Mössbauer and EPR Evidence for an All-Ferrous Fe$_4$S$_4$ Cluster with $S = 4$ in the Fe Protein of Nitrogenase

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The ability to fix dinitrogen is restricted to a small but diverse group of procaryotes that contain the nitrogenase system. This system is composed of two proteins called the Fe protein and the MoFe protein; the latter contains the site of N$_2$ binding and reduction.[1,2] The Fe protein contains a single Fe$_4$S$_4$ cluster bridged between two identical subunits each of which has a single binding site for MgATP. It is the only known reductant capable of reducing the MoFe protein such that the latter can reduce substrates. Our current understanding of nitrogenase is based on in vitro experiments with purified nitrogenase proteins using *dithionite* as the electron donor. Under these conditions the Fe$_4$S$_4$ cluster shuttles between the 2+ and 1+ oxidation states.

In 1994, Watt and Reddy[3] reported that the [Fe$_4$S$_4$]$^{1+}$ Fe protein could be reversibly reduced ($E^{\circ\prime} = -460$ mV vs SHE) to the all-ferrous [Fe$_4$S$_4$]^0 state. This reaction was reported to be pH independent between pH 7.0 and 8.0. [Fe$_4$S$_4$]^0 could be produced using methylviologen, Ti(III)citrate, or the physiological electron donor flavodoxin as reductants but could not be generated when dithionite was used as a reductant.[1,3,4] The proposal for the formation of an [Fe$_4$S$_4$]^0 state was based on two main lines of evidence.[3] First, Coulometric reduction of the [Fe$_4$S$_4$]$^{1+}$ state of the Fe protein showed that a second electron could be added to the protein but did not show whether the reduction was metal-centered.[5] Second, the two-electron reduced Fe protein did not exhibit the $S = \frac{1}{2}$ EPR signal that arises from the [Fe$_4$S$_4$]$^{1+}$ state, but that signal could be elicited by adding 1 equiv of oxidant to the fully reduced protein. These observations suggest, but do not prove, the unprecedented formation[6] of a protein-bound all-ferrous Fe$_4$S$_4$ cluster.

For the Mössbauer and EPR studies we produced the two-electron reduced form of the Fe protein by treating ^{57}Fe-enriched *Azotobacter vinelandii* Fe protein (Av2) with Ti(III) citrate.[8] Figure 1 shows two Mössbauer spectra of Ti(III) citrate-reduced Av2. The zero-field spectra exhibit quadrupole doublets down to 4.2 K. Preliminary analysis of the whole data set (30 spectra) revealed four distinct sites, all with isomeric shift $\delta = 0.68$ mm/s. The simulation of Figure 1A assumes four doublets of equal intensity with $\Delta E_Q = 1.25$, 1.40, 1.75, and 3.08 mm/s. The absence of magnetic features in the 4.2 K zero-field spectrum strongly suggests that the Fe$_4$S$_4$ cluster has integer or zero spin. The isomer shift, δ, is an excellent indicator for the oxidation state of an Fe$_4$S$_4$ cluster; typically, the average value
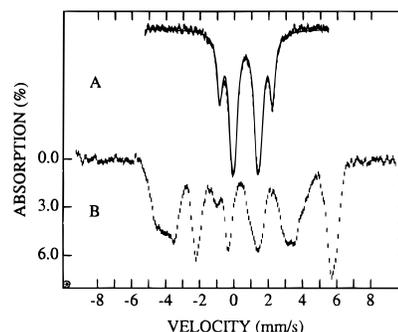

Figure 1. Mössbauer spectra of Ti(III) citrate-reduced Av2 (0.92 mM) recorded at 40 K in zero field (A) and at 1.5 K in a parallel field of 0.05 T (B). Solid line in A is a simulation assuming four doublets with the parameters given in the text.

of δ increases by 0.10−0.12 mm/s per electron added. Table 1 shows that the isomeric shifts of *all* iron sites are larger than those of the ferrous pair ($\delta = 0.59$ mm/s[12]) of the $S = \frac{1}{2}$ state of [Fe$_4$S$_4$]$^{1+}$ Av2. From these observations we conclude that all iron sites of the cluster are ferrous.

As shown in Figure 1B, a weak applied magnetic field elicits substantial ^{57}Fe magnetic hyperfine interactions. Such a response is characteristic of a multiplet with integer electronic spin for which the two lowest levels are nearly degenerate (our studies suggest that the two levels are split by $\Delta \approx 0.02-0.03$ cm^{-1} in zero field). Such systems are expected to yield integer spin EPR signals.[13] Indeed, the cluster exhibits, in parallel mode, a sharp EPR feature (Figure 2, solid line) centered at $g_{\text{eff}} = 16.4$. This resonance can be assigned to a transition between the levels of $M_S = \pm 4$ parentage of an $S = 4$ multiplet with zero-field splitting parameter $D < 0$ (the two levels are mixed by rhombicity with other M_S levels to allow the transition; $0.25 \leq E/D \leq 0.33$). As the temperature is raised, a second, broader feature appears (dashed line); we assign this feature to the $M_S = \pm 3$ doublet.

