Direct Spectroscopic Evidence for the Presence of a 6Fe Cluster in an Iron-Sulfur Protein Isolated from Desulfovibrio desulfuricans (ATCC 27774)*

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A novel iron-sulfur protein was purified from the extract of Desulfovibrio desulfuricans (ATCC 27774) to homogeneity as judged by polyacrylamide gel electrophoresis. The purified protein is a monomer of 57 kDa molecular mass. It contains comparable amounts of iron and inorganic labile sulfur atoms and exhibits an optical spectrum typical of iron-sulfur proteins with maxima at 400, 305, and 280 nm. Mössbauer data of the as-isolated protein show two spectral components, a paramagnetic and a diamagnetic, of equal intensity. Detailed analysis of the paramagnetic component reveals six distinct antiferromagnetically coupled iron sites, providing direct spectroscopic evidence for the presence of a 6Fe cluster in this newly purified protein. One of the iron sites exhibits parameters (ΔEQ = 2.67 ± 0.03 mm/s and δ = 1.09 ± 0.02 mm/s at 140 K) typical for high spin ferrous ion; the observed large isomer shift indicates an iron environment that is distinct from the tetrahedral sulfur coordination commonly observed for the iron atoms in iron-sulfur clusters and is consistent with a penta- or hexacoordination containing N and/or O ligands. The other five iron sites are most probably high spin ferric. Three of them show parameters characteristic for tetrahedral sulfur coordination. In correlation with the EPR spectrum of the as-purified protein which shows a resonance signal at g = 15.3 and a group of signals between g = 9.8 and 5.4, this 6Fe cluster is assigned to an unusual spin state of 9/2 with zero field splitting parameters D = −1.3 cm−1 and E/D = 0.062. Other EPR signals attributable to minor impurities are also observed at the g = 4.3 and 2.0 regions. The diamagnetic Mössbauer component represents a second iron cluster, which, upon reduction with dithionite, displays an intense S = 1/2 EPR signal with g values at 2.00, 1.83, and 1.31. In addition, an EPR signal of the S = 3/2 type is also observed for the dithionite-reduced protein.

Iron-sulfur proteins are a class of proteins containing prosthetic groups composed of iron and sulfur atoms. They play a diversified functional role in biological systems and are involved in electron transfer as well as in direct interaction with substrates (1–5). Extensive spectroscopic investigations have been performed on a variety of iron-sulfur proteins, and a wealth of information has emerged pertaining to the physical properties of the iron-sulfur centers (1–11). Earlier studies have established the presence of four basic structures for the iron-sulfur centers: the FeS4 center, the [2Fe-2S]2+, the [3Fe-4S]3+, and the [4Fe-4S] clusters. A common structural feature shared by these four centers is that each iron atom is tetrahedrally coordinated by four sulfur atoms. With the exception of the FeS4 center found in rubredoxin, in which the iron atom is coordinated by four cysteinyl sulfur atoms (12), all of the other cores contain bridging inorganic sulfur ligands. The terminal ligands for the clusters are cysteinyl sulfur atoms (13–17). Possible distortion from the tetrahedral arrangement caused by structural constraints has been suggested for the FeS4 center found in desulfoflexin (18). Facile interconversion between [4Fe-4S] cluster and [3Fe-4S] cluster has been reported (19–21), and activation of aerobically purified aconitase has been shown to involve the interconversion of [3Fe-4S] cluster into [4Fe-4S] cluster (22, 23).

The [2Fe-2S] cluster can be stabilized in two oxidation states, 2+ and 1+. In the oxidized [2Fe-2S]2+ state, both iron atoms are high spin ferric (S = 5/2) and are antiferromagnetically coupled to form a diamagnetic state. Upon one electron reduction, a localized mixed valent Fe(II)-Fe(III) cluster is formed with a system spin of 1/2 resulting from the antiferromagnetic coupling between the ferrous (S = 2) and ferric (S = 5/2) ions (24, 25).

