Subunit Location of the Iron-Sulfur Clusters in Fumarate Reductase from Escherichia coli

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The subunit location of the [2Fe-2S], [3Fe-4S], and [4Fe-4S] clusters in Escherichia coli fumarate reductase has been investigated by EPR studies of whole cells or whole cell extracts of a fumarate reductase deletion mutant with plasmid amplified expression of discrete fumarate reductase subunits or groups of subunits. The results indicate that both the [2Fe-2S] and [3Fe-4S] clusters are located entirely in the iron-sulfur protein subunit. Information concerning the specific cysteine residues that ligate these clusters has been obtained by investigating the EPR characteristics of cells of the deletion mutant amplified with a plasmid coding for the flavoprotein subunit and a truncated iron-sulfur protein subunit. While the results are not definitive with respect to the location of the [4Fe-4S] cluster, they are most readily interpreted in terms of this cluster being entirely in the flavoprotein subunit or bridging between the two catalytic domain subunits. These new results are discussed in light of the amino acid sequences of the two subunits and the sequences of structurally well characterized iron-sulfur proteins containing [2Fe-2S], [3Fe-4S], and [4Fe-4S] centers.

Fumarate reductase from Escherichia coli is a membrane-bound iron-sulfur flavoenzyme complex composed of four nonidentical subunits that catalyzes the final step in anaerobic respiration with fumarate as the terminal electron acceptor (1, 2). The complex is composed of a membrane extrinsic catalytic domain consisting of a flavoprotein (Fp) subunit, FrdA (66 kDa), and an iron-sulfur protein (Ip) subunit, FrdB (27 kDa), as well as a membrane intrinsic domain consisting of two hydrophobic membrane-anchorizing subunits, FrdC (15 kDa) and FrdD (13 kDa). Recent spectroscopic studies have revealed the presence of three distinct types of Fe-S clusters: cluster 1, [2Fe-2S]^{2+} (E_m = -20 mV); cluster 2, [4Fe-4S]^{2+} (E_m = -320 mV); cluster 3, [3Fe-4S]^{1+} (E_m = -70 mV) (8-11). All three clusters are present in amounts stoichiometric with FAD, and their presence in the solubilized 2-subunit fumarate reductase preparations localizes them to the catalytic domain subunits (8, 10). In accord with their analogous enzymatic functions, fumarate reductase complex from E. coli is very similar in terms of Fe-S number, type, and spectroscopic properties of the prosthetic groups and subunit composition to Complex II of the mammalian respiratory chain (12, 13).

In both mammalian Complex II and the fumarate reductase complex from E. coli, the covalently bound FAD is known to reside in the Fp subunit (FrdA) (14, 15). While there is general agreement that the constituent Fe-S clusters are exclusively in the catalytic domain subunits, there has been no conclusive evidence forthcoming concerning their distribution and location within these two subunits. Indeed, this question has been the source of much controversy with different research groups agreeing that the Fe-S clusters are distributed over both subunits but holding diametrically opposed views concerning the subunit location of clusters 1 and 3. The opposing viewpoints have been summarized in two comprehensive review articles (16, 17), but will not be repeated here, since they are open to reinterpretation in light of our current knowledge of the number and type of Fe-S clusters (12, 13). Perhaps the most convincing evidence that is currently available concerning the subunit location of the constituent Fe-S clusters comes from inspection of the amino acid sequences of the catalytic domain subunits. Complete amino acid sequences have recently been published for the Ip subunit of mammalian Complex II (18), and the Ip and Fp subunits of both the succinate dehydrogenase and fumarate reductase from E. coli (19-22). The observation that conserved sequences of cysteines in "ferredoxin-like" clusters are only observed in the Ip subunit (FrdB) has led to speculation that all three clusters are primarily located in this subunit. However, since assignment of Fe-S cluster location based solely on amino acid sequence

1 In previous literature this cluster has been referred to as the 3Fe center or the [3Fe-xS] center because of the uncertainty concerning the structure and number of acid labile sulfur atoms. While analytical and spectroscopic measurements indicated a common [3Fe-4S] core structure for all known clusters of this type (3, 4), the only available x-ray crystallographic data, for A. vinelandii ferredoxin (5), was interpreted in terms of a [3Fe-3S] core. This discrepancy has recently been resolved by redetermination of the x-ray structure of A. vinelandii ferredoxin by Jensen and co-workers (6) and subsequently by Stout (7), which revealed errors in the original data analysis. The new data analysis is consistent with a [3Fe-4S] core having a structure based on a cubane cluster minus one Fe.

