Evidence for a Three-Iron Center in a Ferredoxin from *Desulfovibrio gigas*

MÖSSBAUER AND EPR STUDIES*

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The tetrameric form of a *Desulfovibrio gigas* ferredoxin, named Fd II, mediates electron transfer between cytchrome c₃ and sulfate reductase. We have studied two stable oxidation states of this protein with Mössbauer spectroscopy and electron paramagnetic resonance. We found 3 iron atoms/monomer and a spin concentration of 0.9 spins/monomer for the oxidized protein. Taken together, the EPR and Mössbauer data demonstrate conclusively the presence of a spin-coupled structure containing 3 iron atoms and labile sulfur. The Mössbauer data show also that this metal center is structurally similar, if not identical, with the low potential center of a ferredoxin from *Azotobacter vinelandii*, a novel cluster described recently (Empetage, M. H., Kent, T. A., Huynh, B. H., Rawlings, J., Orme-Johnson, W. H., and Münck, E. (1980) J. Biol. Chem. 255, 1793-1796).

Recently, we demonstrated the presence of a novel iron-sulfur center in a ferredoxin from *Azotobacter vinelandii* (1). We have given detailed Mössbauer evidence, supported by x-ray diffraction studies of Stout et al. (2), for a new metallo center containing 3 iron atoms (and acid-labile sulfur). For this protein, a detailed data analysis was impeded by the presence of an Fe₃S₄ center. Therefore, the characterization of the new iron-sulfur cluster required a rather unusual data analysis. Here, we report that the new center is also present in a ferredoxin, termed Fd II, from *Desulfovibrio gigas*. Since the latter protein contains only one type of metal center, it affords a precise data analysis.

*D. gigas* ferredoxin is isolated in different oligomeric forms. Fd' II is a tetramer of identical polypeptide subunits, each monomer having 57 amino acids of known sequence (3, 4). It has been shown (5) that Fd II mediates electron transfer between cytchrome c₃ and the sulfate reductase, while another oligomeric form, the trimeric Fd I, serves as a carrier in the phosphoroclastic reaction. The presence of iron and acid-labile sulfur and the EPR characteristics of both Fd I and II have suggested (6) the presence of Fe₃S₄ centers. We will demonstrate below that Fd II has a novel chromophore containing 3 iron atoms. The work described here complements in many respects the studies on the *Azotobacter* ferredoxin (1) which we reported with our co-workers of W. H. Orme-Johnson's group.

MATERIALS AND METHODS

The conditions for growth of *D. gigas* and the isolation of the tetrameric form of ferredoxin (Fd II) have been described previously (7). Throughout this manuscript, iron and spin concentrations will be quoted per monomer. Iron was determined by forming a ferrous complex with 2,4,6-tripyridyl-s-triazine using the procedure described by Fischer and Price (8).

EPR spectra were recorded in Dr. J. D. Lipscomb's laboratory on a Varian E-9 spectrometer fitted with an Oxford Instruments continuous flow cryostat. The Mössbauer spectrometer and methods of data analysis have been described previously (9). All isomeric shifts are quoted relative to iron metal at 295 K.

RESULTS

EPR Studies—We have performed iron analyses of three different Fd II preparations and found 2.97, 3.11, and 2.95 iron atoms/monomer. The sample used for the Mössbauer and EPR studies had 2.97 iron atoms/monomer; the monomer concentration was determined by amino acid analysis of the sample. (The sample was analyzed for eight stable amino acids; their distribution fits the known sequence.) The Mössbauer data gave no evidence for any iron impurities; this allowed us to determine the number of iron atoms per EPR-active center.

An isolated Fd II exhibits a fairly isotropic EPR signal around g = 2. Typical spectra are shown in Fig. 2b of Ref. 6. We have studied the EPR spectra of Fd II extensively in the temperature range from 2 to 12 K. These studies suggest strongly that the material is homogeneous, i.e. only one EPR-active species is present. By quantitating the spectra at 6, 8, and 12 K against a copper-EDTA standard, we found 0.93 ± 0.12 spins/3 iron atoms. The quoted uncertainty is mainly due to the problem of taking EPR spectra of the sample and copper-EDTA at precisely the same temperature.

We have also performed spectral simulations of the EPR spectra. A good representation of the observed spectra was obtained by choosing γ₁ = 2.02, γ₂ = 2.00, and g₁ = 1.97, using gaussians of widths 15, 35, and 80 G at γ₁, γ₂, and γ₃, respectively. At present, these values are tentative.