The [3Fe-4S] cluster can also be stabilized in two oxidation states, 1+ and 0. The [3Fe-4S]1+ cluster contains three high spin ferric ions spin coupled to form a S = 1/2 state (26–28). In the one electron reduced [3Fe-4S]0 cluster, the additional electron is shared by two iron atoms forming a delocalized pair [2Fe2,5+] and a high spin ferric site. Spectroscopic evidence is consistent with the delocalized pair having a spin of...
9/2 coupled to the high spin ferric ion forming a cluster spin of 2 (29).

Three stable oxidation states, 3+, 2+, and 1+, have been observed for the [4Fe-4S] cluster. The 3+ and 2+ states are stable in high potential iron-sulfur proteins whereas the 2+ and 1+ states are detected for bacterial ferredoxins and other 4Fe cluster-containing proteins (30). Both the 3+ and 1+ states are paramagnetic, having a cluster spin of 1/2. The 2+ state is diamagnetic. In all three states, valence delocalized pairs are observed. Theoretical investigations indicate that the concept of valence localization, as well as delocalization, is important for the understanding of the iron-sulfur core electronic states (31, 32).

More recent investigations on iron-sulfur proteins and model compounds (8), however, indicate that the above mentioned physical properties constitute only a limited understanding of iron-sulfur clusters, which in fact are susceptible to ligand and metal substitutions and are flexible in their electronic properties and structures. The presence of noncysteinyl ligand has long been suggested for the [2Fe-2S] cluster in the Rieske iron-sulfur protein (33, 34). The [4Fe-4S]1+ cluster of the reduced iron protein of the nitrogenase system exists in two electronic ground states with spins 1/2 and 3/2, and the relative population of these two states can be altered by solvent (35, 36). In addition to the S = 1/2 and 3/2 states, binding of Mg-ATP to the iron protein induces a small replacement of the inorganic sulfur of the [4Fe-4S] core of certain ferredoxins by selenium generates clusters having ground spin states of 1/2, 3/2, and 7/2 (37-39). Ground spin states of 5/2 and 7/2 have also been observed for the oxidized P cluster in the MoFe proteins of the nitrogenase system under different oxidizing conditions (40-44). The P cluster has been characterized as a [4Fe-4S] cluster in disguise (44) or a 8Fe cluster (41). An iron-sulfur cluster containing 6 iron atoms has also been suggested for a novel protein isolated from Desulfovibrio vulgaris (Hildenborough) (45) and for certain hydrogenases (46, 47).

Incorporation of other metals, M, into the [3Fe-4S] core forming mixed metal clusters of the type [M, 3Fe-4S] introduces localized and delocalized valence resulting in different spin states. For example, increasing d-electron occupancy of the [3Fe-4S]1+ cluster [Co, 3Fe-4S]1+ (S = 1/2, S = 1), [Ni, 3Fe-4S]1+ (S = 3/2) and [Zn, 3Fe-4S]1+ (S = 5/2) (48-50). Naturally occurring mixed metal clusters have also been detected in proteins, such as the [Zn, Fe] cluster in the red kidney bean purpl phosphatase (51) and the M center (a putative [Mo, 6Fe] cluster) in the MoFe protein (52).

This manuscript reports the purification and characterization of a novel iron-sulfur protein isolated from Desulfovibrio desulfuricans (ATCC 27774) and presents spectroscopic evidence for the presence of a 6 iron-containing cluster. The spectroscopic data of the native protein suggest a 9/2 spin state for this Fe6 cluster. The cluster is redox active and can be stabilized in multiple oxidation states. The presence of a second iron cluster in this protein is also indicated from this study.

**EXPERIMENTAL PROCEDURES**

**Growth of Microorganism—**D. desulfuricans (ATCC 27774) was grown in the medium described by Liu and Peck (53). Nitrate rather than sulfate was used as a terminal electron acceptor to promote the production of nitrite reductase and avoid the precipitation of iron sulfide. For the growth of isotopically labeled cells, 200 mg of 57Fe (95% plus enrichment, Du Pont-New England Nuclear) was first dissolved in H2SO4, then in HCl, neutralized, and added to 400 ml of media.

**Preparation of Bacterial Extract—**In a typical preparation 800 g of cells was suspended in 10 mm Tris-HCl, pH 7.6, and ruptured in a French press at 9,000 psi. The extract was centrifuged at 19,000 x g for 30 min and then at 180,000 x g for 75 min.