* This work was supported by National Institutes of Health Grant GM-33806 and a University of Georgia Biotechnology Award (to M. K. J.), National Institutes of Health Grant AI-21678 (to G. P. G.) National Institutes of Health Grant HL-16251, and the Veterans Administration (to G. C., and B. A. C. A.), and by National Science Foundation Grant DMB-8715560 (to G. C., and B. A. C. A.), and by National Science Foundation Grant DMB-8715560 (to G. C., and B. A. C. A.).

1 The abbreviations used are: Fp, flavoprotein subunit; Ip, iron-sulfur protein subunit.
data can be misleading, we have initiated a program of spectroscopic studies of genetically modified *E. coli* fumarate reductase to address this question.

In this work we report EPR studies of whole cells as well as membrane and cytoplasmic fractions of a fumarate reductase deletion mutant of *E. coli* with plasmid-amplified expression of discrete fumarate reductase subunits or groups of subunits. The results support the view that both clusters 1 and 3 are located entirely in the FrdB subunit and provide an indication of the specific cysteinyl residues that are involved in coordinating these clusters. While the results are less conclusive with respect to the location of cluster 2, they are most readily interpreted in terms of this cluster being located in FrdA or bridging between the two catalytic domain subunits. These new results are discussed in light of the location and cysteinyl coordination of the clusters that has been inferred by comparison with the sequences of cysteinyl residues of structurally well-characterized ferredoxins containing [2Fe-2S], [3Fe-4S], and [4Fe-4S] clusters.

**MATERIALS AND METHODS**

**Strains and Plasmids—** *E. coli* mutant strain ECL509 was obtained from E. C. C. Lin (Harvard University) and is a derivative of strain MC4100. This strain was shown to have a deletion in *frd* by the failure to give revertants (23). Moreover, we have screened this strain by Southern hybridization (24). Western blots, and enzyme assay which show that it has a complete deletion of the *frd* operon. The plasmids used to amplify specific fumarate reductase subunits or groups of subunits were derived from pGC1002 which codes for all four subunits of the complex (25). Construction of plasmids pFRD23, pFRD105, pFRD39, pFRD32, and pFRD38 have been described elsewhere (26, 27). DNA sequence analysis of plasmid pFRD105 was performed by the dyeoxy chain termination method of Sanger et al. (28) using double stranded plasmid as template. Primers for initiating DNA synthesis were synthesized complementary to the frdB sequence at base pairs 2708-2816 and 2898-3006 as numbered in Ref. 19. Information concerning the size of these plasmids and the fumarate reductase polypeptides for which they code is given in Fig. 1.

Plasmid-transformed cells of the deletion mutant ECL509 were grown anaerobically at 37 °C on glucose/fumarate mineral salts medium (29) to an *A*~om~ of 0.4-0.5 to induce maximal production and stability of individual subunits (26). Manganese was omitted from the mineral salts medium since it interferes with EPR measurements. Plasmid pFRD39 is under control of a *trp* promoter (27), therefore, to induce maximal production of the FrdB polypeptide, indole acrylic acid was added to the culture medium of cells containing this plasmid when the cell *A*~om~ reached 0.15-0.2 (30), and the cells were harvested as above.

**Analysis of Fe-S Clusters of Cells and Extracts—** Cells were harvested anaerobically and frozen in liquid nitrogen. For EPR studies on whole cells, the cells were thawed and suspended in a minimal volume of 50 mM Tris/HCl buffer, pH 7.4, under argon in a Vacuum Atmospheres glove box (<1 ppm oxygen). To facilitate oxidation or reduction of fumarate reductase Fe-S clusters, cells were ruptured by sonication and centrifuged at 10,000 × *g* for 10 min to provide a crude separation of the cytoplasmic and membrane fractions. Both procedures and the resuspension of the membrane fraction in a minimal volume of 50 mM Tris/HCl buffer, pH 7.4, were carried out under argon. Fumarate- and dithionite-treated samples were prepared anaerobically by making the medium 20 mM in fumarate or 10 mM in dithionite and incubating at room temperature for 30 min prior to freezing in liquid nitrogen. Ferricyanide oxidation of cytoplasmic fractions was performed by anaerobic addition of potassium ferricyanide to a final concentration between 0.1-0.5 mM. Samples were frozen within 5 min of ferricyanide addition.

