Mössbauer Evidence for a Diferrous [2Fe-2S] Cluster in a Ferredoxin from *Aquifex aeolicus*

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Proteins containing [2Fe-2S], [3Fe-4S], and [4Fe-4S] clusters accomplish many important biological tasks.[1] All-ferrous states of these clusters have not yet been observed during normal activity of any of these proteins. However, considerable efforts are being made to produce such fully reduced states, as they are expected to provide insight into the electronic and magnetic properties of multinuclear Fe−S centers such as the P-cluster of nitrogenase. Recently, the all-ferrous $[4Fe\text{-}4S]^0$ state of the Fe protein of nitrogenase has been characterized by a variety of spectroscopic techniques.[2,3] The all-ferrous $[3Fe\text{-}4S]^{2-}$ state has been attained by electrochemical reduction,[4,5] but spectroscopic data have not yet been reported. It has been shown that the [2Fe-2S] clusters of spinach and parsley ferredoxin can be reduced to the diferrous state upon treatment with $[Cr(15\text{-}aneN_4)(H_2O)_2]^{2+}$, $Cr^{II}L$, but no direct spectroscopic evidence for attainment of the diferrous state was provided.[6a,b] Im et al. have shown that $Cr^{II}L$ reduces the $[2Fe\text{-}2S]^{2+}$ cluster to the 1+ form, and that the resulting $Cr^{III}L$ binds to the ferredoxin, presumably at an anionic site of the protein. This binding causes a dramatic upward shift of the redox potential such that a second electron from excess $Cr^{II}L$ reduces the cluster to the $[2Fe\text{-}2S]^0$ state at $E^{0\prime} = -410$ mV vs NHE (for spinach Fd).[6b] We have applied the same procedure to a [2Fe-2S] ferredoxin from the hyperthermophilic bacterium *Aquifex aeolicus (Aa* Fd1),[7] and in this communication we report Mössbauer evidence for the generation of a diferrous [2Fe-2S] cluster.[12]

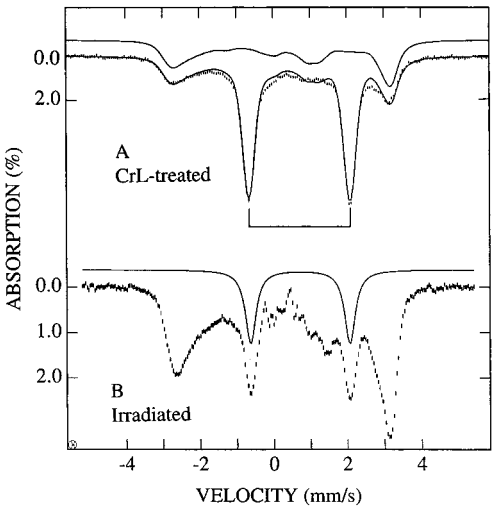

Figure 1. (A) 4.2 K Mössbauer spectrum of Cr^{II}-treated *Aa* Fd1 (2 mM) recorded in a parallel 50 mT field. The solid line drawn through the data is a spectral simulation, consisting of a doublet for $[2Fe\text{-}2S]^0$ (bracket) superimposed on the paramagnetic $[2Fe\text{-}2S]^{1+}$ contribution; the latter is outlined above the data. (B) 4.2 K and 50 mT spectrum of an *Aa* Fd1 sample radiolytically reduced at 77K. For clarity the doublet (40% of Fe) representing nonreduced $[2Fe\text{-}2S]^{2+}$ has been removed from the data. The solid line outlines the contribution of the $[2Fe\text{-}2S]^0$ cluster.

We have recorded Mössbauer spectra of oxidized and dithionite-reduced *Aa* Fd1; details will be reported elsewhere. The spectra of the $[2Fe\text{-}2S]^{1+}$ state were readily simulated, and in the following we will use these simulations, with minor adjustments, to account for the contributions of the $[2Fe\text{-}2S]^{1+}$ state in the spectra of the $Cr^{II}L$-treated protein. Figures 1A and 2A−C show spectra of $Cr^{II}L$-treated *Aa* Fd1. Most prominently, the spectrum of Figure 1A exhibits a quadrupole doublet (bracket, ca. 50% of Fe) with $\Delta E_Q = 2.75$ (3) mm/s and $\delta = 0.71$ (1) mm/s superimposed on a contribution from the $[2Fe\text{-}2S]^{1+}$ state; a spectral simulation for the $[2Fe\text{-}2S]^{1+}$ fraction is shown above the spectrum. The ΔE_Q and δ values of the doublet are strongly indicative of high-spin Fe^{II} sites with tetrahedral sulfur coordination.

