membrane anchors, or blocking the transfer of electrons from the enzyme to quinones. The results of these studies suggest that the succinate-induced inactivation occurs by the production of hydroxyl radicals generated by a Fenton-type reaction following introduction of this mutation into the [3Fe-4S] binding domain. Additional evidence shows that the substrate-induced inactivation requires quinones, which are the membrane-bound electron acceptors and donors for the succinate dehydrogenase and fumarate reductase activities. These data suggest that the [3Fe-4S] cluster is intimately associated with one of the quinone binding sites found in fumarate reductase and succinate dehydrogenase.

During anaerobic respiration in *Escherichia coli*, the fumarate reductase (FRD) complex catalyzes the fumarate-dependent oxidation of menaquinol, thereby allowing production of a transmembrane proton gradient that can be used by the organism to support metabolism (19). The FRD complex is composed of four nonidentical subunits, FrdA, FrdB, FrdC, and FrdD, arranged into two domains: the FrdAB catalytic domain and the FrdCD membrane anchor domain (2, 22). FrdA (66 kDa) contains covalently bound flavin adenine dinucleotide and the active site of the enzyme (38), and the 27-kDa FrdB subunit contains the three distinct iron-sulfur centers present in the enzyme (23). The iron-sulfur clusters, center 1 ($[2Fe\text{-}2S]^{2+,+}$), center 2 ($[4Fe\text{-}4S]^{2+,+}$), and center 3 ($[3Fe\text{-}4S]^{+,0}$), are similar in structure and function in all membrane-bound succinate dehydrogenases (SDHs) and FRDs examined to date (2). The FrdC (15 kDa) and FrdD (13 kDa) subunits are essential for attachment of the catalytic subunits to the inner surface of the cytoplasmic membrane (22) and are also essential for electron transfer reactions involving menaquinol and ubiquinone (8).

The physical and catalytic properties of the FRD complex (FrdABCD) are very similar to those of succinate-ubiquinone oxidoreductase (complex II) from bacteria and mitochondria (2, 14). *E. coli* FRD catalyzes succinate oxidation at rates similar to that of the native SDH of that organism (2) and at about 30 to 40% of the rate at which it reduces fumarate (8). The *frd* gene products will replace SDH in *sdh* mutants when expressed from multicopy plasmids (13). It has been proposed, on the basis of sequence comparison of the catalytic domain subunits of FRD and SDH, that the genes are of common ancestral origin (41). These properties make *E. coli* FRD an excellent model system for studies of both enzyme systems and of their prosthetic groups.

The iron-sulfur clusters of FRD and SDH appear to be organized into two domains in the respective FrdB and SdhB subunits. The first group of four conserved cysteinyl residues are the ligands for center 1, and this [2Fe-2S] cluster can form in the absence of the other clusters (4, 16). The second domain encompasses the [4Fe-4S] and [3Fe-4S] clusters of FRD and SDH. On the basis of site-directed mutagenesis and electron paramagnetic resonance (EPR) studies of FRD and by analogy to the sequence of structurally characterized bacterial ferredoxins, it has been suggested that Cys-148, -151, -154, and -214 bind to the [4Fe-4S] cluster, center 2, and Cys-158, -204, and -210 bind to the [3Fe-4S] cluster, center 3 (2, 14, 23) (Fig. 1).

This communication reports part of an ongoing study to investigate the role of the iron-sulfur clusters of FRD in electron transport. In this study, FrdB Pro-159, which is conserved in all FRDs and SDHs thus far sequenced and situated next to Cys-158, which partially ligates the [3Fe-4S] cluster, was substituted by a Gln or His residue (creating FrdB $Pro^{159}Gln$ or FrdB $Pro^{159}His$) in order to determine if this change would alter the properties of center 3 and electron transport from and to quinones. This proline residue was chosen for mutagenesis because it is conserved and it was thought that alteration of a

* Corresponding author. Mailing address: Molecular Biology Division (151-S), VA Medical Center, 4150 Clement St., San Francisco, CA 94121. Phone: (415) 752-9676. Fax: (415) 750-6959. Electronic mail address: ceccini@itsa.ucsf.edu.

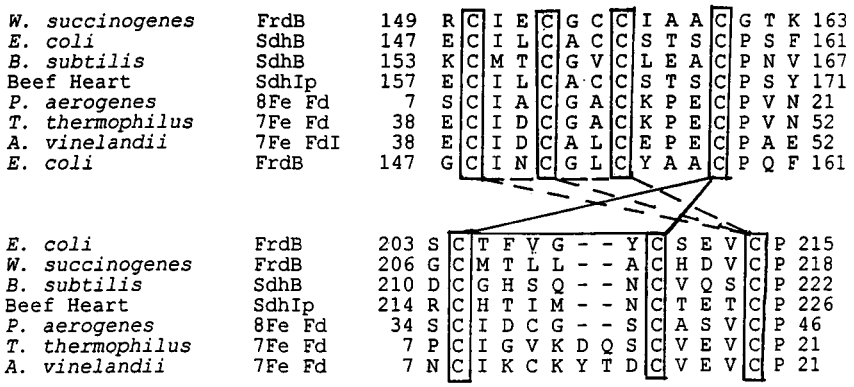

FIG. 1. Comparison of the arrangement of residues ligating the [4Fe-4S] and [3Fe-4S] clusters of FRD and SDH. By reference to the *E. coli* FRD numbering system, Cys-148, -151, -154, and -214 connected by the dashed lines are thought to ligate the [4Fe-4S] cluster. Cys-158, -204, and -210 connected by the solid lines ligate the [3Fe-4S] cluster. The sequence data for *E. coli* FrdB (10), *Wolinella succinogenes* FrdB (21), and SDH from beef heart (42), *Bacillus subtilis* (28), and *E. coli* (11) are compared with those for the 8Fe-ferredoxin (8Fe Fd) from *Peptococcus aerogenes* (3) and the 7Fe-ferredoxin from *Thermus thermophilus* (32) and *Azotobacter vinelandii* (15).

