Effect of Cysteine to Serine Mutations on the Properties of the [4Fe-4S] Center in *Escherichia coli* Fumarate Reductase†

Andrzej T. Kowal,[‡,§] Mark T. Werth,[‡,||] Annamaria Manodori,[⊥,#] Gary Cecchini,[⊥] Imke Schröder,[▽] Robert P. Gunsalus,[▽] and Michael K. Johnson*[,‡]

Department of Chemistry and Center for Metalloenzyme Studies, University of Georgia, Athens, Georgia 30602, Molecular Biology Division, Veterans Administration Medical Center, San Francisco, California 94121, Department of Biochemistry and Biophysics, University of California, San Francisco, California 94143, and Department of Microbiology and Molecular Genetics and Molecular Biology Institute, University of California, Los Angeles, California 90024

Received May 15, 1995; Revised Manuscript Received July 10, 1995[⊗]

ABSTRACT: Site-directed mutants of *Escherichia coli* fumarate reductase in which FrdB Cys^{148}, Cys^{151}, Cys^{154}, and Cys^{158} are replaced individually by Ser have been constructed and overexpressed in a strain of *E. coli* lacking a wild-type copy of fumarate reductase and succinate dehydrogenase. The consequences of these mutations on bacterial growth, enzymatic activity, and the EPR properties of the constituent iron−sulfur clusters have been investigated. The Cys^{154}Ser and Cys^{158}Ser FrdB mutations result in enzymes with negligible activity that have largely dissociated from the cytoplasmic membrane and consequently are incapable of supporting cell growth under conditions requiring a functional fumarate reductase. EPR studies indicate that these effects are associated with loss of both the [3Fe-4S] and [4Fe-4S] clusters. In contrast the Cys^{148}Ser and Cys^{151}Ser FrdB mutations result in functional membrane bound enzymes that are able to support growth under anaerobic and aerobic conditions. EPR studies of these mutants indicate that all three of the constituent Fe-S clusters are assembled, and the redox and spectroscopic properties of the [2Fe-2S] and [3Fe-4S] clusters are unchanged compared to the wild-type enzyme. In both mutants the [4Fe-4S] cluster is assembled with one non-cysteinyl ligand, and the available data suggest serinate coordination. The physicochemical consequences are perturbation of the intercluster spin interaction between the $S = {}^1/_2$ $[4Fe\text{-}4S]^+$ and $S = 2$ $[3Fe\text{-}4S]^0$ clusters and a 60-mV decrease in redox potential for the $[4Fe\text{-}4S]^{2+,+}$ cluster in the FrdB Cys^{148}Ser mutant, and a $S = {}^1/_2$ to $S = {}^3/_2$ spin state conversion for the $[4Fe\text{-}4S]^+$ cluster and a 72-mV decrease in redox potential for the $[4Fe\text{-}4S]^{2+,+}$ cluster in the FrdB Cys^{151}Ser mutant. Taken together with the previous FrdB Cys to Ser mutagenesis results [Werth, M. T., Cecchini, G., Manodori, A., Ackrell, B. A. C., Schröder, I., Gunsalus, R. P., & Johnson, M. K. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87,* 8965−8969; Manodori, A., Cecchini, G., Schröder, I., Gunsalus, R. P., Werth, M. T., & Johnson, M. K. (1992) *Biochemistry 31,* 2703−2712], the results provide strong support for the proposal that all three clusters are located in the FrdB subunit with Cys^{57}, Cys^{62}, Cys^{65}, and Cys^{77} ligating the [2Fe-2S] cluster, Cys^{148}, Cys^{151}, Cys^{154}, and Cys^{214} ligating the [4Fe-4S] cluster, and Cys^{158}, Cys^{204}, and Cys^{210} ligating the [3Fe-4S] cluster. The role of the low potential [4Fe-4S] cluster in mediating electron transfer from menaquinol to the FAD active site is discussed in light of these mutagenesis results.

Escherichia coli fumarate reductase (menaquinol−fumarate oxidoreductase) catalyzes the final step in anaerobic respiration with fumarate as the terminal electron acceptor (Kröger, 1978). The membrane-bound enzyme complex consists of four nonidentical subunits; a membrane extrinsic catalytic domain comprising FrdA (66 kDa) and FrdB (27 kDa) and a membrane-intrinsic hydrophobic domain comprising FrdC (15 kDa) and FrdD (13 kDa). The subunits and prosthetic groups in the catalytic domain are remarkably conserved among bacterial fumarate reductases (FRDs)[1] and bacterial and mammalian succinate dehydrogenases (SDHs), and this general class of oxidoreductase has been the subject of several recent reviews (Ackrell et al., 1991; Hederstedt & Ohnishi, 1992; Kröger, 1992; van Hellemond & Tielens, 1994). FrdA (the flavoprotein or Fp subunit) contains the substrate binding site and the covalently bound 8α-[*N*(3)-histidyl]FAD (Weiner & Dickie, 1979). The combination

† This work was supported by the Veterans Administration and grants from the National Science Foundation (DMB9104297 to G.C.) and the National Institutes of Health (HL16251 to G.C. and R.P.G., and GM51962 to M.K.J.).

* Address correspondence to this author at the Department of Chemistry, University of Georgia, Athens, GA 30602.

‡ Department of Chemistry and Center for Metalloenzyme Studies, University of Georgia.

§ Current address: Institute of Inorganic Chemistry, Technical University of Wroclaw, 50-370 Wroclaw, Poland.

|| Current address: Department of Chemistry, Nebraska Wesleyan University, Lincoln, NE.

⊥ Molecular Biology Division, Veterans Administration Medical Center, San Francisco, and Department of Biochemistry and Biophysics, University of California at San Francisco.

Current address: Department of Medicine, San Francisco General Hospital, UCSF, San Francisco, CA 94110.

▽ Department of Microbiology and Molecular Genetics and Molecular Biology Institute, University of California at Los Angeles.

⊗ Abstract published in *Advance ACS Abstracts,* September 1, 1995.

[1] Abbreviations: FRD, fumarate reductase; SDH, succinate dehydrogenase; Fp, flavoprotein; Ip, iron−sulfur protein; PMS, phenazine methosulfate; BV_{red}, reduced benzyl viologen; Q, quinone; QH_2, quinol; MQ, menaquinone; MQH_2, menaquinol; DMN, 2,3-dimethyl-1,4-naphthoquinone; $DMNH_2$, 2,3-dimethyl-1,4-naphthoquinol; PMS, phenazine methosulfate; DBH, 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone; NHE, normal hydrogen electrode.

0006-2960/95/0434-12284$09.00/0

E. coli	FrdIP	147-GCINCGLCYAACP	203-SCTFVGYCSEVCP
Bovine	SdhIP	157-ECILCACCSTSCP	214-RCHTIMNCTETCP
B. subtilis	SdhIP	153-KCMTCGVCLEACP	210-DCGNSQNCVQSCP
E. coli	SdhIP	147-ECILCACCSTSCP	204-RCHSIMNCVSVCP
P. aerogenes	8Fe Fd	7-SCIACGACKPECP	34-SCIDCGSCASVCP

FIGURE 1: Comparison of the arrangement of cysteine residues in the Ip subunits of fumarate reductase from *E. coli* (Cole et al., 1982), *Wolinella succinogenes* (Lauterbach et al., 1990), and succinate dehydrogenase from beef heart (Yao et al., 1986), *Bacillus subtilis* (Phillips et al., 1987), and *E. coli* (Darlison & Guest, 1984), with those of the 8Fe-ferredoxin from *Peptococcus aerogenes* (now known as *Peptostreptococcus asaccharolyticus*) (Adman et al., 1973). Coordinating cysteine residues are shown in bold face.

of spectroscopic and mutagenesis approaches has identified three distinct types of Fe-S center, each stoichiometric with FAD and located in FrdB (the iron−sulfur or Ip subunit): center 1, $[2Fe\text{-}2S]^{2+,+}$ ($E_m = -79$ mV); center 2, $[4Fe\text{-}4S]^{2+,+}$ ($E_m = -320$ mV); and center 3, $[3Fe\text{-}4S]^{+,0}$ ($E_m = -70$ mV) (Morningstar et al., 1985; Johnson et al., 1985a,b; Werth et al., 1990; Manodori et al., 1992). *E. coli* FRD will catalyze succinate oxidation at 30−40% of the rate at which it reduces fumarate (Cecchini et al., 1986), and the *frd* gene products will replace succinate dehydrogenase in *sdh* mutants when produced from multicopy plasmids (Guest, 1981). These properties coupled with the ease of genetic manipulation make *E. coli* fumarate reductase an excellent model system to investigate the catalytic mechanism and electron transport pathway in this general class of oxidoreductases.