**Protein Purification—**All purification procedures were performed at 4 °C. An amount of 1.5,000 ml of the crude extract was loaded on to a DEAEP-52 column (6 x 32.5 cm) equilibrated with 10 mm Tris-HCl buffer. A linear gradient (0.01-0.5 M) was applied with a total volume of 1 liter. A fraction containing mainly cytochromes was eluted between 0.1 and 0.2 M and was collected in a final volume of 1 liter. The fraction was dialyzed against distilled water overnight and loaded on to another DEAEP-52 column (4 x 30 cm) equilibrated with 10 mm Tris-HCl. A linear Tris-HCl gradient (0.01-0.2 M) was applied with a total volume of 3 liters. A fraction containing cytochromes and a brown protein was collected at an ionic strength of 0.15 M Tris-HCl, concentrated in a Diaflo apparatus with a YM3 membrane and loaded on to a hydroxypatite column (2.5 x 15 cm) equilibrated at the same ionic strength. A linear phosphate gradient (0.001-0.20 M) was applied, and a brown fraction eluted at 80 mm was collected in a total volume of 100 ml. At this stage the protein already shows a distinct visible spectrum resembling that of an iron-sulfur protein. The A600/A690 ratio was 0.24. The fraction was concentrated on a Diaflo apparatus with a YM3 membrane, and its ionic strength was decreased by adding distilled water during concentration. The last step of purification was performed by high performance liquid chromatography (Beckman) on a protein-Pack DEAE SPW (Waters). A linear gradient of 0.01-0.2 M NaCl in 0.01 M phosphate buffer (pH 7) was applied and a brown phosphate fraction was collected when the protein was detected by the Beckman high performance analyzer system 6300 E. The values of threonine, serine, and tyrosine were corrected after extrapolation to zero time hydrolysis. Cysteine and methionine were analyzed after performic acid oxidation as cysteic acid and methionine sulfone, respectively (59). The purified protein was subjected to sequential Edman degradation on an automatic sequenator (Applied Biosystem Analyzer), and the NH2-terminal sequence of the first 36 amino acid residues was determined.

**Electrophoresis—**Polyacrylamide disc electrophoresis was used to establish the protein purity (60). The molecular weight of the purified protein was determined by gel electrophoresis using the Bio-Rad low molecular weight kit as standard for calibration and by gel filtration on a G-50 Sephadex column, according to Whitaker (61), using the following as standards: bovine serum albumin (67,000), ovalbumin (45,000), trypsin (24,000), and horse heart cytochrome c (13,000).

**Spectroscopic Methods—**The UV-visible absorption data were recorded on a Shimadzu UV-265 split-beam spectrophotometer using 1-cm quartz cells. Electron paramagnetic resonance (EPR) measurements were performed on a Bruker EPR 200-SRC spectrometer equipped with an Oxford Instrument continuous flow cryostat. Mossbauer spectra were recorded on either a strong-field or a weak-field spectrometer operating in a constant acceleration mode in a transmission geometry. The zero velocity of the Mossbauer spectra are referred to the centroid of the room temperature spectrum of a metallic iron foil.

**Materials—**Hydroxypatite and low molecular weight protein standards were purchased from Bio-Rad. DEAE-52 was purchased from Whatman. All other chemicals were of reagent grade or the highest available purity.

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''The abbreviations used are: SDS, sodium dodecyl sulfate; T, tesla.
Blue R-250. Similar results are also observed on a 12.5% SDS-polyacrylamide gel electrophoresis slab gel. Both the gel filtration method and the SDS-gel electrophoresis yield the same molecular mass, 57 kDa, for the purified protein, indicating that the protein molecule is a monomer.