residue not directly ligating the iron-sulfur cluster would have less pronounced effects on the properties of the cluster. The results suggest that the enzyme assembles normally, the [3Fe-4S] cluster is still formed, and FRD activity was unimpaired. However, aerobic incubation with succinate caused an inactivation of the mutant FRD complex (FrdABP^{159}QCD) containing the Gln substitution. This inactivation occurred only with the membrane-bound mutant enzyme, and the results suggest that the inactivation is caused by a hydroxyl radical species inactivating the FRD complex. The results support the contention that the [3Fe-4S] cluster is intimately involved in the site(s) of interaction of FRD with quinones and may be part of one of the quinone binding sites (40).

MATERIALS AND METHODS

Strains, plasmids, and phage. Strains, plasmids, and phage used in this study are described in Table 1. *E. coli* DW35 contains a deletion of the entire *frdABCD* operon and a polar mutation in the *sdhC* gene and has no wild-type FRD or SDH activity (40). Plasmid pFrdA$^+$BP^{159}Q was prepared by digestion of pFrdA$^+$BP^{159}QC$^+$D$^+$ (pH3FrdBP^{159}Q) (see below) with *Sal*I and *Hin*dIII and deletion of the 1.3-kb fragment encoding the *frdC*$^+$ and *frdD*$^+$ genes. Ligation of the larger fragment results in a plasmid encoding the soluble catalytic FrdA and FrdB subunits containing the FrdB Pro-159-to-glutamine substitution.

TABLE 1. *E. coli* strains, plasmids, and phage used

Strain, plasmid, or phage	Genotype or description	Reference or source
E. coli strains		
DW35	F$^-$ *zjd*::Tn*10* Δ(*frdABCD*)*18* *sdhC*::*kan araD139* Δ(*argF-lac*)U169 *rpsL150 relA1 flbB5301 deoC1 pfsF25 rbsR*	40
DH5α	F$^-$ ϕ80d/*lacZ*ΔM15 *endA1 recA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1 gyrA relA1* Δ(*lacZYA-argF*) U169 λ$^-$ λ$^-$	5
CJ236	*dut ung thi relA* pCJ105 (Cm^r)	Bio-Rad
MV1190	(Δ*lac-proAB*) *thi supE* Δ(*sr1-recA*) *306*::Tn*10* (Tet^r) [F′ *traD36 proA*B *lacI*^{q}ZΔM15]	Bio-Rad
Plasmids		
pH3	*frd*A$^+$*B*$^+$*C*$^+$*D*$^+$	6
pFrdABP^{159}Q	*frd*A$^+$B^{P159Q}*C*$^-$D$^-$	This study
pH3FrdBP^{159}Q	*frd*A$^+$B^{P159Q}C$^+$D$^+$	This study
pH3FrdBP^{159}H	*frdA*$^+$*B*^{P159H}*C*$^+$*D*$^+$	This study
Phage		
M13mp8		24
M13KW1		39

Site-directed mutagenesis. Site-directed mutagenesis was performed by using an in vitro mutagenesis system from Bio-Rad (Richmond, Calif.) based on the method developed by Kunkel et al. (20). Oligonucleotides for mutagenesis and sequencing were synthesized on a Biosearch model 8700 synthesizer. Oligonucleotides were designed to change FrdB Pro-159 to a glutamine or histidine residue. The mutagenesis was performed with single-stranded M13KW1 DNA as the template. Correct production of the mutation was confirmed by DNA sequence analysis using the dideoxy termination procedure (31) and a Pharmacia (Piscataway, N.J.) sequencing kit. Strains CJ236 and MV1190, supplied by Bio-Rad, were used as hosts for mutagenesis and single-stranded DNA sequence analysis. Following mutagenesis, the 2.0-kb *Eco*RI-*Sal*I fragment containing the *frdB* region was cloned back into the large *Eco*RI-*Sal*I fragment of plasmid pH3 to restore the complete *frdABCD* operon. The plasmid was transformed into *E. coli* DH5α, and the mutation was confirmed by double-stranded DNA sequencing. DNA was prepared by using a Magic miniprep kit from Promega (Madison, Wis.).

Growth of bacteria. Following mutagenesis, plasmids were transformed into *E. coli* DW35 (Δ*frdABCDsdhC*::*kan*) to measure cell growth and for preparation of membranes and enzyme for assay. For anaerobic growth rate determinations, strains were grown on glycerol-fumarate, glucose-fumarate, and glycerol-nitrate minimal medium as previously described (9, 33). Aerobic cell growth was measured in the same minimal medium but with 40 mM succinate as the sole carbon source. Cells used for biochemical studies were grown on anaerobic glucose-fumarate medium to stationary phase, chilled, and harvested by centrifugation, and membrane fractions were prepared as previously described (39). For phage and plasmid manipulations, cells were grown on Luria broth as previously described (6).