The Ip subunits of all fumarate reductases and succinate dehydrogenases sequenced to date show a high degree of homology (Cole et al., 1982; Darlison & Guest, 1984; Yao et al., 1986; Phillips et al., 1987; Lauterbach, et al., 1990). With the exception of *E. coli* SDH, in which one of the cysteines that coordinates the [2Fe-2S] center is replaced by an aspartate, 11 cysteine residues are highly conserved in three ferredoxin-like clusters. The spacing between the first group of four cysteines (Cys^{57}, Cys^{62}, Cys^{65}, and Cys^{77} in *E. coli* FrdB) closely parallels that found in plant-type [2Fe-2S] ferredoxins. EPR and redox studies of mutants in which each of these cysteines has been individually replaced by serine have confirmed this assignment and facilitated understanding of the enzymological consequences of modulating the midpoint potential of the [2Fe-2S] cluster (Werth et al., 1990). The remaining seven cysteines are organized into two groups and spaced analogously to those of an 8Fe ferredoxin, except that the second cysteine in the third group is replaced by a valine, leucine, isoleucine, or serine residue (see Figure 1). By analogy to structurally characterized ferredoxins (Matsubara & Saeki, 1992), it is logical to assign the first three cysteines of the second group and the last cysteine of the third group (Cys^{148}, Cys^{151}, Cys^{154}, and Cys^{214} in *E. coli* FrdB) as ligands of the [4Fe-4S] cluster, and the first two cysteines of the third group and the last cysteine of the second group (Cys^{158}, Cys^{204}, and Cys^{210} in *E. coli* FrdB) as ligands of the [3Fe-4S] cluster. The involvement of the third group of three cysteines in coordinating the [3Fe-4S] or [4Fe-4S] clusters was demonstrated by site-directed mutational studies (Manodori et al., 1992). Cysteine to serine mutations of each of these residues individually resulted in inactive enzyme that had dissociated from the membrane and was deficient in both [3Fe-4S] and [4Fe-4S] clusters. Indirect evidence supporting the above assignment of coordinating cysteines came from the ability to induce a [3Fe-4S] to [4Fe-4S] cluster conversion via a $Val^{207}Cys$ replacement (Manodori et al., 1992). The ability to dramatically decrease the midpoint potential of the $[2Fe\text{-}2S]^{2+,+}$ cluster by mutating a coordinating cysteine to serine (Werth et al., 1990) and the $[3Fe\text{-}4S]^{+,0}$ cluster by converting to a low potential $[4Fe\text{-}4S]^{2+,+}$ cluster (Manodori et al., 1992) has provided direct evidence for the involvement of these two clusters in mediating electron transfer from menaquinol to the FAD active site. However, the role (if any) of the indigenous low-potential $[4Fe\text{-}4S]^{2+,+}$ cluster in mediating electron transfer remains unclear.

In this work, we complete our investigation of cysteine to serine mutations of the conserved cysteines in *E. coli* FrdB, and report on the growth, activity, and physicochemical consequences of mutations involving the second group of cysteines, i.e., $Cys^{148}Ser$, $Cys^{151}Ser$, $Cys^{154}Ser$, and $Cys^{158}Ser$. The results support assignment of Cys^{148}, Cys^{151}, Cys^{154}, and Cys^{214} as ligands of the $[4Fe\text{-}4S]^{2+,+}$ cluster, center 2, and indicate that this cluster can be assembled with one serine ligand. This has enabled investigation of the effects of serine ligation on the spin state of the reduced cluster, intercluster spin−spin interactions, and cluster midpoint potential. The role of the low potential $[4Fe\text{-}4S]^{2+,+}$ cluster in FRDs and SDHs is discussed in light of these new results.

MATERIALS AND METHODS

Strains, Plasmids, and Phage. The *E. coli* strains, plasmids, and phage used in this work are listed in Table 1. Strain DW35 is a sdhCDAB and frdABCD deletion strain, containing a deletion of *frdABCD* operon and a *kan* insertion in the *sdhCDAB* region (*sdhC::kan*) which causes a disruption of the operon (Schröder et al., 1991; Westenberg et al., 1993). Phage M13KW1 was constructed by inserting the 2.0-kb *Eco*R1−*Sal*I fragment from pH3 into the polylinker site of M13mp8 (Werth et al., 1990).

Site-Directed Mutagenesis. Site-directed mutagenesis was performed using the *in vitro* mutagenesis system from Bio-Rad (Richmond, CA), based on the method developed by Kunkel (Kunkel, 1985; Kunkel et al., 1987), using single-stranded M13KW1 DNA as a template. Oligonucleotides for mutagenesis and sequencing were synthesized on a Biosearch model 8700 and were designed to change the FrdB codons for Cys^{148}, Cys^{151}, Cys^{154}, and Cys^{158} to codons for serine residues. The mutants were identified by DNA sequence analysis using the dideoxy termination procedure (Sanger et al., 1977) and a Pharmacia 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 DH5α, and the construct was confirmed by double-stranded DNA sequencing. DNA was prepared using the Qiagen plasmid kit from Diagen (Chatsworth, CA).

Growth of Bacteria. For growth studies and biochemical analysis, the mutant plasmids were transformed into *E. coli* strain DW35 (Δ*frdABCD*, *sdhC::kanDAB*). Wild-type and mutants were grown anaerobically on glycerol/fumarate or glucose/fumarate minimal media or aerobically on minimal succinate medium as previously described (Schröder et al., 1991; Cecchini et al., 1986b). Samples of the membrane or cytoplasmic fractions for biochemical and spectroscopic

Table 1: *E. coli* Strains, Plasmids, and Phage

strain, phage, or plasmids	parent	genotype or phenotype	source
strains			
DW35	MC4100	Δ(*frdABCD*),*sdhC::kan*DAB *rec*A1	*a*
DH5α		F⁻ ϕ80d/*lac*ZΔM15 *end*A1 *rec*A1 *hsd*R17(R_k-m_{k+}) *sup*E44 *thi*-1 *gyr*A *rel*A1Δ(*lac*ZYA-*arg*F)U169 λ⁻	*b*
CJ236		*dut ung thi relA p*CJ105 (Cm^r)	Bio-Rad
MV1190		(Δ*lac-pro*AB) *thi sup*E Δ(*sr*1−*rec*A) 306::Tn10 (tet^r) [F′:*tra*D36 *pro*AB*lac*1^qZΔM15]	Bio-Rad
phage			
M13KW1	M13mp8	*frd*B⁺(ΔACD)	*c*
plasmids			
pH3	pBR322	*frd*A⁺B⁺C⁺D⁺	*d*
pFrdBC^{148}S	pH3	*frd*A⁺B^{C148S}C⁺D⁺	this study
pFrdBC^{151}S	pH3	*frd*A⁺B^{C151S}C⁺D⁺	this study
pFrdBC^{154}S	pH3	*frd*A⁺B^{C154S}C⁺D⁺	this study
pFrdBC^{158}S	pH3	*frd*A⁺B^{C158S}C⁺D⁺	this study

[a] Westenberg et al. (1993). [b] Hannahan (1983). [c] Werth et al. (1990). [d] Blaut et al. (1989).

studies were purified from cells grown anaerobically on a glucose/fumarate medium to stationary phase, harvested by centrifugation, and frozen at −20 °C. For phage and plasmid manipulations, cells were grown on Luria broth and solid media (Blaut et al., 1989).

Purification of Cellular Fractions Enriched in Fumarate Reductase. Sixty grams (wet weight) of *E. coli* DW35 containing the appropriate plasmid were suspended in 300 mL of 50 mM Tris-HCl (pH 7.8), and cytoplasmic and membrane fractions were prepared as described in Cecchini et al. (1986b), except that sonication under an argon atmosphere in a Vacuum Atmospheres glove box (<1 ppm O_2) was used to break the cells in the preparation of cytoplasmic fractions. Subsequent centrifugation and concentration (Amicon ultrafiltration cell) of cytoplasmic fractions was carried out under anaerobic conditions.

Enzyme Assays. Enzyme activity assays were conducted as described in previous work (Cecchini et al., 1986a,b) using reduced benzyl viologen (BV_{red}) and the menaquinol (MQH_2) analog 2,3-dimethyl-1,4-naphthoquinol ($DMNH_2$) as the electron donors for fumarate reduction. The oxidation of succinate was measured with phenazine methosulfate (PMS) and the ubiquinone analog 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone (DBH) as primary electron acceptors. Enzyme concentration are based on the covalently bound histidyl-FAD concentration which was assessed using the published procedures (Salach et al., 1972).

