The High Potential Iron-Sulfur Center in *Escherichia coli* Fumarate Reductase Is a Three-iron Cluster*

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The fumarate reductase complex and soluble enzyme from *Escherichia coli* have been investigated by low temperature magnetic circular dichroism and electron paramagnetic resonance spectroscopies. The results confirm the presence of one [2Fe-2S] cluster and show that the high potential iron-sulfur center is a 3Fe cluster of the type found in bacterial ferredoxins. Since the 3Fe cluster is present in catalytically competent enzyme and does not appear to be involved in any type of cluster conversion under reducing conditions, we conclude that it is an intrinsic component of the functional enzyme. The significance of the results is discussed in relation to the published amino acid sequence and the iron-sulfur cluster composition of bacterial fumarate reductases.

The metabolism of succinate and fumarate in *Escherichia coli* is catalyzed by two genetically distinct membrane-bound iron-sulfur flavoenzymes, succinate dehydrogenase and fumarate reductase, which are synthesized during aerobic and anaerobic growth, respectively (1, 2). Fumarate reductase is the terminal reductase of the anaerobic electron transport chain when *E. coli* is grown on a medium containing fumarate and a nonfermentable carbon source, such as glycerol or formate. The enzyme from *E. coli* is the best characterized fumarate reductase, having been cloned, amplified, sequenced, and isolated in pure form (3–7).

The fumarate reductase complex from *E. coli* contains a large flavoprotein subunit (M, 66,000), A, with covalently bound FAD, a smaller iron-sulfur protein subunit (M, 27,000), B, and two small hydrophobic subunits (M, 15,000 and 13,000), C and D, that are membrane anchors (8) and are required for interaction of the enzyme with quinone in the membrane (7). The nature of the iron-sulfur centers has been addressed by electron paramagnetic resonance spectroscopy (9, 10, 42) and by comparison of the amino acid sequence with those of well-characterized iron-sulfur proteins (3, 11, 12). The results have been interpreted in terms of at least one [2Fe-2S]^{2+} type, located in subunit B, and a high potential iron-sulfur center of uncertain location, but probably of the [4Fe-4S]^{2+} type.

In this work we report low temperature magnetic circular dichroism (MCD) and EPR studies of the fumarate reductase complex and soluble enzyme from *E. coli*. The results confirm the presence of one [2Fe-2S] cluster and provide conclusive evidence that the paramagnetic high potential iron-sulfur center is a 3Fe cluster, similar to those found in bacterial ferredoxins.

**MATERIALS AND METHODS**

*Escherichia coli* 1100 (bgIR thi1 rol1 Nfr Pol) was transformed with plasmid pGC1002, which codes for the entire *fdr* operon (7), and was grown anaerobically on glycerol-fumarate medium (2) to induce maximal levels of the fumarate reductase complex. The cells were broken by one passage through a French pressure cell (15,000 p.s.i.). Unbroken cells were removed by centrifugation at 8,000 × g for 10 min, and the supernatant was centrifuged for 2 h at 100,000 × g to prepare a cytoplasmic membrane fraction. The fumarate reductase complex was isolated by extraction of the membrane fraction (1 g of protein) for 1 h at 4 °C with 100 ml of 100 mM potassium phosphate, pH 6.8, containing 30 mM octyl-β-D-glucopyranoside, 100 μM phenylmethylsulfonyl fluoride, and 1 mM diethiothreitol. The extract was centrifuged for 1 h at 45,000 × g, and the supernatant fraction was dialyzed for 12 h against 40 volumes of 100 mM potassium phosphate, pH 6.8, 1 mM dithiothreitol, with one change of buffer. The extract was then centrifuged at 100,000 × g for 2 h. The resulting pellet, containing the fumarate reductase complex, was stored at −70 °C until used.