Enzyme assays. Cells were grown anaerobically on glucose-fumarate medium as described above and harvested, and membrane and soluble fractions prepared as previously described (8, 39). The reduction of fumarate was measured in anaerobic cuvettes by using reduced benzyl viologen (BV) or reduced 2,3-dimethyl-1,4-naphthoquinone (DMN) as the electron donor for fumarate reduction (40). The oxidation of succinate was measured with phenazine methosulfate (PMS) or decylubiquinone (2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone) (DBH) as the primary electron acceptor. Anaerobic PMS reductase assays were performed under an Argon atmosphere in cuvettes made anaerobic by previously described methods (8). Enzyme concentration was determined as covalently bound histidyl-flavin adenine dinucleotide by using established procedures (29), and protein concentration was determined by the Lowry method.

Materials. BV, PMS, DBH, 2-heptyl-4-hydroxyquinoline *N*-oxide (HOQNO), 2,6-dichlorophenolindophenol (DCIP), deferoxamine mesylate, and 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) were obtained from Sigma (St. Louis, Mo.). Restriction enzymes were purchased from Gibco-BRL (Gaithersburg, Md.) and New England Biolabs (Beverly, Mass.). [^{35}S]dATP was purchased from Amersham Corp. (Arlington, Ill.). All other chemicals were of the highest grade commercially available.

RESULTS

As part of our ongoing studies on the structures and functions of the iron-sulfur clusters of FRD and SDH, we wished to

TABLE 2. Anaerobic and aerobic growth properties of wild-type and *frdB* strains

Strain/plasmid	Generation time (h)			
	Succ, O_2	Gly-Fum	Glc-Fum	Gly-NO_3^-
DW35	NG	NG	0.8	1.0
DW35/pH3	2.1	2.1	0.8	1.0
DW35/pH3FrdBP^{159}H	2.4	2.1	0.8	1.1
DW35/pH3FrdBP^{159}Q	4.6	1.9	0.8	1.0

[a] Growth on succinate was performed aerobically, while all other measurements were done under anaerobic conditions. Succ, O_2, aerobic growth on succinate; Gly- Fum, Glc- Fum, and Gly-NO_3^-, anaerobic growth on glycerol-fumarate, glucose-fumarate, and glycerol-nitrate minimal media, respectively; NG, no growth.

TABLE 3. FRD and SDH activities of wild-type and FrdB mutant enzymes

FrdB substitution	Turnover no.			
	FRD		SDH	
	BV	DMN	PMS	DBH
None (wild type)	27,700	10,700	4,200	2,400
Pro^{159}His	29,200	ND	4,000	ND
Pro^{159}Gln	28,300	9,200	850	350

[a] The indicated compounds were used as electron donor or acceptor. PMS and DBH reductase activities were measured by established methods (8) which involve a 5-min preincubation of the enzyme in the presence of 20 mM succinate at 38°C to remove inhibitory oxaloacetate in order to achieve full enzyme activity (1). ND, not determined.

assess the effects of mutations near the cysteinyl residues known to ligate the [3Fe-4S] cluster of FRD (Fig. 1). Two FrdB Pro-159 substitutions were constructed to investigate the role of the [3Fe-4S] cluster in electron transfer and to determine if these substitutions would have an effect on the formation or properties of this iron-sulfur cluster. It was reasoned that this type of substitution might be particularly informative because direct substitution of the cysteinyl residues ligating the [3Fe-4S] cluster usually results in the formation of enzyme which lacks this iron-sulfur cluster (23). The mutagenized plasmid DNA was transformed into *E. coli* DW35 (40) so that background of wild-type FRD and SDH is eliminated from the subsequent enzymatic analyses.

Growth studies. To determine whether the FrdB mutant enzymes that were constructed were physiologically functional, we measured the doubling time of cells grown anaerobically on glycerol-fumarate medium or aerobically on minimal succinate medium (Table 2). Under these conditions, a functional FRD is required; i.e., *E. coli* DW35 does not grow in either of these media. FRD when expressed from multicopy plasmids is fully capable of functionally replacing SDH in *E. coli* (13). FRD when expressed has, in fact, the same turnover number for succinate oxidation as native *E. coli* SDH (2, 33). As seen in Table 2, the doubling times of *E. coli* DW35 carrying the wild-type FRD plasmid pH3 were 2.1 h during aerobic and anaerobic growth. Doubling times of the FrdB Pro^{159}His mutant were essentially the same as those of the wild type, indicating that this mutant enzyme still functions fully as a bifunctional oxidoreductase. In contrast, the FrdB Pro^{159}Gln substitution resulted in an enzyme complex which functions normally as an FRD but, as evidenced by its aerobic generation time of 4.6 h, is partially impaired in its ability to support aerobic growth of *E. coli.*

Enzyme activity studies. The turnover numbers of wild-type and mutant FRDs are shown in Table 3. The values shown reflect both the FRD activity as measured by anaerobic assays of the oxidation of reduced BV or the menaquinone analog DMN and aerobic assays of SDH activity determined by the reduction of PMS or the ubiquinone analog DBH. Turnover numbers indicate that both mutant enzymes are fully active in both catalytic assays for fumarate reduction, in agreement with the growth rate data (Table 2). In assays of succinate oxidation, the FrdB Pro^{159}His mutant enzyme was found to exhibit normal activity; however, the FrdB Pro^{159}Gln enzyme was found to retain only 15 to 20% of wild-type enzyme activity. The mutant FrdB Pro^{159}Gln enzyme assembled normally into the cytoplasmic membrane of *E. coli*, as judged from levels of covalent flavin adenine dinucleotide found to be associated with the membrane (data not shown), indicating that lack of assembly was not the reason for the lowered activity.