EPR Spectroscopy. Samples for EPR spectroscopy were prepared under an argon atmosphere in a Vacuum Atmospheres glove box (<1 ppm O_2). Cytoplasmic-membrane preparations were resuspended in a minimal volume of 50 mM Tris-HCl buffer, pH 7.8, and cytoplasmic fractions were in the same buffering medium. Fumarate-, succinate-, and dithionite-treated samples of membrane and cytoplasmic fractions were prepared anaerobically by addition of 20 mM fumarate, 20 mM succinate, or 10 mM dithionite followed by incubation for 10 min at room temperature prior to freezing in liquid nitrogen. Cytoplasmic fractions were also oxidized by treatment with 5 mM potassium ferricyanide for 5 min prior to freezing.

EPR redox titrations were performed at ambient temperature (25−27 °C) in the glove box under anaerobic conditions using a 100 mM potassium phosphate buffer, pH 7.2. Mediator dyes were added, each to a concentration of ca. 50 μM, in order to cover the desired range of redox potentials, i.e., methyl viologen, neutral red, safranin, phenosafranin, 2-hydroxy-1,4-anthraquinone, 2-hydroxy-1,4-naphthoquinone, indigodisulfonate, methylene blue, 1,4-naphthoquinone, duroquinone, and 1,2-naphthoquinone-4-sulfonate. Samples were first reduced completely by addition of excess sodium dithionite followed by oxidative titration with potassium ferricyanide. After equilibration at the desired potential, a 0.2-mL aliquot was transferred to a calibrated EPR tube and immediately frozen in liquid nitrogen. Potentials were measured with a platinum working electrode and a saturated calomel reference electrode. All redox potentials are reported relative to the standard hydrogen electrode.

X-band EPR spectra were recorded using a Bruker ESP-300E spectrometer fitted with an Oxford Instruments ESR-9 cryostat (4.2−300 K). Spin quantitations were carried under nonsaturating conditions by double integration with reference to a 1 mM CuEDTA standard.

RESULTS

Growth Properties. Growth studies under conditions requiring a functional fumarate reductase provide a means of addressing if the FrdB mutant enzymes are physiologically competent. The DW35 deletion strain with no complementing plasmids is incapable of anaerobic growth on a glycerol/fumarate medium or aerobic growth on a minimal succinate medium. Complementation with a plasmid encoding for wild-type FrdABCD enzyme allows for growth under these conditions (Table 2). No growth was observed for the FrdB $Cys^{158}Ser$ mutant, whereas the other three mutants investigated in this work, FrdB $Cys^{148}Ser$, $Cys^{151}Ser$, and $Cys^{154}Ser$, still contained functional bidirectional oxidoreductases, albeit with impaired growth properties as judged by doubling times compared to wild-type (see Table 2). While the aerobic growth rates were substantially impaired for all three mutants, anaerobic growth was only marginally affected by the FrdB $Cys^{148}Ser$ and $Cys^{151}Ser$ replacements.

Enzyme Activities and Cellular Location. Analysis of covalently bound FAD was used to determine the cellular location of the wild-type and mutant enzyme. The wild-type enzyme as well the FrdB $Cys^{148}Ser$ and $Cys^{151}Ser$ mutants were located predominantly (75−80%) in the membrane fraction. In contrast, only 5% of the FrdB

Table 2: Growth Properties, Catalytic Activities, and Fe-S Cluster Midpoint Potentials of *E. coli* DW35 with Amplified Expression of Wild-Type or Mutant Fumarate Reductase Operons

	doubling time (h)		turnover number (min^{-1})[c]				midpoint potentials (mV vs NHE)		
encoded subunits	$-O_2$[a]	$+O_2$[b]	BV	DMN	PMS	DBH	$[2Fe\text{-}2S]^{2+,+}$	$[3Fe\text{-}4S]^{+,0}$	$[4Fe\text{-}4S]^{2+,+}$
FrdABCD (wild-type)	2.0	2.1	27000	11700	5100	2800	−79	−70	−300
$FrdAB^{C148S}CD$	3.1	9.5	26500	6700	4500	3200	−70	−86	−360
$FrdAB^{C151S}CD$	3.5	5.6	27400	2700	5400	2000	−67	−85	−372
$FrdAB^{C154S}CD$[d]	5.6	7.3	10400	4800	2800	1200	−75	ND[e]	ND
$FrdAB^{C158S}CD$[d]	NG[f]	NG	<1000	<100	<100	<100	−60	ND	ND

[a] $-O_2$, anaerobic growth on glycerol/fumarate minimal medium. [b] $+O_2$, aerobic growth in succinate minimal medium. [c] Turnover numbers are based on an average of at least two enzyme assays and covalent FAD determinations; BV, BV_{red} fumarate oxidoreductase activity; DMN, $DMNH_2$ fumarate oxidoreductase activity; PMS, succinate PMS oxidoreductase activity; DBH, succinate DBH oxidoreductase activity. [d] Less than 5% of the enzyme in membrane fraction based on covalent FAD content. Activity data are for the membrane fraction and are subject to larger errors (±30%) due to the very low amounts of enzyme present. EPR signals from the Fe-S clusters were not observable above background for membrane samples and the listed midpoint potentials are for the cytoplasmic fraction. [e] ND, not detected. [f] NG no growth.

$Cys^{154}Ser$ mutant enzyme was located in the membrane fraction, and the FrdB $Cys^{158}Ser$ mutant enzyme was found exclusively in the cytoplasmic fraction. Since previous studies have shown that only the membrane-bound enzyme is able to support aerobic and anaerobic cell growth under conditions requiring a functional FRD enzyme (Manodori et al., 1992), the catalytic properties of the cytoplasmic-membrane fractions of the wild-type and mutant FRDs were determined and are presented in Table 2. Turnover numbers based on FAD concentration (i.e., assuming one FAD per enzyme molecule) are shown for FRD activity as measured by oxidation of reduced benzyl viologen or the menaquinol analog $DMNH_2$ and SDH activity as measured by the reduction of PMS or the ubiquinone analog DBH.

The FRD activities of the FrdB $Cys^{148}Ser$ and $Cys^{151}Ser$ mutants using the menaquinol analog as the electron donor were decreased to approximately 50% and 25% of the wild-type values, respectively, whereas the activity with reduced benzyl viologen as the electron donor were like that of the wild-type enzyme. This is in accord with and provides a rationalization for the trend in the anaerobic growth rates, i.e., wild-type > FrdB $Cys^{148}Ser$ > FrdB $Cys^{151}Ser$ (see Table 2). However, the other activities for these mutants are largely unaffected. The absence of a significant decrease in the succinate dehydrogenase activities using PMS or DBH as the electron acceptor is surprising at first sight, in view of the marked affect of this mutation on the rates of aerobic growth. However, it should be remembered that the membrane fractions for enzyme assays are from cells that were grown anaerobically and hence were not exposed to O_2 prior to the assay. Hence the pronounced effect of these mutations on aerobic growth most likely results from instability of one or more of the constituent Fe-S clusters in the mutant enzymes toward prolonged exposure to O_2. The reported turnover numbers for the membrane-bound component of the FrdB $Cys^{154}Ser$ mutant are subject to much larger errors (±30%) due to the low FAD concentration of the membrane fraction. Since this mutant shows significant growth under selective aerobic and anaerobic conditions, it is likely that the mutant enzyme dissociates form the cytoplasmic membrane upon cell disruption. Membrane fractions of the FrdB $Cys^{158}Ser$ mutant were devoid of covalently bound FAD and consequently showed negligible FRD and SDH activities.

The cytoplasmic fractions of both the FrdB $Cys^{154}Ser$ and $Cys^{158}Ser$ mutants contained covalently bound FAD but exhibited <1% of the wild-type FRD and SDH activities in each of the assays used in this work. This suggests loss or modification of one or more of the Fe-S clusters, since the soluble FrdAB enzyme containing all three Fe-S clusters is catalytically competent in fumarate reduction using reduced benzyl viologen as the electron donor and in succinate oxidation using PMS as the electron acceptor (Manodori et al., 1992).

EPR Studies. The consequence of the FrdB mutations on the properties of the Fe-S clusters was assessed by EPR studies of membrane and cytoplasmic fractions of *E. coli* with amplified expression of wild-type and mutant *frd* genes. Parallel studies of the DW35 strain, devoid of wild-type FRD and SDH, were carried out under analogous conditions to facilitate identification of "background" resonances. Further confirmation that the observed resonances arise exclusively from amplified FRD came from quantitation of the EPR signal from reduced center 1, $g = 2.025$, 1.930, and 1.920, in dithionite-reduced samples at 50 K which corresponded to the covalently bound FAD concentration within the combined experimental error of the FAD and spin quantitation (±30%).