Fumarate reductase was obtained from the fumarate reductase complex essentially as described by Davis and Hatefi (13) to resolve beef heart Complex II. The isolation procedure was carried out at 4 °C under strictly anaerobic conditions. Fumarate reductase complex (10 mg/ml) suspended in 50 mM Hepes (pH 7.4) with 20 mM succinate and 1 mM diethiothreitol was treated with 400 mM sodium perchlorate for 4 min and then centrifuged in a Beckman Airfuge (160,000 × g for 2 min). The pellet was resuspended in the same buffer mixture, treated with 800 mM sodium perchlorate for 1 min, and centrifuged at 160,000 × g for 3 min. At this point the supernatant solution was immediately centrifuged through Sephadex G-25 (course) equilibrated with anaerobic Hepes buffer (50 mM), pH 6.8 (14), to remove perchlorate and lower the pH. Unresolved fumarate reductase complex was removed by precipitation with 30% ammonium sulfate. The soluble fumarate reductase was then precipitated by bringing the supernatant to 55% saturation and the pellets stored in liquid N₂ until used for spectroscopic investigations.

For spectroscopic measurements all preparations were in pH 7.8 buffer mixtures (see Table II for details), containing 50% v/v ethylene glycol. Addition of ethylene glycol facilitates formation of an optical glass on freezing samples for MCD measurements. Protein determinations were made on trichloroacetic acid-precipitated material by a modification of the Lowry method (15). The enzyme concentrations used in quantifying MCD, EPR, and absorption spectra are based on the concentration of bound FAD (16), since there is 1 mol of FAD/P.
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mol of enzyme (5). Activity assays were carried out as described elsewhere (7).

The ferredoxin from Thermus thermophilus HB8 was provided by Dr. J. A. Fee (Los Alamos, NM). The sample used for spectroscopic studies was 306 μM in 100 mM potassium phosphate buffer (pH 7.7) with 50% (v/v) ethylene glycol.

Low temperature MCD and EPR and room temperature absorption measurements were carried out using the instrumentation described in Ref. 17. Theoretical MCD magnetization plots were computed as previously described (17). Quantitations of EPR spectra were made using 1 mM CuEDTA (>30 K) or 1 mM metmyoglobin cyanide (<30 K) as standard (18). Checks were made to ensure that these conditions did not produce power saturation in either sample or standard.

RESULTS

Table I shows analytical and assay data for the samples of fumarate reductase complex and soluble enzyme used in the spectroscopic investigations. On the basis of FAD determinations and subunit molecular weights and the assumption of 1 mol of FAD/mol of enzyme (5), the preparations of the complex and soluble enzyme were approximately 52 and 83% pure, respectively. The soluble enzyme was approximately 43% less competent in the succinate-phenazine methosulfate oxidoreductase assay than the complex and thus appeared to have suffered some preparative and/or manipulative damage. The low level of ubiquinone reductase activity exhibited by the soluble enzyme could reflect a minor contamination with unresolved complex (<20% of total FAD present).

Table II summarizes the EPR data, including spin quantitations, for the samples used in MCD studies and provides details of sample preparation. The EPR results are discussed, together with MCD data, in the appropriate section below.

MCD and EPR Studies of Fumarate Reductase Complex

(a) Complex, as Prepared—Fumarate reductase complex, as prepared, exhibits an EPR signal with a sharp peak at g = 2.02, a broad trough around g = 1.97 with an extended tail toward high field, and a distinct shoulder at g = 1.99 (Fig. 1). This signal is characteristic of the oxidized high potential iron-sulfur cluster. This EPR signal corresponds to approximately one cluster/FAD (Table II). Samples exposed to air or ferricyanide exhibit identical EPR signals, but, on prolonged exposure, this center is partially lost (Table II).

Fig. 1 illustrates the close similarity in the form of the EPR spectra of fumarate reductase complex, as prepared, with that of the bacterial ferredoxin from T. thermophilus, as isolated. Both EPR spectra correspond to approximately one spin/molecule and show analogous temperature and microwave power dependence (signal observable only below 30 K and not saturating at powers up to 50 mW at 13 K). Computer

**Table I**

<table>
<thead>
<tr>
<th>Analytical and assay data for samples of fumarate reductase complex and soluble enzyme</th>
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<td>Complex</td>
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<tr>
<td>Analytical data</td>
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<td>FAD content (nmol-mg⁻¹)</td>
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<td>Q⁺ reductase activity</td>
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<td>PMS⁺ reductase activity</td>
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Based on at least two determinations of nonheme Fe, FAD, and protein.