To further define the reason for the reduced activity in succinate oxidation by the FrdB Pro^{159}Gln mutant enzyme complex, the time course of PMS reductase activity was measured in the mutant and compared with that of the wild-type membrane fraction. FRD and SDH as isolated are in a partially deactivated state due to the presence of enzyme containing inhibitory oxaloacetate in a 1:1 stoichiometry. Activity can be rapidly restored to the enzyme by reducing the enzyme complex with dithionite, for example, or more slowly by incubating the enzyme with the substrate succinate at 38°C (1). As shown in Fig. 2, incubation of the FrdB Pro^{159}Gln enzyme complex at 38°C in the absence of 20 mM succinate results in an enzyme complex which retains activity during the 5-min incubation. In contrast, when the FrdB Pro^{159}Gln enzyme complex is incubated with succinate present before the enzyme reaction is initiated with PMS and DCIP, it rapidly loses activity so at the end of the usual 5-min incubation period, the enzyme retains only 15 to 20% of its initial activity. Oxaloacetate is apparently bound to the mutant enzyme complex, as evidenced by the slight increase in activity during the first minute of assay, indicating that activation of the enzyme is occurring. To determine if the enzyme was being inactivated by incubation at 38°C, we incubated both wild-type FRD and the FrdB Pro^{159}Gln mutant for 10 min at that temperature in the absence of substrate prior to initiating the assay. Neither the mutant nor the wild-type enzyme complex was inhibited by

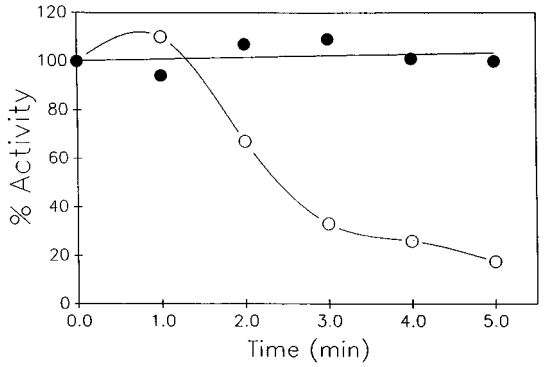

FIG. 2. Effect of incubation time on PMS reductase activity of the FrdB Pro^{159}Gln enzyme. All assays were initiated at the designated time by the addition of 1 mM PMS and 80 μM DCIP with or without the addition of succinate as indicated. FRD-enriched membrane fractions were assayed at 38°C following a preincubation for the designated time in the presence of 20 mM succinate (○), or enzyme was incubated for the designated time and then the reaction was started with 20 mM succinate (●). Each point represents the average of at least three independent measurements (±7%).

TABLE 4. Effects of various agents on enzyme inactivation by succinate of the FrdBPro[159]Gln mutant[a]

Agent added or condition	% Protection against inactivation
Superoxide dismutase, 200 μg/ml	0
Catalase, 50 μg/ml	6
Mannitol, 100 mM	8
DMPO, 10 mM	19
Deferoxamine mesylate, 16 mM	46
HOQNO, 50 nM	88
Anaerobiosis	100
FrdAB Pro[159]Gln[b]	100

[a] Enzyme activity was measured in the succinate PMS reductase assay. The anaerobic PMS reductase assay was done in an anaerobic cuvette prepared similarly to the method used for the BV assay (8).

[b] The soluble form of the enzyme deleted for the hydrophobic C and D anchoring subunits.

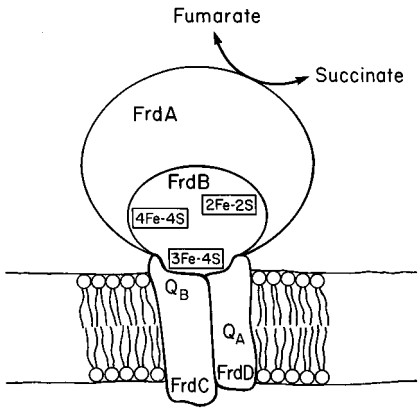

FIG. 3. Topographical model of native FRD. The model shows the close spatial relationship between the [3Fe-4S] cluster in FrdB and the quinone (Q) binding sites in the hydrophobic FrdC and FrdD peptides.

incubation at an elevated temperature (data not shown). The inhibition of the FrdB Pro[159]Gln mutant enzyme complex thus occurred only when the enzyme was incubated aerobically in the presence of its substrate succinate. The identical inhibition pattern was observed when the FrdB Pro[159]Gln mutant enzyme complex was assayed in either the PMS reductase assay or the DBH reductase assay.

Effects of oxygen, electron transport inhibitors, and oxygen radical scavengers on inactivation of the FrdB Pro[159]Gln mutant enzyme complex. Membrane particles enriched for the FrdB Pro[159]Gln complex were assayed for PMS reductase activity by using anaerobic cuvettes to measure enzyme activity. Under these conditions, the enzyme was found to be fully active, exhibiting turnover numbers similar to those observed for wild-type or FrdB Pro[159]His enzyme complex (Table 4).