Fig. 1 shows an optical absorption spectrum of the as-isolated protein which exhibits maxima at 400, 305, and 280 nm, typical of an iron-sulfur protein. The molar extinction coefficient at 400 nm is determined to be within the range of 25-34/cm/mM using protein determination based on the Lowry method. The amino acid composition of this iron-sulfur protein is tabulated in Table I, and its NH2-terminal sequence of the first 36 amino acid residues is presented in Fig. 2. Consistent with the following spectroscopic data, which indicate that this protein may contain two atypical iron-sulfur clusters, the cysteine-rich (11-12 residues) amino acid composition suggests a capacity for binding of two clusters. Since normal sequencing procedure cannot identify cysteine residues, the three unidentified residues in the sequence are most probably cysteines. It is interesting to point out that the position suggests a capacity for binding of two clusters. Since rified iron-sulfur protein from D. desulfuricans is determined to be within the range of 4.7/cm/mM Fe whereas it varies in a large range from 25 to 34 cm/mM protein. This observation could also, of course, simply indicate the difficulty and large uncertainty involved in protein determination. Sulfur analysis in correlation with protein determination using the Lowry method produces a value of 6 ± 1 labile sulfur atoms/protein molecule of 57 kDa.

Mössbauer Data of the As-isolated Protein—Mössbauer spectra of this new iron-sulfur protein in the as-isolated form are shown in Fig. 3. Spectrum A is recorded at 140 K in the absence of a magnetic field. Spectrum B is recorded at 1.5 K with a magnetic field of 50 mT applied parallel to the γ-beam, and spectrum C is at 4.2 K with a parallel field of 3 T. The low temperature spectra will be discussed first. In spectrum B, two spectral components with approximately equal intensity are observed: a central quadrupole doublet (marked by a bracket) and a magnetic spectrum with well resolved peaks extending from -7.0 to +8.0 mm/s. For the central quadrupole doublet, the quadrupole splitting (\(\Delta E_Q\)) and isomer shift (\(\delta\)) are 1.20 ± 0.03 mm/s and 0.46 ± 0.02 mm/s, respectively. Although these parameters are indicative of high spin ferric ions (\(S = 5/2\)), spectra recorded at strong applied field (e.g. Fig. 3C) indicate that this doublet is originating from a diamagnetic species (\(S = 0\)), suggesting that this species is probably an iron cluster (i.e. the paramagnetic iron ions are antiferromagnetically coupled to form a diamagnetic cluster). The nature of this cluster will be discussed at the end of this section. For the magnetic spectral component, well resolved hyperfine peaks are observed, and a wealth of useful infor-

### Table I

| Amino acid composition of the newly isolated iron-sulfur protein from D. desulfuricans (ATCC 27774) |
| Calculation is based upon a molecular mass of 58 kDa. |
|---|
| Asp 56 | Ile 26 |
| Thr 31 | Leu 55 |
| Ser 28 | Tyr 15 |
| Glu 47 | Phe 16 |
| Gly 54 | His 11 |
| Ala 60 | Lys 38-39 |
| Cys 11-12 | Arg 14 |
| Val 38 | Pro 18 |
| Met 13 |
| Total 531-533 |

### Table II

| No. of iron atoms/molecule determined for the novel iron-sulfur protein from D. desulfuricans (ATCC 27774) |
|---|
| \(5^{77}\)Fe-enriched preparations |
| Lowry | Amino acid |
| 1 | 6.9 |
| 2 | 6.1 |
| 3 | 6.6 |
| 4 | 4.8 |
| Unenriched preparations |
| 1 | 6.9 |
| 2 | 8.3 |
| 3 | 7.0 |
| 4 | 6.8 |
| 5 | 5.5 |
| Total 531-533 |

Fig. 1. Optical absorption spectrum of the as-isolated new iron-sulfur protein from D. desulfuricans (ATCC 27774).

Fig. 2. NH2-terminal amino acid sequence of the newly purified iron-sulfur protein from D. desulfuricans (ATCC 27774).
Mössbauer spectra of the as-isolated new iron-sulfur protein from *D. desulfuricans* (ATCC 27774). The data were recorded at 140 K in the absence of a magnetic field (spectrum A), at 1.5 K in parallel applied field of 50 mT (spectrum B), and at 4.2 K in parallel field of 3 T (spectrum C). The solid line in A is a least squares fit to the data, assuming three quadrupole doublets, the solid lines plotted in B and C are spectral simulations of the paramagnetic 6Fe cluster using the parameters listed in Table III. The simulated spectra in B and C are normalized to 48% of the total iron absorption.