No significant EPR signals over and above those of the DW35 background were observed for fumarate-, succinate-, or dithionite-treated membrane fractions of the FrdB $Cys^{154}Ser$ and $Cys^{158}Ser$ mutants. However, the characteristic resonance of the reduced $[2Fe\text{-}2S]^+$ cluster, center 1, was clearly observed in succinate- and dithionite-reduced cytoplasmic fractions of both mutants. Figure 2 shows EPR spectra of the cytoplasmic fraction of the FrdB $Cys^{154}Ser$ mutant after oxidation with ferricyanide and reduction with succinate and dithionite. Almost identical spectra were observed for the cytoplasmic fraction of the FrdB $Cys^{158}Ser$ mutant (data not shown). The characteristic, fast-relaxing resonance of the oxidized $[3Fe\text{-}4S]^+$ cluster, center 3, (i.e., $g = 2.016$, ~1.98, ~1.93, detectable at temperatures <30 K) was not observed in fumarate- or ferricyanide-oxidized samples. Rather, the spectra consists solely of a radical signal centered at $g = 2.003$ that persists at temperatures >100 K. Likewise there was no evidence for the presence of the reduced $[4Fe\text{-}4S]^+$ cluster, center 2. This is manifest in wild-type FRD as a broad fast-relaxing resonance underlying the center 1 resonance in dithionite-reduced samples (see Figure 3) and by the dramatic enhancement of the center 1 spin relaxation that accompanies reduction of center 2 as a result of weak spin−spin interaction (Johnson et al., 1985b; Cammack, et al., 1986). The latter is conveniently assessed by comparing the half-saturation powers ($P_{1/2}$ values) for

Table 3: Spin Quantitations and Power Saturation Behavior of the EPR Signals from *E. coli* DW35 with Amplified Expression of Wild-Type or Mutant Fumarate Reductase Operons

		$S = {}^1/_2$ spin quantitation (spins/FAD)		[2Fe-2S]$^+$ $P_{1/2}$ values (mW)[a]	
encoded subunits	fraction	fumarate-oxidized[b]	dithionite-reduced[c]	succinate-reduced	dithionite-reduced
FrdABCD (wild-type)	membrane	0.8	1.8	0.35	100
FrdAB^{C148S}CD	membrane	0.7	1.9	0.30	80
FrdAB^{C151S}CD	membrane	0.7	1.1	0.40	120
FrdAB^{C154S}CD	cytoplasm	0.0	0.8	0.10	0.20
FrdAB^{C158S}CD	cytoplasm	0.0	0.7	0.05	0.15

[a] EPR half-saturation powers for reduced center 1 at 10 K, based on the intensity of the derivative-shaped feature centered at $g = 1.93$ or the absorption-shaped feature at $g = 2.025$ after subtraction of any underlying features. [b] Spin quantitation at 10 K and 1 mW for the $g = 2.016$, 1.98, 1.93 resonance of oxidized center 3. Values are the average of three determinations. No evidence for a center 3 resonance was apparent in the cytoplasmic fraction of the FrdB Cys^{154}Ser and Cys^{158}Ser mutant after oxidation with 5 mM ferricyanide. [c] Spin quantitation for the entire resonance in the 250−450 mT region under nonsaturating conditions, i.e., 10 K and 0.5 mW for membrane fractions or 15 K and 0.01 mW for cytoplasmic fractions. Values are the average of three determinations.

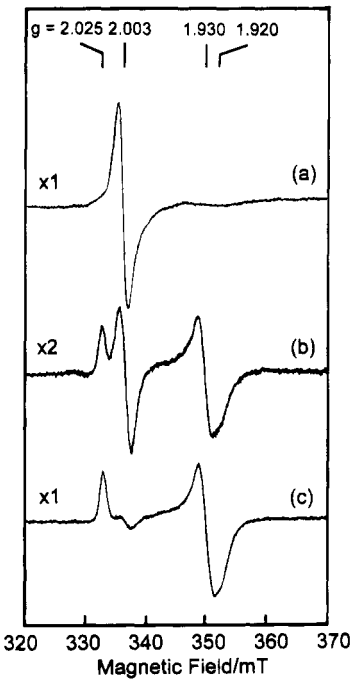

FIGURE 2: EPR spectra of cytoplasmic fraction of *E. coli* DW35 with amplified expression of *frd*AB^{C154S}CD. (a) Oxidized with 5 mM ferricyanide; temperature, 10 K. (b) In the presence of 20 mM succinate; temperature, 50 K. (c) Reduced with 10 mM dithionite; temperature, 50 K. Spectra were recorded at 9.44 GHz with a modulation amplitude of 1 mT and a microwave power of 1 mW. The multiplication factors indicate relative gains.

reduced center 1 in succinate- and dithionite-reduced samples, since center 2 is reduced by dithionite but not by succinate. The $P_{1/2}$ value of reduced center 1 increases by at least two orders of magnitude in wild-type FRD as a result of the reduction of center 2 to the $S = 1/2$ [4Fe-4S]$^+$ state (see Table 3). The dithionite-reduced cytoplasmic fractions of the FrdB Cys^{154}Ser and Cys^{158}Ser mutants showed no evidence of EPR signals underlying that of reduced center 1 and spin quantitations (0.7−0.8 spins/FAD) provide evidence for no more than one $S = {}^1/_2$ Fe-S center. This coupled with the absence of a significant increase in the $P_{1/2}$ value for reduced center 1 on dithionite reduction (see Table 3) attests the absence of a dithionite-reducible center 2.

The results indicate that the FrdB Cys^{154}Ser and Cys^{158}Ser mutations destabilize or prevent assembly of both centers 2 and 3, and this effects dissociation of the membrane-extrinsic catalytic domain (FrdAB) from the membrane anchor subunits (FrdCD). The EPR properties of center 1 are largely unaffected by the loss of these two clusters and dissociation from the membrane. Moreover, the midpoint potentials of center 1 in these mutants, as assessed by dye-mediated EPR redox titrations, are unchanged from WT within the experimental error of the measurement (±20 mV) (see Table 2).

The EPR properties of fumarate-oxidized and succinate-reduced membrane fractions of DW35 with amplified FrdB Cys^{148}Ser and Cys^{151}Ser mutant enzymes were indistinguishable from those reported previously for DW35 with amplified wild-type FRD [see Figure 3 of Manodori et al. (1992)]. Fumarate-oxidized samples showed the characteristic $S = 1/2$ EPR signal ($g = 2.016$, ~1.98, ~1.93) of the oxidized [3Fe-4S]$^+$ cluster, center 3. This resonance accounted for 0.7 spins/FAD in both mutants (see Table 3). Succinate reduction resulted in partial loss of the oxidized center 3 EPR signal and partial reduction of center 1, as evidenced by the appearance of the characteristic $S = {}^1/_2$ EPR signal ($g = 2.025$, 1.930, 1.920) of the reduced [2Fe-2S]$^+$ cluster. Spin quantitation of this resonance at 45 K for succinate- and dithionite-reduced samples indicate that this cluster is approximately 50% reduced by succinate both in the mutants and in the wild-type.

Differences between wild-type and the FrdB Cys^{148}Ser and Cys^{151}Ser mutants were clearly evident in the EPR properties of the dithionite-reduced membrane fractions (see Figure 3). Moreover, the differences relate directly to changes in the properties of the reduced [4Fe-4S]$^+$ cluster, center 2. In the $S = {}^1/_2$ region, the FrdB Cys^{148}Ser mutant showed a complex resonance, quite distinct from that of wild-type, although both integrate to approximately two spins/FAD indicating the presence of two $S = {}^1/_2$ clusters. Temperature-dependence studies facilitated deconvolution of the signal into two overlapping resonances with different relaxation properties (see Figure 4). At 5 K, the resonance is dominated by a fast-relaxing rhombic signal, $g = 2.02$, 1.95, 1.91, which is attributed to center 2, since the slower relaxing resonance from center 1 is saturated under these conditions. In contrast, the faster relaxing resonance of center 2 is too broad to be observed at 45 K, and spin quantitation of the center 1 resonance under these conditions accounted for 1.1 spins/FAD. The line shape of the center 2 resonance was assessed by taking the difference between the spectra at 5 and 13 K at 1 mW microwave power, since the intensity of the saturated center 1 resonance was not significantly perturbed under these conditions. Similar procedures with dithionite-reduced samples of wild-type membrane fractions and the