The observation that inactivation of the FrdB Pro[159]Gln enzyme complex occurs only in the presence of air implicates reactive oxygen species as the cause of this inactivation. To further define the reason for the inactivation of the mutant enzyme complex, oxygen radical scavengers were tested to determine if they had a protective effect. The data in Table 4 show the effects of various agents on the inactivation of the FrdB Pro[159]Gln enzyme complex. Superoxide dismutase, known to consume $O_2^{\cdot -}$, shows no protective effect. Catalase, an H_2O_2 scavenger, had a minor protective effect, as did the $^{\cdot}OH$ scavenger mannitol. A somewhat greater protective effect was seen with DMPO, which has been reported to be a $^{\cdot}OH$ spin trap (35). These data suggest that the primary species responsible for the inactivation of the FrdB Pro[159]Gln enzyme complex in air is $^{\cdot}OH$.

The FrdB Pro[159]Gln substitution might be expected to perturb the environment of the [3Fe-4S] cluster. One mechanism for $^{\cdot}OH$ formation is through metal ion-catalyzed oxidation by the Fenton reaction (27): $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {}^{\cdot}OH$. Therefore, we tested the effect of deferoxamine mesylate, a strong chelator of iron, on inactivation of the mutant enzyme complex. This iron chelator was found to have a significant protective effect against succinate-induced inactivation in the presence of air (Table 4). Thus, it appears that the formation of $^{\cdot}OH$ in the mutant enzyme complex is at least partially iron mediated. The mutant enzyme complex is stable to the presence of air, and the inactivation is observed only after initiation of reduction of the enzyme by the substrate succinate. Succinate will reduce the [3Fe-4S] cluster (16), which in turn will reduce quinones present in the membrane complex used for assay. The inhibitor HOQNO was used to block the reduction of quinones by the enzyme. In kinetic experiments with the wild-type enzyme, it was determined that the K_i of HOQNO in the DBH reductase assay was 34 nM; in contrast, the PMS reductase activity was uninhibited at these concentrations of inhibitor (data not shown).

An approximately 1,000-fold excess of HOQNO was needed before any inhibition of the wild-type enzyme in the PMS reductase assay was observed. Transfer of electrons from the [3Fe-4S] cluster to quinone can thus be specifically inhibited in the membrane-bound FRD complex by nanomolar concentrations of HOQNO. As seen in Table 4, HOQNO gave an 88% protection against succinate-induced air inactivation of the FrdB Pro[159]Gln complex. To determine if only the membrane-bound form of the mutant complex was sensitive to this inactivation, a soluble form of the mutant enzyme was produced by deleting the membrane anchors FrdC and FrdD. This soluble form of FRD is unable to interact with quinones (8). As can be seen in Table 4, the soluble FrdAFrdB Pro[159]Gln is completely protected against succinate-induced air inactivation in the PMS reductase assay. Catalytic assays demonstrate that the soluble FrdAB Pro[159]Gln has a turnover number similar to that of soluble wild-type enzyme (8), in agreement with the finding that a component present in the membrane is required for the succinate induced inactivation.

To determine if the [3Fe-4S] cluster was being modified following succinate-induced air inactivation of the enzyme, the membrane-bound enzyme complex was analyzed by EPR spectroscopy. The EPR properties of the inactivated enzyme showed that the iron-sulfur clusters remained unchanged following this treatment, suggesting that direct modification of the [3Fe-4S] cluster is not responsible for the air-induced inactivation (data not shown) (17).

DISCUSSION

The growth properties and enzymatic activities of the *E. coli* FRD site-directed mutants reported in this work provide further insight into the binding domain of the [3Fe-4S] cluster as well as the interaction of this iron-sulfur cluster with the quinone binding domain(s) of this complex respiratory chain enzyme. A topographical model of FRD (Fig. 3) shows the close interaction between the binding domain of the [3Fe-4S] cluster and one of the two proposed quinone binding sites in the hydrophobic membrane anchor polypeptides. The data reported in this communication suggest that alteration at the [3Fe-4S] cluster domain in FrdB also perturbs the menaqui-

none binding domain in FrdC and FrdD so that the interaction of quinone and iron results in the generation of damaging hydroxyl radicals. These points are discussed below.