Fig. 3.

**FIG. 3.** Mössbauer spectra of the as-isolated new iron-sulfur protein from *D. desulfuricans* (ATCC 27774). The data were recorded at 140 K in the absence of a magnetic field (spectrum A), at 1.5 K in parallel applied field of 50 mT (spectrum B), and at 4.2 K in parallel field of 3 T (spectrum C). The solid line in A is a least squares fit to the data, assuming three quadrupole doublets, the solid lines plotted in B and C are spectral simulations of the paramagnetic 6Fe cluster using the parameters listed in Table III. The simulated spectra in B and C are normalized to 48% of the total iron absorption.

mation can be readily deduced from the spectrum. First, the observed magnetic splitting is large (the total splitting is ~15 mm/s, corresponding to an effective field of 46.6 T) even though the external applied field is small (50 mT), suggesting that the spectrum is originating from a half-integer spin system. Second, the spectrum is independent of the direction of the applied field with respect to the γ-beam, indicating that it arises from a uniaxial system \((g_{||} > g_{\perp}).\) These properties are consistent with a ground Kramers doublet of a half-integer spin system with negative zero-field splitting parameter \((D < 0)\) or \(E/D = 1/3.\) A similar type of spectrum has also been observed for the thionine-oxidized P cluster in the MoFe protein of nitrogenase (44, 63). The electronic spectrum of the oxidized P cluster has been characterized as \(S = 5/2,\) \(D \approx -10 \text{ cm}^{-1}\) and \(E/D \approx 0\) (44). Third, the Mössbauer spectrum of an iron atom associated with a uniaxial system is a simple six-line pattern with intensity ratio of 3:2:1:1:2:3 (if \(\Delta E_Q \approx 0\)). Since the magnetic spectral component contains more than absorption peaks, it must be associated with a multiron cluster. Furthermore, since the spectrum for each iron site is a simple six-line pattern with well defined intensity ratio, it is possible to estimate the minimum number of iron sites in the cluster. We began by first estimating the absorption intensity of the peak at ~7 mm/s and found that it corresponds to 2.0% of the total absorption. Because this peak is the outermost line of a six-line spectrum, the total intensity of the spectrum should be ~8%, which should correspond to one iron site. Because the total absorption of the magnetic component is approximately 50%, it follows then that the magnetic component is probably originating from a cluster containing six iron atoms. Another alternative would be that the magnetic component is originating from two clusters each containing three iron sites. High temperature data (Fig. 3A), however, suggest that this possibility is rather unlikely.

Fig. 3A shows a 140 K spectrum of the as-isolated iron-sulfur protein recorded in the absence of an external field. At this temperature the electronic relaxation time is fast, and the magnetic component observed at low temperature collapses into quadrupole doublets. Most of the doublets of this magnetic 6Fe cluster overlap with the central doublet detected at 1.5 K and cannot be resolved, indicating that most of the iron sites of the cluster are probably high spin ferric. However, a resolved peak is observed at ~2.44 mm/s. Least squares fit of the spectrum with three quadrupole doublets indicates that this peak is the high energy line of a quadrupole doublet with \(\Delta E_Q = 2.67 \pm 0.03 \text{ mm/s}\) and \(\delta = 1.09 \pm 0.02 \text{ mm/s},\) characteristic of a high spin ferrous iron. The observed large isomer shift indicates an iron environment distinct from the tetrahedral FeS₄ structure and is consistent with a penta- or hexacoordination containing N and/or O ligands. The absorption intensity of this doublet is determined to be 8 ± 1.4%. Parameters for the other two doublets at the central portion of the spectrum are: \(\Delta E_Q = 1.09 \pm 0.04 \text{ mm/s}\) and \(\delta = 0.42 \pm 0.02 \text{ mm/s}\) for doublet 1 and \(\Delta E_Q = 0.90 \pm 0.04 \text{ mm/s}\) and \(\delta = 0.21 \pm 0.02 \text{ mm/s}\) for doublet 2. Their absorption intensities are 64 ± 3% and 28 ± 2% for doublets 1 and 2, respectively. Doublet 1 has parameters that are similar to those of the central quadrupole doublet observed at 1.5 K and is therefore attributed to the diamagnetic cluster. However, the intensity observed for doublet 1 is larger than 50%, indicating that a part of the paramagnetic cluster also contributes to this doublet. Doublet 2 and the ferrous doublet are attributed to the paramagnetic cluster.² Because the high energy line of the ferrous doublet is well resolved, the absorption intensity of the ferrous doublet can be determined reliably using this resolved line. Most interestingly, the absorption intensity of the ferrous doublet is estimated to be 8%, identical to that of a single iron site estimated from the low temperature spectrum. If the low temperature magnetic component is originating from two similar clusters each containing three iron atoms, the intensity of the ferrous doublet would be 16% (supposedly a result of two ferrous sites, one for each cluster). Also, one ferrous and two ferric ions cannot couple to form a half-integer spin system. Consequently, by correlating the information obtained from Mössbauer spectra recorded at different temperatures, it is concluded that the magnetic cluster (tentatively labeled as cluster I) is most likely to contain six iron atoms: five ferric and one ferrous ions with the ferrous ion having N and/or O ligands.