Figure 2A shows an 8.0 T spectrum of the $Cr^{II}L$-treated sample recorded at 4.2 K. At this field, the features of the $[2Fe\text{-}2S]^{1+}$ cluster can be simulated quite well with the parameters obtained from fitting the low-field spectra. Figure 2B shows the 8.0 T spectrum of the $\Delta E_Q = 2.75$ mm/s species obtained by subtracting the contribution of the $[2Fe\text{-}2S]^{1+}$ cluster from the spectrum of Figure 2A. The solid line in Figure 2B is a simulation based on the assumption that the $\Delta E_Q = 2.75$ mm/s component is diamagnetic at 4.2 K. The good match of the theoretical and experimental data supports this assumption. Since the values of ΔE_Q and δ imply high-spin ferrous ($S_1 = S_2 = 2$) sites, the observed diamagnetism suggests that this spectral component must belong to an antiferromagnetically coupled $[2Fe\text{-}2S]^0$ cluster. Moreover, our simulations show that two sites of the diferrous cluster have the same ΔE_Q and δ.

Because of the presence of $[2Fe\text{-}2S]^{1+}$ clusters and the large excess of CrL, determination of the exchange coupling constant J of the $[2Fe\text{-}2S]^0$ cluster by magnetic susceptibility is not feasible. However, we were able to establish a lower limit for J by studying 8.0 T Mössbauer spectra at temperatures above 100 K, using the following strategy. The $S = 1$ excited state of the exchange-coupled $[2Fe\text{-}2S]^0$ cluster has energy J ($H = JS_1 \cdot S_2$). At temperatures for which this state is measurably populated, the ^{57}Fe nuclei experience, for fast relaxation of the electronic spin, internal magnetic fields $\mathbf{B}_{int} = -\langle \mathbf{S} \rangle_{th} \cdot \mathbf{A}/g_N\beta_N$, where $\mathbf{A}$ is the

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(7) The *fdx1* gene (entry Aq919a in genome sequence) from *Aquifex aeolicus*[8] has been cloned by PCR and expressed in *Escherichia coli* as previously described for other ferredoxins.[9] *Aa* Fd1 is homologous to plant- and mammalian-type [2Fe-2S] ferredoxins. Its purification and properties will be reported elsewhere. Protein samples for Mössbauer analysis were prepared by growing the cultures in ^{57}Fe-enriched mineral medium[10] and adding a 50-fold excess of $Cr^{II}L$ to the purified protein ($E^{0\prime} = -580$ mV for $Cr^{II}L/Cr^{III}L$ at $[H^+] = 0.40$ M).[11]
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(12) We have also carried out preliminary experiments with ^{57}Fe-reconstituted spinach ferredoxin, and detected $[2Fe\text{-}2S]^0$ clusters (≈25% of Fe) in the Mössbauer spectra. The spectra also contained contributions from $[2Fe\text{-}2S]^{1+}$ clusters (≈50% of Fe) and from adventitious Fe^{II} released from the active site as a consequence of cluster destruction.

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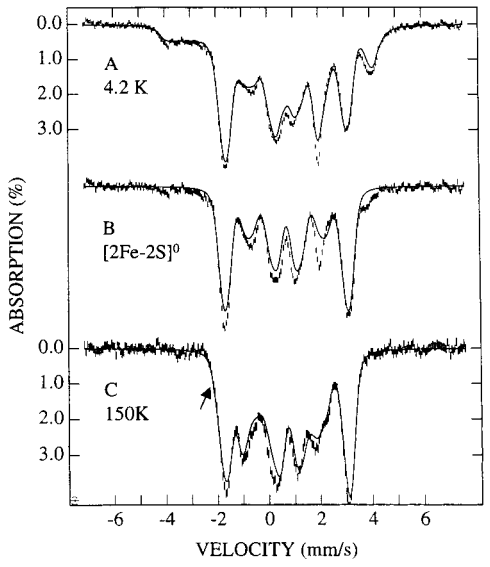