Electron transport has long been recognized as a major intracellular source of damaging oxygen radicals and hydrogen peroxide which can result in the metal-catalyzed production of hydroxyl radical (43). The sequence of reactions in electron transport chains in which quinones stimulate the production of hydroxyl radicals is discussed elsewhere (12, 25). Briefly these reactions involve the production of a semiquinone radical ($Q^{\cdot -}$) in the presence of dioxygen, leading to the formation of superoxide ($O_2^{\cdot -}$) anion which spontaneously dismutates to H_2O_2. Metal-catalyzed hydroxyl radical can then be generated by the reductive breakdown of H_2O_2 mediated by Fe^{2+}, which is the Fenton reaction presented in Results. It has also been suggested that in a hydrophobic environment, hydroxyl radicals can be generated from a direct electron transfer from $Q^{\cdot -}$ to H_2O_2 (26). The data reported in this communication are consistent with hydroxyl radical production mediated by both $Q^{\cdot -}$ and Fe^{2+} as the agents responsible for inactivation of FRD in an aerobic environment. Anaerobic growth of *E. coli* is mediated by menaquinone, whereas aerobically grown cells contain ubiquinone in the membrane (37). The cells used for preparation of the FRD complex are grown anaerobically and thus contain primarily menaquinone as the electron donor for fumarate reduction. Thus, in the FRD-enriched membrane preparations used for this study, menaquinone is presumed to be bound at two sites, termed Q_A and Q_B, in the FrdC and FrdD subunits as part of the FRD redox chain (Fig. 3) (40). The FrdB $Pro^{159}Gln$ mutant enzyme complex when assayed as an SDH (Fig. 2) is rapidly inactivated in an aerobic environment when the enzyme is reduced with its substrate succinate. Addition of succinate to the enzyme would cause reduction of the [3Fe-4S] cluster, which in turn would reduce Q_A to $Q_A^{\cdot -}$ (2, 23, 40). The soluble form of the FrdB $Pro^{159}Gln$ enzyme ($FrdABP^{159}Q$), in which no quinones are bound to the enzyme, is completely insensitive to aerobic inactivation by succinate (Table 4). The inhibitor HOQNO, which blocks electron transfer from the iron-sulfur clusters to the quinone site, also significantly protects (88%) the membrane-bound mutant complex from inactivation. These data are consistent with hydroxyl radicals being produced by a quinone-mediated mechanism. The iron chelator deferoxamine mesylate also provided significant protection (46%) against the inactivation as seen in Table 4. This may be because of chelation of iron present in the membrane environment of the mutant enzyme complex, thus preventing the Fenton reaction. It also might reflect modification of the [3Fe-4S] cluster, since FrdB Pro-159 is next to Cys-158, which is one of the ligands of the cluster. This latter possibility is considered less likely, since preliminary EPR data indicate that the [3Fe-4S] cluster is still formed and reduced by succinate in the FrdB $Pro^{159}Gln$ mutant enzyme complex even following treatment of the enzyme complex with succinate in the presence of air (17). Additionally, since the [3Fe-4S] cluster can still be reduced by succinate, it does not appear that the redox potential of this cluster has been dramatically changed, although it is still possible that the electron transfer rate through the cluster may be altered by modification of an amino acid residue. Superoxide dismutase provides no protection against the inactivation of the mutant enzyme complex, whereas minor protection is provided by catalase, hydroxyl radical scavengers such as mannitol, and the hydroxyl radical spin trap DMPO. That these agents do not provide a greater protection against inactivation in the mutant complex is not surprising, since mannitol and catalase may not be capable of accessing the site in the membrane environment where the hydroxyl radical is being generated. The protection afforded, however, is consistent with hydroxyl radical being the primary reactive species responsible for the inactivation.

On the basis of the thermodynamic and spin relaxation properties of SDH, it has been suggested that a stable ubisemiquinone pair exists in the vicinity of the [3Fe-4S] cluster (30). The data reported here are consistent with the [3Fe-4S] cluster of FRD being similarly tightly associated with one of the $Q^{\cdot -}$ binding sites (Fig. 3). The mutation of FrdB Pro-159 to a Gln residue seems to perturb the $Q^{\cdot -}$ binding site so that a semiquinone radical can participate in the generation of hydroxyl radical. We have previously suggested that the FRD complex contains two sites for quinone interaction similar to sites in the photoreaction center (40). The hydrophobic and tightly bound Q_A site would cycle between Q_A and $Q_A^{\cdot -}$ and take an electron directly from the [3Fe-4S] cluster when the enzyme is reduced by succinate. The somewhat more hydrophilic Q_B site can cycle between Q_BH_2, $Q_B^{\cdot -}$, and Q_B. It would appear that the FrdB Pro-159-to-glutamine mutation perturbs either the Q_A or Q_B site, thus allowing generation of hydroxyl radical which subsequently inactivates the enzyme. Changing FrdB Pro-159 to a histidyl residue had no effect on the catalytic activity of the enzyme. It may be that this substitution does not alter the structure of the protein significantly or that a positively charged His residue helps to stabilize $Q^{\cdot -}$, preventing damage by hydroxyl radicals. That the quinone binding sites and the [3Fe-4S] cluster are intimately associated in SDH and FRD is also supported by the observation that a single amino acid change in the [3Fe-4S] binding domain of SDH from *Ustilago maydis* confers resistance to carboxin, which blocks electron transfer to quinones in eukaryotes (7).

FRD normally is expressed in an anaerobic environment and thus is not expected to be exposed to damaging oxygen species. The wild-type enzyme is quite stable and capable of functioning in an aerobic environment, which is not surprising considering the high degree of structural similarity between FRD and SDH (2, 14). A recent report suggests that FRD may generate more superoxide than SDH (18), although there is no evidence that wild-type FRD is inhibited by an aerobic environment if this is the case. The data reported here suggest that single amino acid substitutions in FRD near the [3Fe-4S] cluster result in an enzyme which either is more sensitive to hydroxyl radicals or has a greater propensity for producing them. By analogy with the high structural similarity between FRD and SDH, we would suggest that perturbation of the [3Fe-4S]-quinone binding domain in SDH results in similar effects. It has been suggested that oxidative damage plays a critical role in causing protein modifications and the mitochondrial dysfunction of aging (34, 36). A mutation such as Pro-159 to Gln in SDH might contribute to increased oxidative damage in the mitochondrion, resulting in a decline in mitochondrial function with age. These data point out the importance of the proper protein environment near the $Q^{\cdot -}$ and [3Fe-4S] sites for normal functioning of SDH and FRD.

ACKNOWLEDGMENTS

We thank B. A. C. Ackrell, and W. S. McIntire for helpful discussions.