² It is important to point out that the central portion of this high temperature spectrum consists of overlapping unresolved quadrupole doublets. Our least squares fit of assuming two doublets for this part of the spectrum is a simple approximation for the data and should not be taken as a unique solution. Nevertheless, it is interesting to note that the intensity and isomer shift determined for doublet 2 are consistent with at least three iron sites in this paramagnetic 6Fe cluster having tetrahedral sulfur coordination and the other three having nitrogen/oxygen ligands. The presence of three iron sites with N/O ligands is also supported by the high temperature Mössbauer data of dithionite-reduced samples, which show three high spin ferrous sites with \(\delta\) larger than 1 mm/s.
have been performed by using the following spin Hamiltonian.

\[ H = D S^2 - \frac{S(S + 1)}{3} \]

\[ + \frac{E}{D} (S^2 - S^2) \]

\[ + \frac{eQv}{4} (I^2 - I(I + 1)/3 + \frac{7}{3}I^2 - I^2) - e\delta \vec{A} \cdot \vec{H} + \delta S \cdot \vec{A} \cdot \vec{I} \]

\[ \text{(1)} \]

The magnetic component is treated as a superposition of six spectral components, each corresponding to an iron site. All six sites share the same electronic state (i.e., the same \( D, E/D, \) and \( S \)) but have different magnetic hyperfine coupling \( A \) tensors and electric field gradient \( V \) tensors. Based on the EPR data presented below, we have assigned a spin \( S = 9/2 \) and an \( E/D = 0.062 \) for this magnetic cluster. However, it is important to note that the major conclusion discussed above (i.e., the magnetic component representing a 6Fe cluster) does not depend on the specific value of \( S \) as long as it is a half-integer larger or equal to 3/2. Once the spin and \( E/D \) values are assigned, the magnetic hyperfine \( A \) values can be determined from the 50 mT spectrum, and the value of \( D \) can be evaluated from the strong field spectra. The values of \( \Delta E_q \) and \( \delta \) are estimated from the high temperature data. Consequently, analysis of the magnetic component basically becomes assigning proper partitions of the well resolved peaks to the respective iron sites. After a series of educated guesses and trial simulations we were able to decompose the magnetic component into six spectral components successfully. The solid lines plotted in Fig. 3, B and C, are the superpositions of spectral simulations of the six iron sites using the parameters listed in Table III. Each theoretical spectrum is normalized to 48% of the total iron absorption. Good agreement between theory and experiment is observed, demonstrating that the data are consistent with the assumption that (i) each iron site contributes to 8% of the total absorption, and (ii) these iron sites share the same electronic state (i.e., the iron sites belonging to one cluster). Of particular interest are the opposite signs of the \( A \) values observed for the iron sites. This observation supports the anisotropic nature of this spin-coupled 6Fe cluster.