The 1.5 K Mössbauer spectrum of Figure 1B is associated with the $M_S = \pm 4$ doublet. This spectrum depends on more than 20 unknowns, and we have not yet fully analyzed the data. Spectra recorded in strong applied magnetic fields (not shown) indicate that at least three, perhaps all four, Fe sites have magnetic hyperfine tensors with negative components. Applied fields as small as 0.01 T elicit full magnetic hyperfine splittings for all four Fe sites suggesting that the $S = 4$ spin is common to all sites. The spin manifold of [Fe$_4$S$_4$]^0 contains 15 multiplets with $S = 4$. We are currently studying which combinations of the six exchange coupling constants produce an $S = 4$ ground multiplet having properties compatible with the Mössbauer data.

† University of California.
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(4) Thiriot, D. M.S. Thesis, Brigham Young University, Provo, UT, 1995.
(5) Watt and Reddy[3] suggested that the electron might be localized on sulfur atoms.
(6) A synthetic all-ferrous Fe$_4$S$_4$ cluster with phosphine ligands has been reported.[7]
(7) Goh, C.; Segal, B. M.; Huang, J.; Lang, S. R.; Holm, R. H. *J. Am. Chem. Soc.* **1996**, *118*, 11844−11853.

(8) Purified[9] *A. vinelandii* ^{57}Fe protein was concentrated to >100 mg/mL using a centricon 30 (Amicon). Sodium dithionite was removed using an anaerobic Sephadex G-25 column. Freshly prepared Ti(III) citrate[10] was added to give a final concentration of 7−8 mM. For the UV/vis and CD samples, the Ti(III) citrate was removed with a 1 × 18 cm Sephadex G-25 column. All steps were carried out in a Vacuum Atmospheres drybox. All columns were equilibrated with Chelex-treated[11] 0.05 M Tris-HCl (pH 8.0), 0.25 M in NaCl. Ti and Fe analyses were carried out at the University of Minnesota Soil Testing and Research Analytical Laboratories, St. Paul, MN 55108-6089. It should be noted that if CD spectra are recorded in the presence of sodium citrate, which is present in excess in the stock solution of Ti(III) citrate,[10] high protein concentrations are required to lower the background CD of citrate in the <370 nm region. It should also be noted that all Fe protein samples were fully active in a standard nitrogenase assay[9] and that the fully reduced protein is stable for days at room temperature.
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Table 1. ΔE_Q and δ Values for $Av2$ [Fe$_4$S$_4$]$^{2+,1+,0}$ Cluster at 4.2 K

cluster state	ΔE_Q (mm/s)	δ (mm/s)[b]	no. of sites
[Fe$_4$S$_4$]$^{2+}$ $S = 0$[a]	1.22	0.45	3
	0.83	0.44	1
[Fe$_4$S$_4$]$^{1+}$ $S = 1/2$[a]	1.6	0.59	2
	0.98	0.53	2
[Fe$_4$S$_4$]^{0} $S = 4$	3.08	0.68	1
	$\approx$1.5	0.68	3

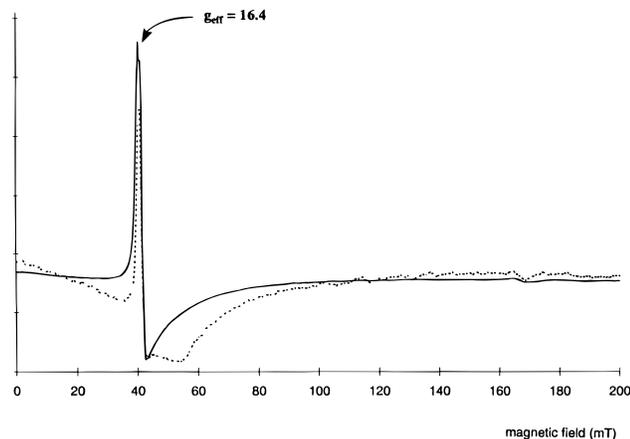[a] Data from ref 12. [b] Shift relative to Fe metal at 298 K.

Figure 2. X-band EPR spectra of Ti(III) citrate-reduced $Av2$ at $\approx$2 K (solid line) and at $\approx$16 K (dashed). Conditions: 0.92 mM $Av2$ enriched with ^{57}Fe; dual-mode cavity in parallel mode; 1.26 mW microwave power; 3 mT modulation amplitude; 9.303 GHz.