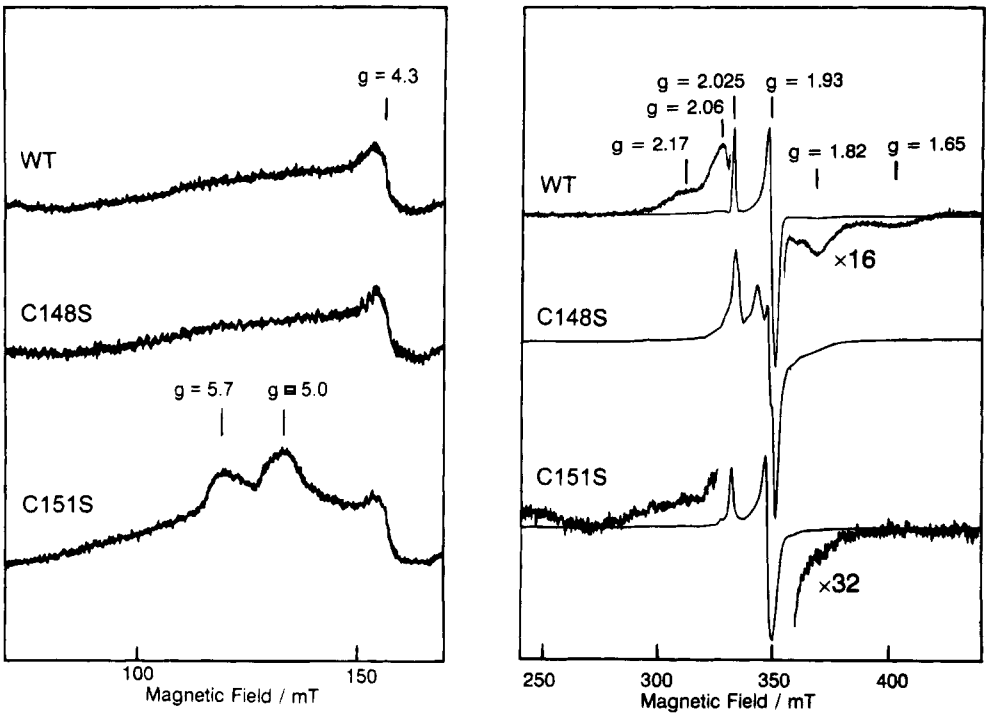

FIGURE 3: EPR spectra of dithionite-reduced membrane fractions of *E. coli* DW35 with amplified expression of *frd*ABCD (WT), *frd*AB^{C148S}CD (C148S), and *frd*AB^{C151S}CD (C151S). (Left panel) Low-field region at a temperature of 4.2 K and a microwave power of 10 mW. (Right panel) $S = {}^1/_2$ region at a temperature of 10 K and a microwave power of 1 mW. Expansions of the low- and high-field region are overlaid for the WT and C151S samples. All spectra were recorded with a microwave frequency of 9.45 GHz and a modulation amplitude of 1 mT.

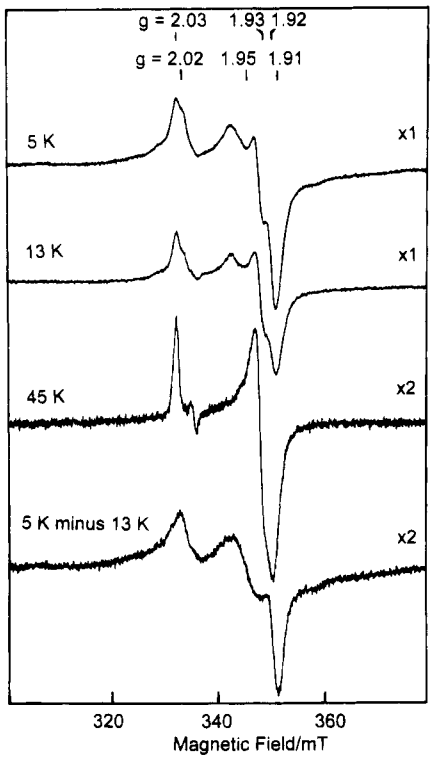

FIGURE 4: Temperature-dependence of the EPR spectrum of dithionite-reduced membrane fractions of *E. coli* DW35 with amplified expression of *frd*AB^{C148S}CD. Spectra were recorded at the indicated temperatures with a microwave power of 1 mW, a microwave frequency of 9.45 GHz, and a modulation amplitude of 1 mT. The multiplication factors indicate the relative gains of the spectra. The bottom spectrum is the 5 K minus 13 K difference spectrum.

purified FrdABCD complex (Johnson et al., 1985b; Cammack et al., 1986) revealed a much broader and more complex resonance for reduced center 2 (see Figure 3). The broadness and complexity of the reduced center 2 resonance in wild-type samples has been attributed to spin−spin interaction between the $S = 2$ [3Fe-4S]^0 cluster and the $S = {}^1/_2$ [4Fe-4S]$^+$ cluster (Johnson et al., 1985b; Cammack et al., 1986; Salerno & Yan, 1987), and this is strongly supported by EPR studies of the FrdB Val^{205}Cys mutant in which center 3 is converted into a [4Fe-4S] cluster (Manodori et al., 1992). Hence the EPR results for the FrdB Cys^{148}Ser mutant are interpreted in terms of assembly of a [4Fe-4S]$^{2+,+}$ cluster with one serinate ligand. The reduced cluster maintained a $S = {}^1/_2$ ground state, and the effects of the change in cluster ligation are apparent by the perturbation of the [3Fe-4S]^0 ↔ [4Fe-4S]$^+$ intercluster spin interaction. Weak spin−spin interaction between the $S = {}^1/_2$ [2Fe-2S]$^+$ and $S = {}^1/_2$ [4Fe-4S]$^+$ clusters is not manifest by a change in the line shape of the individual resonances but is clearly evident in the relaxation enhancement of reduced center 1 that accompanies reduction of center 2. The $P_{1/2}$ value of center 1 is increased more than 200-fold by dithionite reduction of succinate-reduced samples (see Table 3), showing that this intercluster spin−spin interaction is not significantly perturbed by the FrdB Cys^{148}Ser replacement.

Very different EPR behavior for reduced center 2 was observed in the FrdB Cys^{151}Ser mutant. Dithionite-reduced membrane fractions of this mutant showed no additional resonances other than that of reduced center 1 in the $S = {}^1/_2$ region (see Figure 3). Spin quantitations under nonsaturating conditions at several temperatures between 7 and 45 K accounted for 1.1 ± 0.2 spins/molecule. However, evidence for the presence of a dithionite-reducible center 2 was clearly apparent by the 300-fold increase in the center 1 $P_{1/2}$ value that accompanied dithionite reduction (see Table 3). This prompted a thorough search of the EPR spectrum in the low-

field region at low temperatures (4.2 K) and high microwave powers (10 mW) to look for evidence of a species with $S > {}^1/_2$ ground states that could be responsible for the relaxation enhancement of center 1. Figure 3 shows that, in addition to the $g = 4.3$ feature that is attributed to adventitiously bound high-spin Fe^{3+} ion ($S = {}^5/_2$) and is observed in both the wild-type and FrdB $Cys^{148}Ser$ membranes, the FrdB $Cys^{151}Ser$ mutant exhibited resonances at $g = 5.7$ and 5.0 that are indicative of a $S = {}^3/_2$ $[4Fe\text{-}4S]^+$ cluster (Lindahl et al., 1985; Conover et al., 1990). Temperature-dependent studies over the range 4.2−10 K indicate that these resonances arise from different doublets of the ground state manifold, with the $g = 5.7$ feature originating from the lower doublet. The effective g values are readily interpreted in terms of a $S = {}^3/_2$ spin Hamiltonian with $D < 0$ and $E/D = 0.20$, where E and D are the rhombic and axial zero-field splitting parameters, respectively; e.g., using an isotropic real-g-value of 2.0, these parameters predict $g_{x,y,z} = 5.8, 1.0, 1.2$ for the lower doublet and $g_{x,y,z} = 1.8, 5.0, 2.8$ for the upper doublet of the zero-field components of the ground state manifold. Only the low-field, absorption-shaped component of each doublet is clearly observed, but this is not surprising given the breadth of the resonances. We conclude that center 2 is assembled as a $[4Fe\text{-}4S]^{2+,+}$ cluster in the FrdB $Cys^{151}Ser$ mutant, but that the replacement of a cysteinate ligand by a serinate ligand results in a change of spin state of the reduced cluster from $S = {}^1/_2$ to ${}^3/_2$.

The effects of the FrdB $Cys^{148}Ser$ and $Cys^{151}Ser$ mutations on the redox potentials of centers 1, 2, and 3 were investigated by dye-mediated EPR redox titrations (see Figure 5 and Table 2). Within the experimental error of the measurement (±20 mV), the redox potentials of centers 1 and 3 were not significantly perturbed in the mutant enzymes. However, the redox potential of center 2 decreased by 60 mV to −360 mV in the FrdB $Cys^{148}Ser$ mutant and by 72 mV to −372 mV in the FrdB $Cys^{151}Ser$ mutant. The redox titrations also provided confirmation that the relaxation enhancement of center 1 results directly from spin−spin interaction with the $S = {}^3/_2$ $[4Fe\text{-}4S]^+$ cluster in the FrdB $Cys^{151}Ser$ mutant. The midpoint potential for the relief of center 1 power saturation coincides with that obtained for center 2 by monitoring the intensity of the $g = 5.0$ resonance of the $S = {}^3/_2$ species (see Figure 5).