EPR spectra were recorded on an IBM ER200D EPR spectrometer interfaced to an IBM 9031 computer for data handling and manipulation. Low temperatures were obtained with an Oxford Instruments ESR-9 cryostat positioned in a TE102 cavity resonating at X-band frequencies. Spin quantitations were carried out by double integration under nonsaturating microwave powers using 1 mm CuEDTA as the reference and correcting for *g*-values according to Aasa and Vranggard (31).

**RESULTS**

EPR spectra recorded at 50 K of whole cells of the *E. coli* frdABC deletion mutant, ECL509, with plasmid-amplified expression of fumarate reductase subunits are shown in Fig. 2. ECL509 (*ΔfrdABC*) is completely deficient in the *frd* operon as shown by Southern DNA-DNA hybridization (24) using various *frd* genes as probes. In addition, no fumarate reductase complex polypeptides are produced as judged by Western blots using antibody prepared against the complex or by enzyme assay. However, when this strain is transformed with *frd* plasmids, Western blots indicate that the corresponding fumarate reductase polypeptides are produced and are of the appropriate size (data not shown). The strains containing plasmids pGC1002, pFRD23, pFRD105, or pFRD39, that code for FrdABC, FrdAB**, or FrdAB*, respectively, all showed an almost axial EPR signal, *g* = 2.025 and *g* = 1.928, characteristic of the S = 5/2 [2Fe-2S]** cluster, center 1 (8-10), in addition to an isotropic radical signal centered at *g* = 2.003. The invariant nature of the EPR characteristics argues against any change in the specific residues that coordinate center 1 in the mutant forms of the enzyme. Identical EPR spectra were obtained after breaking open the cells and separating the membrane and cytoplasmic fractions, as described under "Materials and Methods" section. As expected, the most intense EPR signals were observed in the membrane fraction for cells with the plasmid coding for FrdABC, and in the cytoplasmic fraction for cells with plasmids coding for FrdAB, FrdAB*, and FrdB. EPR spectra for cells of the *frdABC* deletion mutant grown in the absence of any plasmids, see Fig. 2, or with plasmids pFRD38 and pFRD92 (data not shown), which code for FrdD and FrdCD, respectively, only exhibited a radical signal centered at *g* = 2.003, irrespective of the temperature or microwave power we conclude, therefore, that the [2Fe-2S] cluster, center 1, is located exclusively in FrdB.

To be more specific concerning the individual cysteinyl residues involved in ligating center 1, it was necessary to determine the exact nature of the *frdB* deletion contained on pFRD105 that codes for FrdAB**. Thus, the nucleotide sequence of pFRD105 was determined, and the sequence of amino acids encoded is shown in Fig. 3. Analysis of the
sequence shows that the FrdB* polypeptide is 197 amino acids in length and contains the first 167 residues of FrdB plus an additional 30 residues encoded by the DNA sequence contained on the vector pBR322. The FrdB* polypeptide produced has a predicted molecular mass of 21,670 daltons and Western blots of cytoplasmic extracts of ECL509 transformed with pFRD105 show that a peptide of this size is indeed present in the cytoplasm. Since the EPR data show that center 1 is present in cells amplified with the plasmid coding for FrdAB*, cysteine residues 205, 211, and 215 in FrdB can be ruled out as potential ligands for this center.

EPR studies of E. coli FRD both as the 4-subunit complex and 2-subunit soluble enzyme have shown that the [4Fe-4S] cluster, center 2, can be reduced by dithionite to a form with an S = ½ ground state that exhibits an EPR signal at temperatures below 20 K (10, 11). However, the EPR resonance is very broad, spanning at least 150 mT, probably as a result of spin-spin interaction with the paramagnetic reduced form of center 3 (S = 2) (10, 13, 36). The broadness of this resonance makes it difficult to detect with certainty in whole cells or whole cell extracts since resonances from a variety of paramagnetic centers (constituents of enzymes and proteins that are not amplified by the plasmids coding for fumarate reductase subunits) are apparent at the high spectrometer gains required to observe reduced center 2. However, redox titrations of mammalian Complex II (37) and E. coli fumarate reductase complex (11) reveal that a marked enhancement of the spin relaxation of center 1 occurs concomitant with the appearance of the EPR signal from reduced center 2. This phenomenon is attributed to spin-spin interaction between reduced center 1 (S = ½) and reduced center 2 (S = ½). Therefore, to establish the presence or absence of center 2, the spin relaxation properties of center 1 have been compared for whole cell extracts as isolated and reduced with dithionite.