The observations that the cluster has integer spin and that the isomer shifts of all iron sites are characteristic of ferrous irons provide compelling evidence for an [Fe$_4$S$_4$]^0 cluster. The values of δ show that the additional electron is not delocalized into the ligands.[15] Considering that the cluster spans the interface of a homodimer, it is somewhat surprising that the iron sites appear (roughly) in a 3:1 ratio. A 3:1 ratio is also observed for the state P^N of the P-clusters of the MoFe protein for which an all-ferrous state has been proposed.[16]

The reduction of the [Fe$_4$S$_4$]$^{1+}$ cluster to the all-ferrous state[8] results in an easily observable color change from brown to red. This transition results in a decrease in absorbance throughout most of the visible region, consistent with reduction, and in the appearance of a new feature at 520 nm (Figure 3A). Figure 3B shows the CD of $Av2$ in the [Fe$_4$S$_4$]$^{1+}$ and in the [Fe$_4$S$_4$]^0 states. The [Fe$_4$S$_4$]^0 spectra display distinct features that can

(14) Our data suggest that $|D| < 1$ cm^{-1} and that three iron sites have A_z values between -8 and -11 MHz. For an $S = 4$ system with $\Delta \approx 0.02-0.03$ cm^{-1}, the Earth's magnetic field induces for the ground doublet hyperfine fields of $0.3-0.5$ T, leading to broadening of the lines. This broadening is essentially absent at 40 K (Figure 1A).

(15) The Fe^{2+} site of rubredoxin has $\delta = 0.70$ mm/s. The smaller δ value (0.59 mm/s) of the Fe^{2+}Fe^{2+} pair in the 1+ state of $Av2$ indicates transfer of d-electron density to the Fe$^{2.5+}$Fe$^{2.5+}$ (0.53 mm/s) pair whose δ has increased by 0.08 mm/s relative to that of the Fe$^{2.5+}$Fe$^{2.5+}$ pair in the 2+ state (0.45 mm/s).

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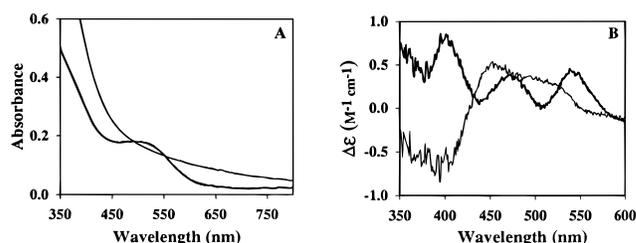

Figure 3. (A) UV/vis spectra of Ti(III) citrate-reduced[8] [Fe$_4$S$_4$]^0 $Av2$ (thick line) and dithionite-reduced [Fe$_4$S$_4$]$^{1+}$ $Av2$ (thin line), both at 57 μM. (B) Visible region CD spectra of [Fe$_4$S$_4$]^0 $Av2$ (thick line) and [Fe$_4$S$_4$]$^{1+}$ $Av2$ (thin line), both at 760 μM. All spectra were baseline-subtracted.

now serve as a signature for the presence of an [Fe$_4$S$_4$]^0 cluster and can be used in experiments aimed at studying the interaction of nucleotides with this new form of the Fe protein. The observation of an absorption band at 520 nm is unusual for ferrous FeS$_4$ sites. Thus, neither Fe^{2+} rubredoxin[17] nor the fully reduced Fe^{2+}Fe^{2+} form of spinach ferredoxin[18] exhibits a band in the visible region. Further, Watt and Reddy[3] did not observe the 520 nm band for Fe protein reduced by two electrons using methylviologen. The question thus arises whether the red color of the all-ferrous cluster is due to adventitiously bound Ti(III) citrate. However, samples that have been analyzed[8] and shown to contain <0.05 atoms of Ti/molecule of Fe protein retain the red color, eliminating the possibility that the 520 nm band arises from Ti bound to the protein. The color change is also completely reversible; reoxidation restores the brown color and the original spectrum. Presently, we cannot rule out the possibility that citrate, which is present in excess in Ti(III) citrate stock solutions,[8] is bound to the protein.

The data reported here confirm the earlier conclusion of Watt and Reddy[3] that the Fe protein can be reduced to an all-ferrous state. This state can also be produced using the hydroquinone form of the physiological electron donor flavodoxin.[1,4] Since flavodoxin is known to serve as a one-electron donor under these conditions,[1] this observation suggests a reason for why the Fe protein is a dimer with a single Fe$_4$S$_4$ cluster: thus, two molecules of flavodoxin may have to bind for the delivery of two electrons. The two-electron-transfer capability of the Fe protein also suggests a reason for why the P-clusters,[1,2,16] the putative electron acceptors within the MoFe protein, are double cubanes rather than simpler clusters: they might function as two-electron rather than one-electron acceptors. If the Fe protein shuttles between the 2+ and the 0 states *in vivo*, then our current understanding of how nitrogenase functions is incorrect and all aspects of the reaction mechanism need to be reexamined.

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