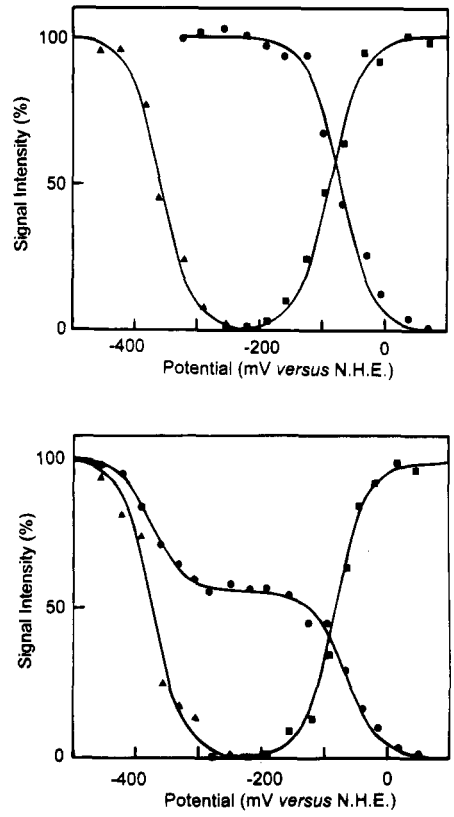

FIGURE 5: EPR-monitored redox titrations for membrane-fractions of *E. coli* DW35 with amplified expression of *frd*$AB^{C148S}CD$ and *frd*$AB^{C151S}CD$. Solid lines are best fits to single or sequential one-electron Nernst plots with midpoint potentials as indicate below for each data set. (Upper panel) $Cys^{148}Ser$ mutant; ■, $g = 2.016$ intensity at 10 K and 1 mW ($E_m = -86$ mV); ●, peak-to-trough intensity of the $g = 1.93$ component at 45 K and 1 mW ($E_m = -70$ mV); ▲, intensity at $g = 1.96$ (minus intensity at −200 mV) at 10 K and 1 mW ($E_m = -360$ mV). (Lower panel) $Cys^{151}Ser$ mutant; ■, $g = 2.016$ intensity at 10 K and 1 mW ($E_m = -85$ mV); ●, peak-to-trough intensity of the $g = 1.93$ component at 10 K and 1 mW ($E_m = -67$ mV and −372 mV); ▲, $g = 5.0$ intensity (minus intensity at −200 mV) at 4.2 K and 10 mW ($E_m = -372$ mV).

DISCUSSION

The work reported herein completes a series of studies on the effects of mutating each of the codons for the conserved cysteines in the Ip subunit of *E. coli* FRD to serine residues. The results taken together provide insight into the role, ligation, and spatial arrangement of the [2Fe-2S], [3Fe-4S], and [4Fe-4S] clusters in FRDs and SDHs. In addition, they have played a major role in defining the physicochemical consequences of non-cysteinyl ligation on each of these ubiquitous classes of Fe-S clusters. The new results presented herein are discussed below in light of the previous mutagenesis results (Werth et al., 1990; Manodori et al., 1992) with particular emphasis on the role and properties of the low-potential [4Fe-4S] cluster in FRDs and SDHs and the consequences of non-cysteinyl ligation on the properties of this class of Fe-S cluster in general.

The 11 cysteines that are highly conserved in the Ip subunits of FRDs and SDHs are found in three groups. Mutagenesis experiments have demonstrated that the first four (Cys^{57}, Cys^{62}, Cys^{65}, and Cys^{77} in *E. coli* FRD) ligate the [2Fe-2S] cluster and that the cluster is assembled, albeit with modified EPR and redox properties, when each is replaced separately by a serine residue (Werth et al., 1990). The remaining seven cysteines are in two groupings, one comprising of four (Cys^{148}, Cys^{151}, Cys^{154}, and Cys^{158} in *E. coli* FRD) and the other comprising three (Cys^{204}, Cys^{210}, and Cys^{214} in *E. coli* FRD). All three clusters are assembled when either of the first two cysteines, Cys^{148} and Cys^{151}, are substituted by serine, but only the [2Fe-2S] cluster is assembled when each of the last five cysteines, Cys^{154}, Cys^{158}, Cys^{204}, Cys^{210}, and Cys^{214} is individually mutated to serine [this work and Manodori et al. (1992)]. Together with the spin coupling data which indicate the closest spatial proximity for the [3Fe-4S] and [4Fe-4S] clusters (Johnson et al., 1985b; Cammack et al., 1986; Salerno & Yan, 1987; Manodori et al., 1992), this supports the existence of separate [2Fe-2S] and [3Fe-4S]/[4Fe-4S] domains within the Ip subunit. Assembly of the cluster in the [2Fe-2S] domain can occur without assembly of either cluster in the [3Fe-4S]/[4Fe-4S] domain. However, the available mutagenesis data indicate that cluster assembly within the [3Fe-4S]/[4Fe-

4S] domain is an all or nothing situation. Moreover, assembly of the clusters in the [3Fe-4S]/[4Fe-4S] domain appears to be a requirement for attachment of the membrane extrinsic FrdAB subunits to the membrane intrinsic FrdCD subunits.

The observation that the $Cys^{148}Ser$ and $Cys^{151}Ser$ replacements alter the EPR and redox properties of the $[4Fe\text{-}4S]^{2+,+}$ cluster without perturbing the EPR and redox properties of the $[3Fe\text{-}4S]^{+}$ cluster implicates these residues as ligands to center 2. The remaining five cysteines in this domain are implicated in coordinating either the [3Fe-4S] or [4Fe-4S] cluster since both clusters are lost as a result of Cys → Ser replacements. Further insight into the residues coordinating specific clusters comes from the sequence homology with structurally characterized 8Fe Fds (see Figure 1), and the observation that conversion of the [3Fe-4S] cluster to a [4Fe-4S] cluster is accomplished via the $Val^{207}Cys$ replacement (Manodori et al., 1992). In 8Fe Fds, the clusters are coordinated by two $C\text{-}X_2\text{-}C\text{-}X_2\text{-}C\text{-}X_3\text{-}CP$ groups of cysteines with the first three of one group and the last one in the other group providing the ligands to individual clusters (Matsubara & Saeki, 1992). On the basis of the sequence and structural data, it is the second cysteine in this sequence that is not ligated or replaced by a non-cysteinyl residue in Fds that contain or are most easily converted to a [3Fe-4S] cluster (Holm, 1992; Matsubara & Saeki, 1992). Hence, the mutagenesis and sequence homology data provide compelling evidence for Cys^{148}, Cys^{151}, Cys^{154}, and Cys^{214} ligating the [4Fe-4S] cluster and Cys^{158}, Cys^{204}, and Cys^{210} ligating the [3Fe-4S] cluster.

The consequences of replacing coordinating cysteines to serines on the properties of biological [2Fe-2S] clusters have been systematically explored both in well characterized Fds (Cheng et al., 1994; Fujinaga et al., 1993; Meyer et al., 1994) and in one complex multicluster enzyme, i.e., *E. coli* FRD (Werth et al., 1990). In most cases, the cluster can be assembled with one serine replacing one of the cysteines. In one case, the $Cys^{49}Ser$ mutant of *Anabaena* Fd, direct evidence for serinate coordination has been provided by an X-ray crystal structure (Holden et al., 1994). Serinate coordination at the Fe^{2+} site of a localized valence $[2Fe\text{-}2S]^{+}$ cluster has been found to have a pronounced effect on the g value anisotropy of the $S = {}^1/_2$ ground state (Werth at al., 1990; Fujinaga et al., 1993; Cheng et al., 1994). Furthermore, there are now two examples, the $Cys^{56}Ser$ and $Cys^{60}Ser$ mutants of *Clostridium pasteurianum* 2Fe Fd, in which serinate coordination effects spin state conversion to yield valence-delocalized $[2Fe\text{-}2S]^{+}$ clusters with $S = {}^9/_2$ ground states (Crouse et al., 1995).