The results are shown in Fig. 4 as log-log plots of (signal amplitude)/P versus P, where P is the microwave power. Under nonsaturating conditions such plots should be horizontal straight lines with the onset of power saturation being marked by a downward deviation from linearity. While the presence of dithionite does not significantly perturb the form or intensity of the EPR signal from center 1 in any of the cell extracts investigated, it does effect marked enhancement of spin relaxation for cell extracts with plasmid amplified FrdABCD, FrdAB, and FrdAB* subunits, see Fig. 4. From this we infer that center 2 is present in these polypeptides, demonstrating that cysteinyl residues 205, 211, and 215 in FrdB cannot be ligands for center 2.

No significant relaxation enhancement of center 1 was apparent for the cytoplasmic fraction of cells amplified with FrdB on addition of dithionite, see Fig. 4. This negative result is more difficult to interpret. For example the center may be present but no longer reducible by dithionite or, if it is reduced, the absence of FrdA may result in a conformational change that results in decreased spin-spin interaction between centers 1 and 2. Alternatively, center 2 may not be present in the plasmid-amplified FrdB subunit. However, even this interpretation does not necessarily mean that center 2 is located in FrdA. Rather, it could imply that the presence of FrdA is required for assembly of this cluster or that it is
potassium ferricyanide and EPR spectra of the native and amplified expression of FrdB. Consequently, oxidation was attempted using potassium ferricyanide and EPR spectra of the native and ferricyanide-treated cytoplasmic fraction of cells with amplified expression of FrdB were presented in Fig. 5c. Oxidation with 6 mM ferricyanide under anaerobic conditions resulted in the complete loss of the EPR signal from reduced center 1 and the appearance of a resonance indicative of oxidized center 3. Samples treated with lower concentrations of ferricyanide exhibited EPR spectra that were a superposition of these two resonances. Spin quantitations revealed that the EPR signal of oxidized center 3 accounted maximally for only 20% of that of reduced center 1 in the native extract. The signal intensity was further reduced by prolonged (>5 min) incubation with ferricyanide or exposure to air. However, such behavior is consistent with assignment to oxidized center 3, since this cluster is known to undergo facile degradation on prolonged exposure to ferricyanide or oxygen in soluble fumarate reductase preparations (8). Further evidence that the EPR resonance observed on ferricyanide-oxidation is endogenous to FrdB came from control experiments with cytoplasmic fractions of the deletion mutant which showed no such resonance on incubation with equivalent concentrations of ferricyanide. Taken together these observations suggest that the EPR signal in ferricyanide-oxidized cytoplasmic extracts of cells with amplified expression of FrdB originates from oxidized center 3 of fumarate reductase. This being the case, we conclude that center 3 is located entirely in FrdB.

DISCUSSION

The results presented above provide new insights concerning the location of the Fe-S clusters in E. coli fumarate reductase. Moreover, in light of the close similarity in cluster content and subunit composition, the conclusions are likely to be applicable to bacterial and mammalian succinate dehydrogenase enzymes. Thus far it has not been possible to separate and isolate Fp and Ip subunits of any fumarate reductase or succinate dehydrogenase and keep the constituent Fe-S clusters intact (2, 13). Consequently, prior to this investigation, ideas on the location of the Fe-S clusters rested largely on comparisons of conserved sequences with those of structurally well-characterized Fe-S proteins. In the following discussion, the picture that has emerged from sequence comparisons is described and assessed in light of the results presented above.

Complete amino acid sequences for both Fp and Ip subunits of E. coli fumarate reductase and succinate dehydrogenase and Bacillus subtilis succinate dehydrogenase have been deduced from the DNA sequence (19-22, 38), while that of the Ip subunit of bovine heart succinate dehydrogenase has been determined by direct means (18). Comparison of this sequence data reveals that 11 cysteine residues in the Ip subunit (10 in E. coli succinate dehydrogenase) are conserved in three ferredoxin-like clusters, see Fig. 6. In contrast the cysteine residues in the Fp subunit are more randomly dispersed with none being conserved. This has led to the suggestion that all three clusters may be located exclusively in the Ip subunit (9, 13, 36). Circumstantial support for this hypothesis comes from EPR studies which have revealed evidence for weak spin-spin interactions between the paramagnetic forms of centers 1 and 2, 2 and 3, and 1 and 3, indicating that the clusters are in close spatial proximity to one another (11, 13, 36).