Mol of succinate oxidized per min per mol of fumarate reductase (38°C). The samples were taken anaerobically from MCD cells shipped on dry ice from Baton Rouge to San Francisco.

Q, ubiquinone.

PMS, phenazine methosulfate.

**Table II**

<table>
<thead>
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<th>EPR data for samples of fumarate reductase complex and soluble enzyme</th>
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<td>g values</td>
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<td>Complex</td>
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<td>Soluble enzyme</td>
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Signal observed only below 30 K. Spin quantitation at 13 K and 1 mW microwave power versus a metmyoglobin cyanide standard. g values based on computer simulation of similar signal observed in T. thermophilus ferredoxin, as isolated (19).

Complex solubilized anaerobically in 100 mM potassium phosphate buffer, pH 7.8, containing 50% (v/v) ethylene glycol and 3% (v/v) Triton X-100.

As prepared complex exposed to air for 2 h at room temperature.

As prepared complex treated with a 15-fold stoichiometric excess of potassium ferricyanide for 30 min at room temperature; ferricyanide removed by gel filtration using Sephadex G-25.

Signal observed up to 120 K; spin quantitation at 70 K and 1 mW microwave power versus a CuEDTA standard; g values taken at peak maxima, crossover, and peak minima.

Complex solubilized anaerobically in 100 mM potassium phosphate, pH 7.8, containing 50% (v/v) ethylene glycol, 100 mM succinate, and 1 mM dithiothreitol; reduced under anaerobic conditions with a 10-fold stoichiometric excess of sodium dithionite.

**Fig. 1.** EPR spectra of E. coli fumarate reductase complex, as prepared, and T. thermophilus ferredoxin, as isolated. Conditions: 13 K, 1 mW microwave power, 0.63 mtesla modulation amplitude, frequency 8.99 GHz. Upper spectrum: fumarate reductase complex, as prepared. Enzyme concentration 47 μM; buffer mixture as in Table II, footnote b. Lower spectrum: T. thermophilus ferredoxin, as isolated.
simulation of the spectrum observed for *T. thermophilus* ferredoxin, as isolated, affords principal *g* values of *g*<sub>x</sub> = 2.015, *g*<sub>y</sub> = 1.98, and *g*<sub>z</sub> = 1.83 (19). Mössbauer spectroscopy (20) and, more recently, MCD studies<sup>2</sup> have unambiguously identified this EPR signal in *T. thermophilus* ferredoxin as originating from an oxidized 3Fe center. Other well-characterized oxidized 3Fe centers in aconitase and ferredoxins from *Desulfurobacterium gigas* and *Azotobacter vinelandii* exhibit similar, but more isotropic, EPR signals (21). The close similarity of the EPR spectra shown in Fig. 1 suggests the presence of one oxidized 3Fe cluster in the fumarate reductase complex. However, since bacterial [4Fe-4S]<sup>±±</sup> centers have somewhat similar EPR spectra in the oxidized state, this observation is not conclusive and requires confirmation using more discriminating spectroscopic techniques such as low temperature MCD.

Room temperature absorption and low temperature MCD spectra, 300–800 nm, of the fumarate reductase complex as prepared are shown in Fig. 2. At low temperatures the MCD spectra are dominated by intense Soret transitions (positive peak at 400 nm and negative trough at 414 nm) from an oxidized cytochrome. The presence of cytochrome is also manifest by the weak shoulder at 414 nm in the absorption spectrum. The Soret MCD spectra are indicative of low spin oxidized cytochrome, either *c*, or some combination thereof (22–24). The MCD intensity indicates that the cytochrome:FAD ratio is approximately 1:10 in this preparation. Subsequently we observed that the quantity of cytochrome in different Triton X-100 extracts is variable, never exceeding 10% of the FAD concentration, as judged by the MCD spectrum. It seems likely, therefore, that the cytochrome is not an intrinsic part of the plasmid-encoded fumarate reductase complex but is present as an impurity originating from some other component of the anaerobic respiratory chain of *E. coli*. Temperature-dependent MCD transitions in the regions 300–360 and 440–800 nm arise, for the most part, from a chromophore other than the cytochrome. MCD magnetization data for the transition at 465 nm (Fig. 3) indicate an EPR-active S = ½ ground state for this chromophore. The experimental data are well fitted by theoretical magnetization data based on the EPR-determined *g* values for the oxidized high potential cluster. These transitions are, therefore, attributed to the oxidized high potential iron-sulfur center.