This study was supported by the Department of Veterans Affairs, National Science Foundation grant MCB 9104297, and National Institutes of Health grant HL-16251.

REFERENCES

1. **Ackrell, B. A. C., B. Cochran, and G. Cecchini.** 1989. Interaction of oxaloacetate with *Escherichia coli* fumarate reductase. Arch. Biochem. Biophys. **268:**26–34.

2. **Ackrell, B. A. C., M. K. Johnson, R. P. Gunsalus, and G. Cecchini.** 1992. Structure and function of succinate dehydrogenase and fumarate reductase, p. 229–297. *In* F. Muller (ed.), Chemistry and biochemistry of flavoenzymes, vol. III. CRC Press, Boca Raton, Fla.
3. **Adman, E. T., L. C. Sieker, and L. H. Jensen.** 1973. The structure of a bacterial ferredoxin. J. Biol. Chem. **258:**3987–3996.
4. **Ævarsson, A., and L. Hederstedt.** 1988. Ligands to the 2Fe iron-sulfur center of succinate dehydrogenase. FEBS Lett. **232:**298–302.
5. **Bethesda Research Laboratories, Inc.** 1986. BRL pUC host: *E. coli* DH5α™ competent cells. Focus **8:**9–10.
6. **Blaut, M., K. Whittaker, A. Valdovinos, B. A. C. Ackrell, R. P. Gunsalus, and G. Cecchini.** 1989. Fumarate reductase mutants of *Escherichia coli* that lack covalently bound flavin. J. Biol. Chem. **264:**13599–13604.
7. **Broomfield, P. L. E., and J. A. Hargreaves.** 1992. A single amino acid change in the iron-sulfur protein subunit of succinate dehydrogenase confers resistance to carboxin in *Ustilago maydis*. Curr. Genet. **22:**117–121.
8. **Cecchini, G., B. A. C. Ackrell, J. O. Deshler, and R. P. Gunsalus.** 1986. Reconstitution of quinone reduction and characterization of *Escherichia coli* fumarate reductase activity. J. Biol. Chem. **261:**1808–1814.
9. **Cecchini, G., C. R. Thompson, B. A. C. Ackrell, D. Westenberg, N. Dean, and R. P. Gunsalus.** 1986. Oxidation of reduced menaquinone by the fumarate reductase complex in *Escherichia coli* requires the hydrophobic FrdD peptide. Proc. Natl. Acad. Sci. USA **83:**8898–8902.
10. **Cole, S. T., T. Grundström, B. Jaurin, J. J. Robinson, and J. H. Weiner.** 1982. Location and nucleotide sequence of the *frdB*, the gene coding for the iron-sulfur protein subunit of fumarate reductase in *Escherichia coli*. Eur. J. Biochem. **126:**211–216.
11. **Darlison, M. G., and J. R. Guest.** 1984. Nucleotide sequence encoding the iron-sulfur protein subunit of the succinate dehydrogenase of *Escherichia coli*. Biochem. J. **223:**507–517.
12. **Dikalov, S. I., G. V. Rumyantseva, A. V. Piskunov, and L. V. Weiner.** 1992. Role of quinone-iron(III) interaction in NADPH-dependent enzymatic generation of hydroxyl radicals. Biochemistry **31:**8947–8953.
13. **Guest, J. R.** 1981. Partial replacement of succinate dehydrogenase function in phage and plasmid-specified fumarate reductase in *Escherichia coli*. J. Gen. Microbiol. **122:**171–179.
14. **Hederstedt, L., and T. Ohnishi.** 1992. Progress in succinate:quinone oxidoreductase research, p. 163–198. *In* L. Ernster (ed.), Molecular mechanisms in bioenergetics. Elsevier Science Publishers, B.V., Amsterdam.
15. **Howard, J. B., T. W. Lorsbach, D. Ghosh, K. Melis, and C. D. Stout.** 1983. Structure of *Azotobacter vinelandii* 7Fe ferredoxin. Amino acid sequence and electron density maps of the residues. J. Biol. Chem. **258:**508–522.
16. **Johnson, M. K., A. T. Kowal, J. E. Morningstar, M. E. Oliver, K. Whittaker, R. P. Gunsalus, B. A. C. Ackrell, and G. Cecchini.** 1988. Subunit location of the iron-sulfur clusters in fumarate reductase from *Escherichia coli.* J. Biol. Chem. **263:**14732–14738.
17. **Johnson, M. K., H. Sices, and G. Cecchini.** Unpublished data.
18. **Kargalioglu, Y., and J. A. Imlay.** 1994. Importance of anaerobic superoxide dismutase synthesis in facilitating outgrowth of *Escherichia coli* upon entry into an aerobic habitat. J. Bacteriol. **176:**7653–7658.
19. **Kröger, A.** 1978. Fumarate as terminal acceptor of phosphorylative electron transport. Biochim. Biophys. Acta **505:**129–145.
20. **Kunkel, T. A., J. D. Roberts, and R. A. Zakour.** 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. Methods Enzymol. **154:**367–382.
21. **Lauterbach, F., C. Körtner, S. P. J. Albracht, G. Unden, and A. Kröger.** 1990. The fumarate reductase operon of *Wolinella succinogenes*. Sequence and expression of the *frdA* and *frdB* genes. Arch. Microbiol. **154:**386–393.
22. **Lemire, B. D., J. J. Robinson, and J. H. Weiner.** 1982. Identification of membrane anchor polypeptides of *Escherichia coli* fumarate reductase. J. Bacteriol. **152:**1126–1131.