In contrast, no systematic studies have been performed on the effects of replacing coordinating cysteines by serines on the properties of [4Fe-4S] clusters in simple Fds. However, three possibilities have emerged from published studies in more complex systems. There are now several examples in which a single Cys to Ser mutation is reported to prevent cluster assembly, e.g., *Azotobacter vinelandii* nitrogenase Fe-protein (Howard et al., 1989) and *E. coli* nitrate reductase (Augier et al., 1993a). In these enzymes mutational analysis of the cysteine residues has been used as a means of identifying ligating cysteine residues. For [4Fe-4S] clusters coordinated by a Fd arrangement of cysteines, i.e., $C\text{-}X_2\text{-}C\text{-}X_2\text{-}C\text{-}X_n\text{-}CP$, Cys to Ser or Asp mutations involving the second cysteine are often accompanied by cluster degradation to yield [3Fe-4S] clusters, e.g., *E. coli* DMSO reductase (Rothery & Weiner, 1991) and clusters F_A and F_B in PsaC of photosystem I (Zhao et al., 1992) . To our knowledge, there are only two reports of [4Fe-4S] clusters that are assembled intact after replacement of a coordinating cysteine by a serine. These are cluster F_X in PsaB of photosystem I (Warren et al., 1993) and cluster II in *E. coli* nitrate reductase (Augier et al., 1993b). The cluster F_X example is marked by dramatic changes in the g value anisotropy, whereas the nitrate reductase example is based largely on spin quantitation data since intercluster spin coupling prevents the observation of a well-resolved EPR signal.

The results presented here demonstrate that the [4Fe-4S] in *E. coli* FRD is assembled when either of the first two cysteines in the $C\text{-}X_2\text{-}C\text{-}X_2\text{-}C\text{-}X_3\text{-}CP$ sequence are changed to serines. However, the mutations have very different consequences in terms of the physicochemical properties of the resultant $[4Fe\text{-}4S]^{2,++}$ clusters. In the FrdB $Cys^{148}Ser$ mutant, the cluster maintains a $S = {}^1/_2$ ground state but exhibits a simple rhombic line shape ($g = 2.02, 1.95, 1.91$) rather than the complex line shape that has been attributed to intercluster spin−spin interaction involving the $S = 2$ $[3Fe\text{-}4S]^{0}$ cluster in the wild-type. Hence the mutation perturbs and most likely weakens this intercluster spin−spin interaction, so that the effects are no longer manifest in a pronounced broadening and change in the line shape of the $S = {}^1/_2$ resonance. In contrast the FrdB $Cys^{151}Ser$ mutant enzyme provides the first example of a mutagenesis-induced $S = {}^1/_2$ to ${}^3/_2$ spin state conversion for a $[4Fe\text{-}4S]^{+}$ cluster. While the structural and environmental factors that determine the ground state spin of biological and synthetic $[4Fe\text{-}4S]^{+}$ clusters in general are not well defined (Carney et al., 1988, 1989; Lindahl et al., 1987; Onate et al., 1993), studies of the native forms of *Desulfovibrio africanus* FdIII and *Pyrococcus furiosus* Fd have shown that replacement of a the second cysteine in the highly conserved $C\text{-}X_2\text{-}C\text{-}X_2\text{-}C\text{-}X_n\text{-}CP$ arrangement by an aspartic acid residue leads to $[4Fe\text{-}4S]^{+}$ clusters with predominantly $S = {}^3/_2$ ground states (George et al., 1989; Conover et al., 1990). We conclude that oxygenic coordination, in the form of serinate or aspartate, at the Fe site that would be customarily be coordinated by the second Cys residue in the conventional Fd arrangement can be a determining factor for producing $S = {}^3/_2$ $[4Fe\text{-}4S]^{+}$ clusters. In view of the difficulty of observing EPR signals from $S = {}^3/_2$ $[4Fe\text{-}4S]^{+}$ clusters, the absence of the characteristic $S = {}^1/_2$ EPR resonance clearly cannot be used alone as a reliable criterion for loss of a cluster as a result of a cysteine mutation.

As yet there is no direct evidence for serinate coordination of the [4Fe-4S] cluster in the *E. coli* FrdB $Cys^{148}Ser$ and $Cys^{151}Ser$ mutant enzymes, and it is not possible to rule out H_2O or OH^- coordination. Below we summarize the considerations that favor serinate coordination. Since unperturbed [3Fe-4S] and [2Fe-2S] clusters are assembled in these mutants, no other cysteine residues are available in the FrdB subunit for cluster-driven protein reorganization of the type characterized in a cysteine mutant of *A. vinelandii* FdI (Iismaa et al., 1991). Hence the [4Fe-4S] clusters in these mutants must have at least one non-cysteinyl ligand. Recent ^1H NMR (Calzolai et al., 1995) and ^1H ENDOR (Telser et al., 1995) studies of *P. furiosus* Fd argue strongly in favor of cluster coordination by the aspartate residue that

occupies the equivalent position to Cys^{151} in FrdB. Consequently there is precedence for oxygenic coordination of a [4Fe-4S] cluster by an amino acid side chain. Precedence for serinate coordination of biological Fe-S clusters comes from the X-ray crystal structures of the nitrogenase P-clusters (Kim & Rees, 1992, 1994) and the $Cys^{49}Ser$ mutant of *Anabaena* [2Fe-2S] Fd (Holden et al., 1994). Finally the 60−72-mV decrease in the redox potential for the $[4Fe-4S]^{2+,+}$ clusters in the FrdB $Cys^{148}Ser$ and $Cys^{151}Ser$ mutant enzymes is consistent with serinate coordination, since this would tend to stabilize the oxidized form relative to cysteinate coordination. The effects of the Cys to Ser mutations on the redox potentials of the $[4Fe-4S]^{2+,+}$ clusters that are assembled in PsaB of photosystem I and *E. coli* nitrate reductase have not been determined. However, this anticipated trend in redox potentials is borne out by the available data for $[2Fe-2S]^{2+,+}$ clusters (Werth et al., 1990; Cheng et al., 1994).

Finally we turn our attention to the implications of these results for the role of the low-potential $[4Fe-4S]^{2+,+}$ cluster, center 2, in mediating electron transfer through the Ip subunits of FRDs and SDHs. This work and our previous mutagenesis studies (Werth et al., 1990; Manodori et al., 1992) demonstrate that the redox potential of each individual cluster can be perturbed by specific mutations without significantly affecting the potential of other two clusters. This argues that the low potential of the $[4Fe-4S]^{2+,+}$ cluster as measured by equilibrium redox titrations is an intrinsic potential rather than an apparent potential that results from anticooperative redox interactions between the clusters. Without cooperative redox interaction it is difficult to envisage how a cluster with an intrinsic midpoint potential approximately 250 mV lower than either the succinate/fumarate or Q/QH_2 couple is able to mediate electron transport. Such considerations suggest that the [4Fe-4S] cluster plays a purely structural role, since it is clearly required for the assembly of the [3Fe-4S] cluster, and thereby the structural integrity of the Ip subunit and its ability to bind to the membrane anchor peptides.

A logical prediction of this proposal would be that further lowering the potential of center 2 while preserving the structural integrity of the enzyme complex and the redox potentials of centers 1 and 3 should have little affect on the activity or growth rates under conditions requiring a functional fumarate reductase. However, the FrdB $Cys^{148}Ser$ and $Cys^{151}Ser$ mutagenesis results show that lowering the potential of center 2 by 60 or 72 mV, respectively, results in decreased growth rates and FRD activities using the menaquinol analog as the electron donor that are 50% and 25% of the wild-type, respectively. These mutagenesis results suggest that center 2 is at least located in the electron transport pathway between centers 1 and 3. However, whether or not the cluster itself undergoes redox cycling during electron transfer remains an unanswered question.

At this junction it is worth noting that an interesting parallel appears to exist between arrangement of and redox properties of the three Fe-S clusters in FRD with those of the Fe-S subunit of *Desulfovibrio gigas* NiFe-hydrogenase. The recent X-ray crystal structure of this enzyme (Volbeda et al., 1995) shows that the high-potential $[3Fe-4S]^{+,0}$ cluster is located between the two low-potential $[4Fe-4S]^{2+,+}$ clusters that are clearly involved in the electron transport pathway between the Ni active site and the electron donor/acceptor. However, it seems unlikely that the $[3Fe-4S]^{+,0}$ cluster, which has a redox potential at least 200 mV higher than that of the H^+/H_2, $[4Fe-4S]^{2+/+}$, or donor/acceptor couples can be actively involved with mediating electron transport. This raises the possibility that "insulating" clusters, such as the [3Fe-4S] cluster in *D. gigas* NiFe-hydrogenase and the [4Fe-4S] cluster in FRDs and SDHs, placed at strategic positions in a multicluster electron transfer pathway, may play important roles in controlling the rates or directionality of electron transfer in these metalloenzymes.

REFERENCES

Ackrell, B. A. C., Johnson, M. K., Gunsalus, R. P., & Cecchini, G. (1991) in *Chemistry and Biochemistry of Flavoenzymes* (Muller, F., Ed.) Vol. III, pp 229−297, CRC Press, Boca Raton, FL.