To obtain the MCD spectrum of the oxidized high potential iron-sulfur in the absence of cytochrome, use was made of the observation that different preparations of the complex had differing amounts of cytochrome. The form of the low temperature MCD spectrum of the cytochrome alone was thus obtained by differencing the MCD spectra of two different preparations of the complex, under identical conditions, after appropriate normalization to equalize the size of the features due to the oxidized high potential cluster (Fig. 4). Subtraction of the cytochrome spectrum from the MCD spectrum of the enzyme as prepared, under analogous conditions, gave the MCD spectrum of the oxidized high potential iron-sulfur center (Fig. 4). For comparison, the previously unpublished MCD spectrum of *T. thermophilus* ferredoxin as isolated is shown under similar conditions in Fig. 4. The close similarity of the spectra in terms of both form and intensity provides convincing evidence that the fumarate reductase complex contains one oxidized 3Fe cluster. Oxidized tetranuclear high potential iron-sulfur clusters have MCD characteristics quite distinct from those in Fig. 4 (21, 25, 26).

(b) Dithionite-reduced Complex—On reduction of the sample with dithionite, the EPR signal of the oxidized high potential cluster disappears and is replaced by a slower relaxing signal, *g* = 2.026, 1.934, 1.920 (Fig. 5). This signal is observable without significant broadening up to 120 K and consistently accounts for slightly more than one spin/FAD. The *g* values and relaxation behavior are similar to those of cluster S1 in mammalian succinate dehydrogenase, which has been characterized as a [2Fe-2S]<sup>±±</sup> cluster by EPR (27, 28), linear electric field effect EPR (29), CD and MCD (17) spectroscopy.

Fig. 6 shows room temperature absorption and low temperature MCD spectra of the dithionite-reduced complex. The absorption spectrum is featureless, monotonically increasing toward higher energy, except for shoulders at 320 nm, arising

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from excess dithionite, and 428 and 556 nm, arising from reduced cytochrome. The low temperature MCD spectra reveal numerous temperature-dependent transitions in the wavelength region investigated. The temperature-independent derivative centered at 556 nm (shown by arrows in Fig. 6), superimposed on a positive temperature-dependent band, is therefore assigned to the low spin cytochrome impurity apparent in the as prepared complex, since low spin Fe(II) ($S = 0$) cytochrome $\delta$ or $\epsilon$ exhibit temperature-independent MCD transitions with a sharp derivative centered at approximately 555 nm as the dominant spectral feature (24). The positive band at 440 nm is typical of that observed for a trace of high spin (S = 2) Fe(II) heme (22, 30). MCD magnetization data (not shown) confirm that the MCD intensity at 440 nm is due in part to high spin Fe(II) heme. Temperature-dependent MCD transitions in all other regions are attributed to paramagnetic iron-sulfur clusters.

In an attempt to assess the contribution to the MCD intensity from different clusters, MCD magnetization data were recorded at 715, 680, and 320 nm (Fig. 7). Reduced 3Fe clusters are unique among all known iron-sulfur centers in terms of their MCD magnetization data. They all (including the reduced 3Fe center in T. thermophilus ferredoxin$^a$) have an intense positive MCD band centered around 715 nm which exhibits magnetization plots that are well fitted by $g_0 = 8.0$ and $g_\perp = 0.0$ at temperatures below 2 K (21, 26). Transitions are, therefore, considered to originate from the $S_a = S_b = \pm 2$ doublet of a $S = 2$ ground state having predominantly axial zero field splitting with $D < 0$ (where $D$ is the axial zero field splitting parameter). The excellent agreement between the experimental magnetization data at 715 nm and the theoretical data for an EPR-silent reduced 3Fe cluster (solid line in Fig. 7) provides conclusive evidence for a reduced 3Fe center in the dithionite-reduced fumarate reductase complex. Reduced 3Fe clusters have low temperature MCD spectra that are considerably more intense than any other known type of iron-sulfur cluster, under comparable conditions (21, 26, 31).