To show the high resolution of the magnetic component and to illustrate better the theoretical assignments for the six iron sites, we have removed the contribution of the diamagnetic component from the raw data (spectra B and C shown in Fig. 3) and plotted the resulting spectra in Fig. 4 (spectra A and B) along with the theoretical simulations for the magnetic component (solid lines plotted over the experimental data). Good agreement between experiment and theory is observed for both weak and strong field spectra. Simulations for each iron site at a parallel applied field of 50 mT are shown on top of spectrum A. It is important to comment that except for the sixth iron site, the assignment for the other five iron sites is determined with justifiable certainty. With the well resolved absorption peaks detected in the low temperature spectra and the isomer shifts determined from the high temperature data, the assignment for the iron sites 1–3 is rather straightforward. After the assignment for the first three sites is made, there are only four ways for pairing the remaining two sets of \( \Delta E_q \) and \( \delta \) values with the two unsigned six-line spectra. The assignment presented here is the only way that agrees with both the weak and strong field spectra, and one of the iron sites, namely, site 4, has to be assigned with a large \( \delta \) value consistent with that of a high spin ferrous iron. It is also important, however, to point out that the spectral component of the sixth iron site is unresolved from the central quadrupole doublet and that the presence of a sixth site is based on the intensity analysis presented in the previous paragraphs.

As mentioned in the beginning of this section, the central quadrupole doublet observed in Fig. 3B originates from a diamagnetic iron cluster. Consequently, the central doublet and the magnetic component could represent two different iron clusters or, alternatively, the same cluster at different oxidation states. Based on the following arguments, the former explanation appears to be more plausible, and the second cluster is labeled as cluster II.

Assuming that there exists only one cluster/molecule and that the two Mössbauer spectral components represent two different oxidation states of the same cluster, then the as-

### Table III

| Iron site | \( \Delta E_q \) | \( \delta \) | \( \gamma \) | \( A/\gamma d \delta \) (T) |
|-----------|----------------|-------|------|-------------------|
| 1         | -1.0           | 0.40  | 8.5  | -10.0             |
| 2         | -1.0           | 0.40  | 3.0  | -7.8              |
| 3         | -1.0           | 0.27  | 3.0  | +7.0              |
| 4         | +2.67          | 1.15  | 5.0  | +5.5*             |
| 5         | -1.0           | 0.27  | 0    | -4.0              |
| 6         | -1.0           | 0.27  | 0    | -1.5              |

*The magnetic hyperfine \( A \) tensor for a high spin ferrous ion is generally anisotropic. In this analysis, however, an isotropic \( A \) tensor is assumed for the ferrous site because the system is uniaxial, and the spectrum is sensitive only to the \( A \) value along the uniaxis.

**Fig. 4. Mössbauer spectra of the paramagnetic 6Fe cluster.**

The spectra are prepared from the raw data shown in Fig. 3 (see text). The experimental conditions are A, 1.5 K with a parallel field of 50 mT; and B, 4.2 K with a parallel field of 3 T. For comparison, spectral simulations for the 6Fe cluster are plotted as solid lines over the experimental data. Simulated 1.5 K spectra for the six individual iron sites at an applied field of 50 mT are shown on top of spectrum A. These spectra are plotted in a descending order with respect to increasing absolute value of the magnetic hyperfine coupling constant \( A \). The iron sites are labeled in an order corresponding to decreasing absolute value of \( A \) (see Table II).
purified protein must be at a potential close to the midpoint redox potential of the cluster since the absorption intensities of the two components are equal. At this region a small variation of the potential can cause a substantial change in the relative concentration of the two states. A shift of 60 mV away from the midpoint potential would change the relative concentration from 1 to 0.1 according to a simple Nernst behavior. Preliminary Mössbauer measurements of samples poised at different redox potentials indicate that this is not the case and are in support of the presence of two distinct clusters.