The EPR studies of the E. coli frdABCD deletion mutant, ECL509, with the plasmid-amplified FrdB subunit provide the first definitive spectroscopic evidence that the [2Fe-2S] cluster, center 1, resides exclusively in the Ip subunit. Previously, Hederstedt et al. (39) had used a similar approach to address this question by investigating the EPR characteristics of well-defined Fp and Ip defective mutants of B. subtilis succinate dehydrogenase. However, no clear cut answer was forthcoming since center 1 appeared to be assembled only in...
Location of Fe-S Clusters in E. coli Fumarate Reductase

Fig. 5. EPR spectra of an isolated and oxidised sample of E. coli frdABCD deletion mutant, ECL509, with plasmid amplified expression of ABCD, AB*, and B. Conditions of measurement: temperature, 13 K; microwave power, 1 mW; frequency 9.41 GHz; modulation amplitude, 0.60 mT; time constant, 1.2 s; scan time, 600 s. a, membrane fraction of cells amplified for ABCD subunits before and after anaerobic oxidation by fumarate (20 mM). b, cytoplasmic fraction of cells amplified for AB* subunits before and after anaerobic oxidation with fumarate (20 mM). c, cytoplasmic fraction of cells amplified for B subunit before and after anaerobic oxidation with ferricyanide (6 mM). The multiplication factors indicate the relative spectrometer gains for the two spectra in each panel.

![Diagram](image)

**FIG. 6. Comparison of the arrangement of cysteine residues in the FrdB subunit of E. coli fumarate reductase (20) and iron-sulfur protein (Ip) subunit of beef heart succinate dehydrogenase (18) with those of the 2Fe ferredoxin from S. platensis (32), the 8Fe ferredoxin from P. aerogenes (53), and the 7Fe ferredoxins from A. vinelandii (34) and T. thermophilus (55). Dashes indicate breaks in the sequence to facilitate alignment of cysteine residues. Numbers refer to the residues immediately preceding and following those shown.**

The observation that center 1 is present in cells of the deletion mutant amplified with FrdAB* restricts the ligating cysteiny1 residues for this cluster to Cys-58, -63, -66, -78, -149, -152, -155, and -159. The possibility of non-cysteiny1 coordination of cluster 1 is unlikely in light of the recent electron spin echo envelope modulation studies of bovine heart succinate dehydrogenase (13, 40). Moreover, the arrangement of the first 4 cysteinyl residues, i.e., Cys-X-X-X-Cys, is analogous to that found in all plant type ferredoxins (41), see Fig. 6. That these cysteines ligate the cluster in plant-type ferredoxins has been established via the x-ray structure of Spirulina platensis ferredoxin (32). Therefore, it seems likely that center 1 is coordinated by Cys-58, -63, -66, and -78 in FrdB. While this is clearly the best working hypothesis at present, it should be noted that not all of the available information is consistent with this assignment. For example, the third cysteinyl residue in this group is replaced by an aspartyl residue in E. coli succinate dehydrogenase (22). Furthermore, the midpoint potential and spectroscopic characteristics of center 1 in fumarate reductase and succinate dehydrogenase enzymes are not identical to those of a plant-type ferredoxin. The midpoint potentials for a plant-type ferredoxin are approximately 400 mV more positive than the 2Fe-2S ferredoxin and the low temperature magnetic CD (8, 42) and EPR spectra more closely resemble adrenodoxin than any other known type of biological [2Fe-2S] center. The available amino acid sequence data for simple [2Fe-2S] proteins, in particular the arrangement of cysteinyl residues, suggest at least four unrelated types of primary structure for this type of cluster (43). These are exemplified by plant-type ferredoxins, adrenodoxin and putidaredoxin, the [2Fe-2S] ferredoxin from Clostridium pasteurianum, and Rieske protein. In light of the...
differences in spectroscopic and redox properties of center 1 in fumarate reductase and succinate dehydrogenase compared to those of other biological [2Fe-2S] clusters, the possibility of coordination by a novel arrangement of cysteinyl residues cannot be excluded at the present time.