23. **Manodori, A., G. Cecchini, I. Schröder, R. P. Gunsalus, M. T. Werth, and M. K. Johnson.** 1992. [3Fe-4S] to [4Fe-4S] cluster conversion in *Escherichia coli* fumarate reductase by site-directed mutagenesis. Biochemistry **31:**2703–2712.
24. **Messing, J.** 1983. New M13 vectors for cloning. Methods Enzymol. **101:**20–78.
25. **Mordente, A., G. E. Martorana, E. Meucci, S. A. Santini, and G. P. Littarru.** 1992. Enzyme inactivation by metal-catalyzed oxidation of coenzyme Q_1. Biochim. Biophys. Acta **1100:**235–241.
26. **Nohl, H.** 1990. Is redox-cycling ubiquinone involved in mitochondrial oxygen activation? Free Radical Res. Commun. **8:**307–315.
27. **Nutter, L. M., E. O. Ngo, G. R. Fisher, and P. L. Gutierrez.** 1992. DNA strand scission and free radical production in menadione-treated cells. J. Biol. Chem. **267:**2474–2479.
28. **Phillips, M. K., L. Hederstedt, S. Hasnain, L. Rutberg, and J. R. Guest.** 1987. Nucleotide sequence encoding the flavoprotein and iron-sulfur protein subunits of the *Bacillus subtilis* PY79 succinate dehydrogenase complex. J. Bacteriol. **169:**864–873.
29. **Salach, J., W. H. Walker, T. P. Singer, A. Ehrenberg, P. Hemmerich, S. Ghisla, and U. Hartmann.** 1972. Studies on succinate dehydrogenase. Site of attachment of the covalently bound flavin to the peptide chain. Eur. J. Biochem. **26:**267–278.
30. **Salerno, J. C., and T. Ohnishi.** 1980. Studies on the stabilized ubisemiquinone species in the succinate-cytochrome *c* reductase segment of the intact mitochondrial membrane system. Biochem. J. **192:**769–781.
31. **Sanger, F., S. Nicklen, and A. R. Coulsen.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
32. **Sato, S., K. Nakazawa, K. Hon-Nami, and T. Oshima.** 1981. Purification, some properties and amino acid sequence of *Thermus thermophilus* HB8 ferredoxin. Biochim. Biophys. Acta **668:**277–289.
33. **Schröder, I., R. P. Gunsalus, B. A. C. Ackrell, B. Cochran, and G. Cecchini.** 1991. Identification of active site residues of *Escherichia coli* fumarate reductase by site-directed mutagenesis. J. Biol. Chem. **266:**13572–13579.
34. **Shigenaga, M. K., T. M. Hagen, and B. N. Ames.** 1994. Oxidative damage and mitochondrial decay in aging. Proc. Natl. Acad. Sci. USA **91:**10771–10778.
35. **Sinha, B. K., A. G. Katki, G. Batist, K. H. Cowan, and C. E. Myers.** 1987. Differential formation of hydroxyl radicals by adriamycin in sensitive and resistant MCF-7 human breast tumor cells: implications for the mechanism of action. Biochemistry **26:**3776–3781.
36. **Stadtman, E. R.** 1993. Oxidation of free amino acids and amino acid residues in proteins by radiolysis and by metal-catalyzed reactions. Annu. Rev. Biochem. **62:**797–821.
37. **Unden, G.** 1988. Differential roles for menaquinone and demethylmenaquinone in anerobic electron transport of *E. coli* and their FNR-independent expression. Arch. Microbiol. **150:**499–503.
38. **Weiner, J. H., and P. Dickie.** 1979. Fumarate reductase of *Escherichia coli*. Elucidation of the covalent-flavin component. J. Biol. Chem. **254:**8590–8593.
39. **Werth, M. T., G. Cecchini, A. Manodori, B. A. C. Ackrell, I. Schröder, R. P. Gunsalus, and M. K. Johnson.** 1990. Site-directed mutagenesis of conserved cysteine residues in *Escherichia coli* fumarate reductase: modification of the spectroscopic and electrochemical properties of the [2Fe-2S] cluster. Proc. Natl. Acad. Sci. USA **87:**8965–8969.
40. **Westenberg, D. J., R. P. Gunsalus, B. A. C. Ackrell, H. Sices, and G. Cecchini.** 1993. *Escherichia coli* fumarate reductase *frdC* and *frdD* mutants. Identification of amino acid residues involved in catalytic activity with quinones. J. Biol. Chem. **268:**815–822.
41. **Wood, D., M. G. Darlinson, R. J. Wilde, and J. R. Guest.** 1984. Nucleotide sequence encoding the flavoprotein and hydrophobic subunits of the succinate dehydrogenase of *Escherichia coli*. Biochem. J. **222:**519–534.
42. **Yao, Y., S. Wakabayashi, S. Matsuda, H. Matsubara, L. Yu, and C.-A. Yu.** 1986. Amino acid sequence of the iron-sulfur protein subunit of beef heart succinate dehydrogenase, p. 240–244. *In* H. Matsubara (ed.), Iron-sulfur protein research. Japan Scientific Societies Press, Tokyo.
43. **Zhang, Y., O. Marcillat, C. Giulivi, L. Ernster, and K. J. A. Davies.** 1990. The oxidative inactivation of mitochondrial electron transport chain components and ATPase. J. Biol. Chem. **265:**16330–16336.