EPR Spectra—Fig. 5 shows EPR spectra of the iron-sulfur protein in its as-isolated form recorded at different temperatures with a microwave power of 2 milliwatts. The spectra show (i) a resonance peak at \( g = 15.3 \); (iii) a group of resonances in the region between \( g = 9.8 \) and \( g = 5.4 \); (iii) a signal at \( g = 4.3 \); and (iv) several signals in the \( g = 2 \) region. The signal at \( g = 4.3 \) is typical for adventitiously bound ferric ions and may represent a minor impurity in the sample. The signals at the \( g = 2 \) region are most probably originating from \( S = 1/2 \) species. At least two sets of \( g \) values can be identified in this region because their temperature-dependent relaxation behaviors are different. At temperatures above 25 K, one set of \( g \) values at 2.02, 1.98, and 1.95 is detected (spectrum D), and at lower temperatures an additional set of signals at \( g = 1.97, 1.94, \) and 1.90 is observed. Spin quantification of these signals amounts to not more than 0.07 spin/molecule. Such \( S = 1/2 \) species are expected to yield magnetic Mössbauer spectra that are strongly dependent on the direction of the applied field. The fact that no such spectra are detected in the Mössbauer measurements is consistent with these species being present in minute quantities. Since the Mössbauer measurements do indicate the presence of a magnetic component correlating to a half-integer spin system, it is reasonable to assume that the other EPR resonances are associated with the magnetic 6Fe cluster. For a half-integer spin system, the smallest spin value that could yield a \( g \) value of 15.3 is \( S = 9/2 \). This spin system forms five Kramers doublets, and their principal \( g \) values can be calculated using the first two terms of Equation 1 assuming an isotropic \( g \) tensor of 2.0. Table IV lists the calculated principal \( g \) values and the energies for all five Kramers doublets using zero-field parameters \( D = -1.3 \text{ cm}^{-1} \) obtained from the Mössbauer analysis and \( E/D = 0.062 \). According to this analysis, the \( g = 15.3 \) signal could be arising from the highest doublet.

### Table IV

| Doublet | Energy (cm\(^{-1}\)) | \( g_x \) | \( g_y \) | \( g_z \) |
|---------|---------------------|-------|-------|-------|
| 1       | 0.0                 | 0.0   | 0.0   | 18.0  |
| 2       | 10.4                | 0.01  | 0.01  | 13.97 |
| 3       | 18.1                | 0.54  | 0.51  | 9.89  |
| 4       | 23.1                | 5.73  | 5.80  | 5.22  |
| 5       | 26.4                | 3.72  | 15.30 | 1.31  |

Fig. 5. EPR spectra of the as-isolated iron-sulfur protein from \( D. \) desulfuricans (ATCC 27774). The spectra are recorded at temperatures of 4.5 K (A), 7.5 K (B), 16 K (C), and 30 K (D). Other experimental conditions are: microwave frequency, 9.46 MHz; microwave power, 2 milliwatts; modulation amplitude, 1 mT; receiver gain, \( 1.6 \times 10^6 \) for A and \( 1.0 \times 10^6 \) for the other spectra.

Fig. 6. EPR spectra of the dithionite-reduced iron-sulfur protein from \( D. \) desulfuricans (ATCC 27774). The spectra are recorded at temperatures of 4.5 K (A), 7.5 K (B), 16 K (C), and 30 K (D). Other experimental conditions are: microwave frequency, 9.46 MHz; microwave power, 2 milliwatts; modulation amplitude, 1 mT; receiver gain, \( 4.0 \times 10^6 \).
transition probability is almost nil. The temperature depend-
ance from an excited state.3

tions or by the situation.

onite or by

the EPR data of the most reduced protein are presented. Fig. 6

can exist in two. The redox data are extremely complicated

sulfuricans and have not yet been completely analyzed. Here, only the

EPR spectrum. A preliminary redox titration study monitored

atmosphere, and the reduced protein exhibits an interesting

In correlation with the EPR data, this 6Fe cluster in the as-

isolated protein is assigned to an unusual spin state of 9/2. One of the iron atoms of this cluster is high spin ferrous and

exhibits Mössbauer parameters indicative of N and/or O

ligation. A second iron cluster is also implicated in the Möss-

bauer data. This cluster is diamagnetic in the as-isolated protein. Upon reduction with dithionite, it exhibits an intense

S = 1/2 EPR signal with g values similar to those of a [6Fe-

6S] prismane cluster. An additional EPR signal of a spin 3/2

system is also observed. The origin of this EPR signal is
currently unclear.

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