Mössbauer Results—Fig. 1A shows a Mössbauer spectrum of oxidized Fd II taken at 77 K. The solid line is a least squares fit to the spectrum assuming that each of the three iron ions yields the same spectrum. The quality of the fit, the symmetry of the spectrum, and the sharpness of the absorption lines (0.28 mm/s full width) support this assumption. The parameters for the quadrupole splitting, ΔE_Q = 0.54 ± 0.03 mm/s, and the isomeric shift, δ = 0.27 ± 0.03 mm/s are practically the same as those observed for rubredoxin (10), suggesting a tetrahedral coordination of (predominantly) sulfur atoms.
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Fig. 1. Zero field Mössbauer spectra of 2.2 mm Fd II from D. gigas. The sample had 26 nm iron with 56Fe in natural abundance. A spectrum of oxidized, EPR-active Fd II taken at 77 K. B, spectrum of reduced Fd II at 4.2 K. The solid lines are the result of fitting quadrupole doublets to the data. Note that the presence of Doublet II is not due to a fraction of oxidized material; at 4.2 K, the Mössbauer spectrum of oxidized Fd II shows paramagnetic hyperfine structure and it extends over a velocity range of 6 mm/s (see Fig. 3).

Fig. 2 shows a spectrum taken at 1.5 K. The data are best understood by comparing the Fd II spectrum with the corresponding one of the Azotobacter ferredoxin (Fig. 2 of Ref. 1). The subspectra of Sites 1 and 2 are nicely resolved for the Azotobacter protein, while the spectrum of Site 3 is masked by the absorption of the high potential center. In Fd II, the resolution between Sites 1 and 2 is poorer because the internal magnetic fields at these sites differ less. The spectrum of Site 3, on the other hand, is clearly discernible. Taken together, the data reveal three distinct sites. We have computed theoretical spectra of the three subcomponents (see Fig. 2) from the spin Hamiltonian $(S = \frac{3}{2})$

$$\hat{H} = g \beta \cdot \mathbf{B} \cdot \mathbf{S} + \mathbf{A} \cdot \mathbf{I} - g_\beta \mathbf{H} \cdot \mathbf{I} + \frac{eQ}{12} [I_z^2 - I(I + 1) + \eta(I_x^2 - I_y^2)]$$

In Equation 1, all symbols have their conventional meaning; the $g$-tensor is known from the EPR data. The spectrum of Site 1 (Fig. 2, solid line) has essentially the same magnetic splitting ($A_1 = 27$ MHz, $A_2 = 44$ MHz) as the corresponding one of the Azotobacter ferredoxin. The splitting of the Site 2 spectrum (Fig. 2, dashed line) is substantially larger in Fd II ($A_1 = 29$ MHz, $A_2 = 16$ MHz), accounting for the poorer resolution. As suggested previously (1), the spectrum of Site 3 (Fig. 2, dotted line) is characterized by a very small magnetic splitting ($A_1 = A_2 = 3.5$ MHz). The parameters quoted are quite tentative and certainly not unique. It is clear, however, that the magnetic interactions are fairly anisotropic.

Fig. 1B shows a spectrum of dithionite-reduced Fd II taken at 4.2 K in zero magnetic field. Doubles I and II (indicated by the brackets) are observed with parameters identical with those found (by a somewhat intricate analysis) for the Azotobacter ferredoxin. Both doublets are symmetric and they exhibit sharp absorption lines. The solid lines in Fig. 1B are the result of least squares fitting two doublets to the data, with the reasonable assumption that the intensities of the high and low energy lines of each doublet are the same. Most interestingly, two species represented by Doubles I and II are found to be in the ratio 2:1. (The fit yields for the ratio, somewhat fortuitously, $2.01 \pm 0.03$.) Furthermore, $\Delta E_0 = 1.47 \pm 0.03$ mm/s and $\delta = 0.46 \pm 0.02$ mm/s for Site I, and $\Delta E_0 = 0.47 \pm 0.02$ mm/s and $\delta = 0.30 \pm 0.02$ mm/s for Site II.

The iron associated with Doublet II is high spin ferrous in character. For the ferredoxin from A. vinelandii, we have listed arguments that Doublet II must be part of a spin-coupled structure (1). This contention is further supported by studies of reduced Fd II in applied magnetic fields. We have studied the reduced Fd II sample at 4.2 K in fields up to 60 kG; a spectrum taken in a 10-kG field is shown in Fig. 3.

The most distinctive property of reduced Fd II is that a field of only a few hundred gauss elicits substantial broadening of both doublets due to induced magnetic hyperfine interactions. This unusual behavior suggests strongly that the magnetic spectra of both Sites I and II are controlled by the same electronic spin $S$. The broadening proves that reduced Fd II is paramagnetic, i.e. $S > 0$. The features of the observed spectra allow us to draw the following conclusions: 1) The electronic spin relaxation rate at 4.2 K is slow compared to the nuclear precession frequencies. 2) The unknown electronic spin $S$ is an integer. 3) Since the magnetic hyperfine fields saturate already in weak applied fields, the lowest electronic spin levels are two closely spaced states of energy separation $\Delta$ (we found $\Delta = 0.35$ cm$^{-1}$).