Since the MCD intensity at 715 nm is consistent with that expected for one reduced 3Fe cluster, transitions from this center would be expected to dominate a MCD spectrum that has contributions from several different types of paramagnetic iron-sulfur clusters.

On the basis of MCD studies of succinate dehydrogenase (17), the paramagnetic [2Fe-2S]$^{1+}$ cluster that is observed in the EPR would be expected to show positive temperature-dependent MCD transitions with maxima at 680 and 550 nm and an intense negative band at 330 nm. Magnetization data at 550 nm were not recorded, because of interference due to the cytochrome, but the experimental data at 680 and 320 nm are consistent with the MCD intensity arising from a mixture of $S = \frac{1}{2}$ and $S = 2$ chromophores. Theoretical data constructed for 60% $S = 2$ and 40% $S = \frac{1}{2}$ at 320 nm, and 75% $S = 2$ and 25% $S = \frac{1}{2}$ at 680 nm provide excellent fits to the experimental data. Although the theoretical $S = \frac{1}{2}$ plot shown in Fig. 7 (broken line) is based on the EPR-determined $g$ values for the [2Fe-2S]$^{1+}$ cluster, any other $S = \frac{1}{2}$ iron-sulfur cluster with $g$ values around 2 would give rise to a similar magnetization curve. Hence, we cannot conclude that the $S = \frac{1}{2}$ contribution to the MCD intensity at these wavelengths is exclusively due to the [2Fe-2S]$^{1+}$ cluster.

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![Fig. 3. MCD magnetization plot for fumarate reductase complex, as prepared.](image)

![Fig. 4. MCD spectra of the cytochrome and high potential iron-sulfur center in fumarate reductase complex, as prepared, and the oxidized three-iron center in T. thermophilus ferredoxin.](image)
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FIG. 5. EPR spectra of fumarate reductase complex and soluble enzyme after dithionite reduction. Conditions: 70 K, 1 mW microwave power, 0.63 mtesla modulation amplitude, frequency 8.99 GHz. Upper spectrum: dithionite-reduced fumarate reductase complex; concentration 42 μM. Lower spectrum: dithionite-reduced soluble fumarate reductase; concentration 53 μM. Buffer mixtures and conditions of reduction as described in Table II, footnotes f and g.

MCD and EPR Studies of Soluble Fumarate Reductase

The oxidized 3Fe center in fumarate reductase was found to be extremely labile in the soluble two subunit enzyme. Similar behavior has been observed for the equivalent center in mammalian succinate dehydrogenase (17, 32-34). As with the latter enzyme, fumarate reductase is isolated in the presence of excess succinate to ensure that this center is kept at least partially reduced. Spectroscopic studies of the soluble enzyme, for comparison with the complex, are reported for the dithionite reduced form only.

Dithionite-reduced Enzyme—The dithionite-reduced soluble enzyme has an EPR signal identical to that observed in the complex (Fig. 5). Since the signal integrates to just less than one spin/FAD, we conclude that the [2Fe-2S] center is essentially unaltered in the soluble enzyme.

Room temperature absorption and low temperature MCD spectra (Fig. 8) show no evidence of any reduced cytochrome impurity. Temperature-dependent MCD transitions, originating solely from paramagnetic iron-sulfur clusters, are observed throughout the spectral region investigated. Magnetization data at 1.53 K measured at 715, 680, 550, 406, and 320 nm are shown in Fig. 9. In agreement with MCD studies of the dithionite-reduced complex, magnetization data are consistent with a reduced 3Fe cluster as the sole contributor to the MCD intensity at 715 nm. In contrast, the MCD intensity at 550 nm originates almost exclusively from an iron-sulfur center having an $S = \frac{1}{2}$ ground state. By comparison with the spectrum of reduced S1 in succinate dehydrogenase, we conclude that the [2Fe-2S]$^{2+}$ cluster is responsible for the majority of the MCD intensity at this wavelength. The magnetization data at 680, 406, and 320 nm agree well with theoretical data constructed for 65% $S = 2$ and 35% $S = \frac{1}{2}$, 70% $S = 2$ and 30% $S = \frac{1}{2}$, and 45% $S = 2$ and 55% $S = \frac{1}{2}$, respectively.