As indicated in the results section, the EPR studies reported here are not conclusive concerning the subunit location of center 2. However, the studies of cells with plasmid-amplified FrdAB* limit the cysteinyl residues that are available for ligating this cluster and hence provide a test of the coordination arrangement that is suggested from sequence comparisons of structurally characterized 8Fe and 7Fe ferredoxins. The second and third groups of cysteinyl residues in the Ip subunits of all succinate dehydrogenase and fumarate reductase enzymes sequenced thus far are highly conserved and show remarkable homology to the arrangement of ligating cysteinyl residues in the 8Fe (2 × [4Fe-4S]) ferredoxin from *Peptococcus aerogenes*, see Fig. 3, except for the replacement of the second cysteine in the third group by a nonpolar residue: isoleucine (*E. coli* succinate dehydrogenase (22)), and bovine heart succinate dehydrogenase (18)), valine (*E. coli* fumarate reductase (20)), or serine (*B. subtilis* succinate dehydrogenase (38)).

X-ray structures for two ferredoxins containing [4Fe-4S] clusters have been established, the 8Fe ferredoxin from *P. aerogenes* (33) and the 7Fe ferredoxin from *Azotobacter vinelandii* (6, 7). In both instances, and in the case the 8Fe ferredoxins such as that from *Bacillus stearothermophilus* which contain one [4Fe-4S] cluster and only has 4 cysteinyl residues (44), each [4Fe-4S] cluster is ligated by a Cys-X-Cys-X-Cys-X-X-Cys arrangement, with the fourth cysteine at some distance and invariably followed by a prolyl residue. Therefore it should be realized, in connection with Fig. 6, that individual Cys-X-X-X-Cys and Cys-45, -42, -41, and -18, in *P. aerogenes* and Cys-45, -42, -39, and -20, in *A. vinelandii*. Thus, while Cys-X-X-Cys-X-Cys-X-X-X-Pro sequences are found in almost all 8Fe and 7Fe ferredoxins, the first group of three cysteines and the last cysteine ligate different clusters. Indeed calculations of the conformational energy associated with a [4Fe-4S] center and the position of cysteine residues in a polypeptide chain (45) have indicated that the first 3 cysteine residues can be close together, but the fourth needs to be at a distance for the formation of a stable chromophore. It is, therefore, very unlikely that the second group of 4 cysteine residues in FrdB can provide all four ligands for a single [4Fe-4S] cluster. Consequently, the observation that the [4Fe-4S] cluster, center 2, is present in FrdAB*, which is deficient in the third group of conserved cysteines in FrdB, argues against the cysteine coordination that would be inferred from sequence comparisons with [4Fe-4S] ferredoxins. Furthermore, these investigations give no evidence to suggest that center 2 is assembled in cells amplified with the plasmid coding only for the FrdB subunit. Assuming that the cysteinyl coordination for center 2 and that the first group of 4 cysteinyl residues in FrdB ligate center 1, this leaves two possibilities for the location of center 2. Either it is located entirely in FrdA or it is bridging between subunits FrdA and FrdB.

Finally, we turn our attention to the location of center 3. EPR studies of oxidized samples of cytoplasmic extracts of cells with amplified expression of FrdB and FrdAB* are most readily interpreted in terms of center 3 being located exclusively in the FrdB subunit with coordination by 1 or more of the last group of 3 cysteinyl residues. The revised x-ray structure of the 7Fe ferredoxin from *A. vinelandii* indicates that only 3 cysteinyl residues are required to coordinate a [3Fe-4S] cluster. In this ferredoxin the [3Fe-4S] cluster is ligated by Cys-8, -15, and -49 (6, 7). As with [4Fe-4S] clusters, the distant cysteinyl residue, Cys-49, is followed by a proline. That Cys-11 is not required is further supported by the observation that the equivalent cysteine is replaced by valine in the sequence of other 7Fe ferredoxins, such as that from *Thermus thermophilus* (35), see Fig. 6. Clearly, there are two distinct arrangements of cysteines in FrdB that are consistent with both the data presented herein and are in accord with sequence data for other [3Fe-4S]-containing ferredoxins (46): one involves ligation by Cys-205, -211, and -159, and the other by Cys-149, -155, and -215. At present it is not possible to decide between these two alternatives.

The results presented and discussed above illustrate the potential of combined spectroscopic and genetic approaches for elucidating the location of the multiple Fe-S clusters fumarate reductase and succinate dehydrogenase. While the results thus far do not permit definitive conclusions with respect to the specific cysteine that are ligating the clusters, they do provide the first experimental evidence that centers 1 and 3 are located entirely in Ip subunit as well as suggesting that center 2 either resides exclusively in the Ip subunit or bridges between the two catalytic domain subunits. More discriminating experiments involving EPR and magnetic CD studies of *E. coli* fumarate reductase mutants with site-specifically mutated cysteine residues are in progress to address the remaining unresolved questions.

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