Analysis of the data reveals that the two equivalent iron sites of Doublet I remain indistinguishable in strong applied fields, i.e. the spectrum in Fig. 3 is a superposition of two spectra with an intensity ratio of 2:1. Furthermore, the iron nucleus associated with Site II experiences a positive internal magnetic field, proving this nucleus to be a member of a spin-coupled structure. The line assignments of the spectrum in Fig. 3 were facilitated by comparing the spectra of Fd II with those of the $[S_3MoS_2Fe(PhS)_2]^-$ anion presently under study in our laboratory (in cooperation with Dr. B. A. Averill). The

Fig. 3. Mössbauer spectrum of reduced Fd II recorded at 4.2 K in a magnetic field of 10 kG applied parallel to the observed $\gamma$-radiation. Shown also is a theoretical curve and a decomposition of the spectrum into two subcomponents with intensities 2:1. The subcomponent corresponding to Site I is traced by the solid line. For Site II, a positive internal magnetic field is observed. For convenience, the spectra were computed from a spin Hamiltonian with $S = 2$. 

FIG. 2. Mössbauer spectrum of oxidized Fd II taken at 1.5 K in a field of 600 G applied parallel to the observed $\gamma$-radiation. The solid line plotted over the data is a superposition of three spectra computed from Equation 1 with the parameters quoted in the text.
values for $\Delta E_q$ and $\delta$ of the latter compound match those found for Site I; the high field spectra have a close resemblance also. The electronic system has an easy axis of magnetization and in applied fields up to 10 kG the Mössbauer spectra of Sites I and II are characterized by internal fields of $-237$ kG and $+250$ kG, respectively. In stronger fields, anisotropies of the magnetic hyperfine interactions become apparent. The results of spectral simulations are shown in Fig. 3; also shown is a decomposition of the spectrum into subcomponents. Although the fits to the data are excellent, the ambiguity commonly associated with multiparameter fits requires more detailed studies. At present, the main result is the fact that the magnetic behavior of the iron sites is describable by a common electronic spin, i.e. a spin-coupled cluster is strongly suggested.

**DISCUSSION**

In the following, we will review the main evidence in support of a spin-coupled cluster containing 3 iron atoms. The present preparations consistently yield 3 iron atoms/monomer (or 12 iron atoms/holo-protein). Our studies show that the material is pure; there is no evidence for iron impurities from either EPR or Mössbauer spectroscopy. The magnetic Mössbauer spectra of oxidized Fd I1 observed at $4.2$ K show three distinct iron sites and they attest that the sites belong to EPR-active centers. The EPR data reveal the presence of only one type of paramagnetic center; a spin quintuplet yields 0.9 spins/3 iron atoms. Taken together, the data show that 3 iron atoms belong to the EPR-active center.

Upon reduction by 1 electron, the center becomes EPR-silent (7). The Mössbauer data reveal that reduced Fd II is paramagnetic, i.e. the electronic ground state of the clusters has $S > 0$. The Mössbauer spectra demonstrate two distinct iron environments which are present in the ratio of 2:1. The features of the magnetic Mössbauer spectra and their response to applied magnetic fields suggest a common electronic spin, i.e. the sites are subsites of a spin-coupled cluster. Spin coupling is indicated by the observation of positive and negative magnetic hyperfine fields. Thus, both the oxidized and the reduced states independently yield evidence for a three-iron center.

The observed isomeric shifts suggest that the irons have tetrahedral environments of sulfur atoms. This, however, does not rule out the possibility that a site might have one oxygenic or nitrogenous ligand; no model complexes are available for comparisons. It is interesting to note that the three irons are distinguishable in the oxidized state of the cluster (there are three distinct Mössbauer spectra at 4.2 K). In the reduced state, however, 2 iron atoms (the ones yielding Doublet I) are indistinguishable even in strong applied fields. The increased isomeric shift and the quadruple splitting suggest that the 2 iron atoms of Doublet I share the electron that enters the complex upon reduction; both sites are roughly at the oxidation level Fe$^{2+}$. It is noteworthy that the Fd II spectroscopically resembles closely the *Azotobacter ferredoxin*; yet, the midpoint potentials of the three-iron centers differ by 280 mV.

Besides the tetrameric Fd II, the basic subunit can form a trimeric protein, termed Fd I (7). EPR studies of reduced Fd I have elicited signals qualitatively similar to those observed for reduced Fe$_3$Si centers (6). A Fd I sample appropriate for Mössbauer studies is anticipated with considerable interest.

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Note Added in Proof—We have studied a Fd I sample with EPR and Mössbauer spectroscopy. A typical EPR signal with principal $g$ values at $g = 1.92$, $g = 1.94$, and $g = 2.07$. This species yields at 4.2 K a magnetic Mössbauer spectrum almost identical to that observed for the reduced ferredoxin from *Bacillus stearothermophilus* (11), a protein with an established Fe& center. Upon oxidation, the EPR signal vanishes with a concomitant change of the magnetic Mössbauer spectrum into a quadrupole pattern. High field studies prove that this pattern results from iron atoms in diamagnetic sites. Thus, a Fe$_3$Si center is strongly implicated. We found that approximately 30% of the iron was present as a three-iron center. Chromatographically, however, the sample was homogeneous (Fd I and Fd II are easily separated).

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