Comparison with the magnetization for the dithionite-reduced complex at 680 and 320 nm suggests a decrease in the amount of reduced 3Fe cluster in the soluble enzyme.

More direct evidence for a diminution in the quantity of 3Fe clusters in the soluble enzyme compared to the complex is provided by the comparison of the dithionite-reduced MCD spectra under identical conditions (Fig. 10). The 50% decrease in the band centered at 715 nm suggests that approximately one-half of the 3Fe centers have been lost during preparation of the soluble enzyme.

DISCUSSION

The results presented in this work provide unambiguous evidence for one [2Fe-2S] and one 3Fe cluster in the fumarate reductase complex. Recent spectroscopic studies of Complex II from the mammalian respiratory chain (29) and reconstitutively active soluble preparations of succinate dehydrogenase (17) have demonstrated the presence of a 3Fe center, S3, in mammalian succinate dehydrogenase. It seems likely that a 3Fe center is an intrinsic component of all succinate dehydrogenase and fumarate reductase enzymes and that analogous EPR signals in other bacterial fumarate reductases (e.g. from Vibrio succinogenes (35, 36)) and bacterial succinate
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MCD magnetization plots for dithionite-reduced fumarate reductase complex. Wavelengths: 715 nm (+), 680 nm (X), 320 nm (O). Temperature 1.53 K, magnetic fields between 0 and 4.5 tesla. Solid line is theoretical magnetization curve for $g_1 = 8.0$ and $g_L = 0.6$. Broken line is theoretical magnetization curve for $g_1 = 2.026$, $g_L = 1.927$, $m_1/m_2 = -1$.

As yet it has not been possible to prove unambiguously that any 3Fe center is a necessary functional component of an in vivo enzyme, although this may well be the case in nitrate reductase (31), succinate dehydrogenase (17), and fumarate reductase. Activation of aconitase has been shown to be associated with conversion of a 3Fe center to a [4Fe-4S] cluster, under reducing conditions (35, 39). The intensity of the MCD bands from the reduced 3Fe center in the complex argues against any significant cluster conversion involving this center in fumarate reductase. We conclude that the 3Fe center in fumarate reductase is able to sustain catalytic activity. Future experiments in this laboratory will concentrate on investigating the possibility of cluster conversions and their effect on the activity of the fumarate reductase as well as attempts to establish the nature of the clusters in whole cells of E. coli.

The results reported here necessitate a re-evaluation of the iron-sulfur cluster composition of E. coli fumarate reductase. Previous EPR studies have been interpreted in terms of two [2Fe-2S] clusters (midpoint potentials -50 and -285 mV), only one of which is reducible by succinate, in addition to the high potential cluster (10). The evidence for two [2Fe-2S] centers resides in the observed doubling of the EPR spin intensity on addition of dithionite to the succinate-reduced enzyme and the marked relaxation enhancement of the EPR signal on dithionite reduction. The latter phenomena was rationalized by invoking spin interaction between the two $S = 1/2$ [2Fe-2S]$^{1+}$ centers. Although the samples of fumarate reductase complex and soluble enzyme used in the work reported here showed identical behavior, the spin quantitation of the dithionite-reduced samples (0.9–1.2 spins/FAD) argues against two [2Fe-2S]$^{1+}$ clusters. Rather we conclude that this cluster is only partially reduced (~50%) by succinate in fully activated preparations of the complex and in soluble enzyme. Analogous behavior has been reported for the fumarate reductase complex from V. succinogenes (35). The equivalent center in succinate dehydrogenase, S1, is fully reduced by succinate, but its midpoint potential is between 20 and 50 mV more positive (10, 42).

Recently we have shown that the spin relaxation enhancement of center S1 in succinate dehydrogenase on dithionite

![Graph](image-url)
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Fig. 9. MCD magnetization plots for dithionite-reduced soluble fumarate reductase. Wavelengths: 715 nm (+), 680 nm (×), 550 nm (C), 495 nm (O), 390 nm (O). Temperature 1.53 K, magnetic fields between 0 and 4.5 tesl. Solid line is theoretical magnetization curve for $g_1 = 8.0$ and $g_1 = 0.0$. Broken line is theoretical magnetization curve for $g_1 = 2.026, g_1 = 1.927, m_+ / m_- = -1$.