Figure 2. 8.0 T Mössbauer spectra of $Cr^{II}L$-treated *Aa* Fd1 (same sample as in Figure 1A) recorded at 4.2 (A) and 150 K (C). The solid lines drawn through the data of parts A and C are spectral simulations. For the simulation of the $[2Fe\text{-}2S]^{1+}$ cluster spectra we have assumed slow and fast electronic spin relaxation at 4.2 and 150 K, respectively. The arrow of part C points to the spectral contribution of the Fe^{II} site of the $[2Fe\text{-}2S]^{1+}$ cluster. (B) 8.0 T Mössbauer spectrum of $[2Fe\text{-}2S]^0$ obtained by subtracting the simulated 8.0 T spectrum of the $[2Fe\text{-}2S]^{1+}$ cluster from the data of part A; the solid line in part B is a spectral simulation for the $[2Fe\text{-}2S]^0$ cluster assuming diamagnetism and two indistinguishable Fe sites with $\Delta E_Q = 2.75$ mm/s, $\eta = 1$, and $\delta = 0.71$ mm/s. The small deviations between theory and experiment at +2 and + 3.8 mm/s reflect an imperfect fit for the $[2Fe\text{-}2S]^{1+}$ cluster.

magnetic hyperfine tensor of a ferrous site and $\langle \mathbf{S} \rangle_{th}$ is the thermally averaged cluster spin.[13] Neglecting zero-field splittings, $\langle \mathbf{S} \rangle_{th}$ is isotropic and, for $\beta B \ll kT$, $\langle S_z \rangle_{th}$ is in good approximation given by $\langle S_z \rangle_{th} = (-2g\beta B/kT)/(3 + e^{J/kT})$; the contribution of the $S = 2$ state of the spin ladder is negligible in the present case. For $J = 80$ cm^{-1}, $B = 8.0$ T, and $g = 2$, $\langle S_z \rangle_{th} \approx -0.028$ at 150 K. All high-spin $Fe^{II}S_4$ sites studied to date in rubredoxins and $[2Fe\text{-}2S]^{1+}$ clusters have **A**-tensors for which one component has the magnitude $A/g_N\beta_N \geq 20$ T (this is expected because at least one component of the traceless spin-dipolar contribution to the **A**-tensor must add to the isotropic contact term). With the above choices for **B** and **J**, $A/g_N\beta_N = -20$ T would produce an internal field $B_{int} = -0.56$ T at 150 K. The presence of such a field would decrease the magnetic splittings at 150 K relative to 4.2 K and would be readily detected in the spectrum of Figure 2C. Comparison of the observed splittings of the $[2Fe\text{-}2S]^0$ cluster at 4.2 and 150 K provides no evidence for the presence of a detectable ($\approx$0.2 T) internal magnetic field at 150 K.[14] To establish a lower limit for J, we have performed spectral simulations based on an exchange-coupled spin Hamiltonian that includes zero-field splittings ($-7 \leq D_i \leq 7$ cm^{-1}, $i = 1, 2$) and ^{57}Fe hyperfine tensors at arbitrary orientations. On the basis of these simulations, we conservatively estimate that $J > 80$ cm^{-1} for the diferrous $[2Fe\text{-}2S]^0$ cluster.

In a recent study of the Fe protein of nitrogenase, Yoo et al.[2] have shown that the $[4Fe\text{-}4S]^0$ cluster state can be produced at 77 K by radiolytic reduction in a synchrotron X-ray beam; this state was indistinguishable from the one obtained by chemical reduction with Ti(III) citrate, thus ruling out the possibility of extensive structural rearrangements caused by the reductant. Following the same rationale, $[2Fe\text{-}2S]^{2+}$ *Aa* Fd1 was irradiated under similar conditions and analyzed by Mössbauer spectroscopy. The spectrum of this irradiated sample (Figure 1B) contained 40% of the iron as $[2Fe\text{-}2S]^{2+}$ ($\Delta E_Q = 0.67$ mm/s, $\delta = 0.27$ mm/s), 50% as $[2Fe\text{-}2S]^{1+}$, and again a doublet (10%, outlined in Figure 1B) with $\Delta E_Q = 2.75$ mm/s and $\delta = 0.71$ mm/s. A spectrum recorded at 4.2 K in an applied field of 1.0 T showed that this $\Delta E_Q = 2.75$ component of the irradiated sample is diamagnetic like that of the $Cr^{II}L$ treated sample. Thus, as in the case of the [4Fe-4S] cluster of the Fe protein of nitrogenase, a diferrous cluster has been produced by irradiation of [2Fe-2S] *Aa* Fd1, showing that the most reduced redox level can be reached without structural rearrangement.

Magnetic susceptibility data of $[2Fe\text{-}2S]^{2+,1+}$ clusters are scarce. For spinach Fd, $J = 366$ and 200 cm^{-1} have been reported for the 2+ and 1+ states, respectively.[16] The observation that $J >$ 80 cm^{-1} for $[2Fe\text{-}2S]^0$ shows that the diferrous cluster state exhibits substantial antiferromagnetic exchange. In the absence of reliable J-values for ferrous ion pairs in more complex Fe−S clusters, the present estimate can serve as a useful guide.

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