Adman, E. T., Sieker, L. C., & Jensen, L. H. (1973) *J. Biol. Chem. 248*, 3987−3996.

Augier, V., Asso, M., Guigliarelli, B., More, C., Bertrand, P., Santini, C.-L., Blasco, F., Chippaux, M., & Giordano, G. (1993a) *Biochemistry 32*, 5099−5108.

Augier, V., Guigliarelli, B., Asso, M., Bertrand, P., Frixon, C., Giordano, G., Chippaux, M., & Blasco, F. (1993b) *Biochemistry 32*, 2013−2023.

Blaut, M., Whittaker, K., Valdovinos, A., Ackrell, B. A. C., Gunsalus, R. P., & Cecchini, G. (1989) *J. Biol. Chem. 264*, 13599−13604.

Calzolai, L., Gorst, C. M., Zhao, Z.-H., Teng, Q., Adams, M. W. W., & La Mar, G. N. (1995) *Biochemistry* (in press).

Cammack, R., Patil, D. S., & Weiner, J. H. (1986) *Biochim. Biophys. Acta 870*, 545−551.

Carney, M. J., Papaefthymiou, G. C., Spartalian, K., Frankel, R. B., & Holm, R. H. (1988) *J. Am. Chem. Soc. 110*, 6084−6095.

Carney, M. J., Papaefthymiou, G. C., Frankel, R. B., & Holm, R. H. (1989) *Inorg. Chem. 28*, 1497−1503.

Cecchini, G., Ackrell, B. A. C., Deshler, J. O., & Gunsalus, R. P. (1986a) *J. Biol. Chem. 261*, 1808−1814.

Cecchini, G., Thompson, C. R., Ackrell, B. A. C., Westenberg, D. J., Dean, N., & Gunsalus, R. P. (1986b) *Proc. Natl. Acad. Sci. U.S.A. 83*, 8898−8902.

Cheng, H., Xia, B., Reed, G. H., & Markley, J. L. (1994) *Biochemistry 33*, 3155−3164.

Cole, S. T., Grundström, T., Jaurin, B., Robinson, J. J., & Weiner, J. H. (1982) *Eur. J. Biochem. 126*, 211−216.

Conover, R. C., Kowal, A. T., Fu, W., Park, J.-B., Aono, S., Adams, M. W. W., & Johnson, M. K. (1990) *J. Biol. Chem. 265*, 8533−8541.

Crouse, B. R., Meyer, J., & Johnson, M. K. (1995) *J. Am. Chem. Soc.* (in press).

Darlison, M. G., & Guest, J. R. (1984) *Biochem. J. 223*, 507−517.

Fujinaga, J., Gaillard, J., & Meyer, J. (1993) *Biochem. Biophys. Res. Commun. 194*, 104−111.

George, S. J., Armstrong, F. A., Hatchikian, E. C., & Thomson, A. J. (1989) *Biochem. J. 264*, 275−284.

Guest, J. R. (1981) *J. Gen. Microbiol. 122*, 171−179.

Hannahan, D. (1983) *J. Mol. Biol. 166*, 557−580.

Hederstedt, L., & Ohnishi, T. (1992) in *Molecular Mechanisms in Bioenergetics* (Ernster, L., Ed.) pp 163−198, Elsevier Science Publishers, New York.

Holden, H. M., Jacobson, B. L., Hurley, J. K., Tollin, G., Oh, B.-H., Skjeldal, L., Chae, Y. K., Cheng, H., Xia, B., & Markley, J. L. (1994) *J. Bioenerg. Biomembr. 26*, 67−88.

Holm, R. H. (1992) *Adv. Inorg. Chem. 38*, 1−71.

Howard, J. B., Davis, R., Moldenhauer, B., Cash, V. L., & Dean, D. (1989) *J. Biol. Chem. 264*, 11270−11274.

Iismaa, S. E., Vázquez, A. E., Jensen, G. M., Stephens, P. J., Butt, J. N., Armstrong, F. A., & Burgess, B. K. (1991) *J. Biol. Chem. 266*, 21563−21571.

Johnson, M. K., Morningstar, J. E., Cecchini, G., & Ackrell, B. A. C. (1985a) *Biochem. Biophys. Res. Commun. 131*, 653−658.

Johnson, M. K., Morningstar, J. E., Cecchini, G., & Ackrell, B. A. C. (1985b) *Biochem. Biophys. Res. Commun. 131*, 756−762.

Kim, J., & Rees, D. C. (1992) *Science 257*, 1677−1682.

Kim, J., & Rees, D. C. (1994) *Biochemistry 33*, 389−397.
Kröger, A. (1978) *Biochim. Biophys. Acta 505*, 129−145.
Kröger, A. (1992) *Arch. Microbiol. 158*, 311−314.
Kunkel, T. A., Roberts, J. D., & Zakour, R. A. (1987) *Methods Enzymol. 154*, 367−382.
Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 488−492.
Lauterbach, F., Körtner, C., Albracht, S. P. J., Unden, G., & Kröger, A. (1990) *Arch. Microbiol. 154*, 386−393.
Lindahl, P. A., Day, E. P., Kent, T. A., Orme-Johnson, W. H., & Münck, E. (1985) *J. Biol. Chem. 260*, 11160−11173.
Lindahl, P. A., Teo, B.-K., & Orme-Johnson, W. H. (1987) *Inorg. Chem. 26*, 3912−3916.
Manodori, A., Cecchini, G., Schröder, I., Gunsalus, R. P., Werth, M. T., & Johnson, M. K. (1992) *Biochemistry 31*, 2703−2712.
Matsubara, H., & Saeki, K. (1992) *Adv. Inorg. Chem. 38*, 223−280.
Meyer, J., Fujinaga, J., Gaillard, J., & Lutz, M. (1994) *Biochemistry 33*, 13642−13650.
Morningstar, J. E., Johnson, M. K., Cecchini, G., Ackrell, B. A. C., & Kearney, E. B. (1985) *J. Biol. Chem. 260*, 13631−13638.
Onate, Y. A., Finnegan, M. G., Hales, B. J., & Johnson, M. K. (1993) *Biochim. Biophys. Acta 1164*, 113−123.
Phillips, M. K., Hederstedt, L., Hasnain, S., Rutberg, L., & Guest, J. R. (1987) *J. Bacteriol. 169*, 864−873.
Rothery, R. A., & Weiner, J. H. (1991) *Biochemistry 30*, 8296−8305.
Salach, J., Walker, W. H., Singer, T. P., Ehrenberg, A., Hemmerich, P., Ghisla, S., & Hartmann, U. (1972) *Eur. J. Biochem. 26*, 267−278.
Salerno, J. C., & Yan, X. (1987) in *Cytochrome Systems: Molecular Biology and Bioenergetics* (Papa, S., Chance, B., & Ernster, L., Eds.) pp 467−471, Plenum Press, New York.
Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463−5467.
Schröder, I., Gunsalus, R. P., Ackrell, B. A. C., Cochran, B., & Cecchini, G. (1991) *J. Biol. Chem. 266*, 13572−13579.
Telser, J., Smith, E. T., Adams, M. W. W., Conover, R. C., Johnson, M. K., & Hoffman, B. M. (1995) *J. Am. Chem. Soc. 117*, 5133−5140.
van Hellemond, J. J., & Tielens, A. G. M. (1994) *Biochem. J. 304*, 321−331.
Volbeda, A., Charon, M.-H., Piras, C., Hatchikian, E. C., Frey, M., & Fontecilla-Camps, J. C. (1995) *Nature 373*, 580−587.
Warren, P. V., Smart, L. B., McIntosh, L., & Golbeck, J. H. (1993) *Biochemistry 32*, 4411−4419.
Weiner, J. H., & Dickie, P. (1979) *J. Biol. Chem. 154*, 8590−8593.
Werth, M. T., Cecchini, G., Manodori, A. M., Ackrell, B. A. C., Schröder, I., Gunsalus, R. P., & Johnson, M. K. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 8965−8969.
Westenberg, D. J., Gunsalus, R. P., Ackrell, B. A. C., Sices, H., & Cecchini, G. (1993) *J. Biol. Chem. 268*, 815−822.
Yao, Y., Wakabayashi, S., Matsubara, H., Yu, L., & Yu, C.-A. (1986) in *Iron−Sulfur Protein Research* (Matsubara, H., Katsube, Y., & Wada, K., Eds.) pp 240−244, Japan Science Society Press, Tokyo.
Zhao, J. D., Li, N., Warren, P. V., Golbeck, J. H., & Bryant, D. A. (1992) *Biochemistry 31*, 5093−5099.

BI951098A