Fig. 10. Comparison of low temperature MCD spectra for dithionite-reduced fumarate reductase complex and soluble enzyme. Conditions: temperature 1.53 K, magnetic field 4.5 tesla. ---, complex; - - - - - - - - , soluble enzyme.

reduction is due to spin coupling between the [2Fe-2S]" center and a [4Fe-4S]^+ cluster that becomes reduced and paramagnetic only on reduction with dithionite" (17). EPR studies of the dithionite-reduced fumarate reductase complex at temperatures below 15 K and microwave powers up to 50 mW also show features to high and low field of the signal from the [2Fe-2S]^+ center that are attributable to a [4Fe-4S]^+ cluster. Spin coupling between the reduced binuclear and tetranuclear clusters would then explain the relaxation phenomena. The MCD data reported in this work are not inconsistent with the presence of a $S = \frac{1}{2}$ [4Fe-4S]^+ center in the dithionite-reduced samples. The intensity of the MCD spectrum from the [4Fe-4S]^+ center would be expected to be between four and six times weaker than that of the reduced 3Fe cluster (31) and hence would not be readily observed. In succinate dehydrogenase, the form of the low temperature MCD spectrum of the [4Fe-4S]^+ was obtained by differencing the spectra of dithionite and succinate-reduced samples (17). Indeed it was this difference spectrum that first suggested that this center was a tetranuclear cluster. Equivalent manipulation of fumarate reductase MCD data is not feasible, since our experiments indicate that both the [2Fe-2S] and 3Fe centers are only partially reduced by succinate.

Complete amino acid sequences have been determined for the flavoprotein and iron-sulfur protein subunits of succinate dehydrogenase and fumarate reductase from E. coli (3, 6, 12, 40). The iron-sulfur protein subunits of both enzymes show striking homology. Both contain 11 cysteine residues, with 10 being conserved in three clusters that closely resemble those found in bacterial ferredoxins. In contrast, the corresponding flavoprotein subunits have cysteine residues (11 in succinate dehydrogenase, 10 in fumarate reductase) that are more randomly dispersed and do not adopt distinctive ferredoxin-like clusters, with only one occupying the same position in both polypeptides. It is interesting to note that all three types of iron-sulfur cluster in fumarate reductase can be accommodated within the B subunit.

As pointed out by Cummauck (11), there is a marked similarity between the first cluster of cysteines, I, in the B subunit of fumarate reductase (residues 57, 62, 65, and 77) and the arrangement of cysteines bound to [2Fe-2S] centers in plant and cyanobacterial ferredoxins. The arrangement of cysteines in the two remaining clusters in the B subunit of fumarate reductase (II, residues 148, 151, 154, and 158; III, residues 204, 210, 214, and 218) closely resemble those found for [4Fe-4S] centers in bacterial ferredoxins (12). However, since facile interconversion between 3Fe and [4Fe-4S] clusters is now well established for many bacterial ferredoxins and aconitase (21), the same sequences must also be able to accommodate 3Fe clusters. The number of cysteines required to coordinate a 3Fe cluster is presently unknown and awaits further structural elucidation of this type of center. Since a 3Fe center is readily formed from the [4Fe-4S] cluster in Bacillus stearothermophilus (21), a maximum of four cysteines is necessary. Recent spectroscopic results suggest a common [3Fe-4S] stoichiometry for all 3Fe clusters, and two plausible structures have been advanced (41). These structures require 3 or 4 cysteines to complete tetrahedral sulfur coordination about Fe. Thus, while the possibility of some noncysteine coordination or cysteine residues being supplied by the flavoprotein subunit of the enzyme cannot be ruled out, it should be realized that they are not necessarily obligatory for the proposed cluster composition.

On the basis of the cluster composition and sequence data, we tentatively suggest that all three iron-sulfur clusters in E. coli fumarate reductase are primarily located in the iron-sulfur

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subunit, with the possibility of one or two clusters bridging between the iron-sulfur and flavoprotein subunits. Future spectroscopic studies will be designed to test this hypothesis.

Acknowledgment—We thank Dr. J. A. Fee for providing samples of Thermus thermophilus ferredoxin for spectroscopic investigations.

REFERENCES