Mössbauer Study of Cys56Ser Mutant 2Fe Ferredoxin from *Clostridium Pasteurianum*: Evidence for Double Exchange in an [Fe$_2$S$_2$]$^+$ Cluster

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Ferredoxins containing Fe$_2$S$_2$(cys)$_4$ clusters have been studied intensively for the past 30 years.[1] The iron−sulfur cluster of these electron transport agents undergoes redox transitions between an Fe^{3+}Fe^{3+} form and a mixed-valence Fe^{2+}Fe^{3+} state. In the latter state, designated [Fe$_2$S$_2$]$^+$, the local spins ($S_1 = 2$, $S_2 = {}^5/_2$) are coupled by strong antiferromagnetic Heisenberg−Dirac−van Vleck (HDvV) exchange (>150 cm^{-1} $S_1 \cdot S_2$) to yield a cluster ground state with $S = {}^1/_2$.[2] Mössbauer spectroscopy has revealed two distinct, valence-trapped Fe^{2+} and Fe^{3+} sites.[3] The parameters listed in Table 1 are typical for such sites. In contrast, [Fe$_3$S$_4$]^0 and [Fe$_4$S$_4$]$^{+,2+,3+}$ clusters contain *delocalized* Fe$^{2.5+}$Fe$^{2.5+}$ pairs.[4] In addition to HDvV exchange, the sites of the delocalized pairs are coupled by double exchange (spin-dependent resonance delocalization).

Among the mutated forms of *C. pasteurianum* Fe$_2$S$_2$ ferredoxin,[5] the Cys60Ser and Cys56Ser variants have been reported by Crouse et al.[6] to contain a mixture of clusters with $S = {}^1/_2$ (Fd$_{1/2}$, $g = 2.01$, 1.92, 1.88) and $S = {}^9/_2$ (Fd$_{9/2}$, $g_0 = 2.03$, $D = -1.0$ cm^{-1}, $E/D = 0.13$ for the Cys56Ser mutant). Crouse et al. raised the possibility that the $S = {}^9/_2$ ground state in Fd$_{9/2}$ is the result of double exchange[7] within a valence-delocalized cluster.[6] Here, we demonstrate that this state is indeed *valence-delocalized*.

We have studied the Mössbauer spectra of ^{57}Fe-enriched samples[8] in the oxidized and reduced states, without and with glycerol. The highest fraction (28%) of Fd$_{9/2}$ was obtained for a sample with 30% (v/v) glycerol.[9] The following spectral properties of Fd$_{9/2}$ are relevant for our analysis. Up to 8 K, only the ground Kramers doublet of Fd$_{9/2}$ is measurably populated.[10] For $E/D = 0.13$,[6] its calculated effective g values are $g_x \approx g_y \approx 0$, $g_z = 18.25$. In weak applied magnetic fields, H, or for $H = 0$, such a doublet gives rise to a Mössbauer spectrum consisting of six absorption lines with a 3:2:1:1:2:3

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(1) For a review on protein-bound Fe$_2$S$_2$ clusters, see: Cammack, R. *Adv. Inorg. Chem.* **1992,** *38,* 281.
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(8) Enrichment was achieved by overexpressing the Fd gene in *E. coli*[5] grown in a ^{57}Fe-containing synthetic medium.

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Table 1. Hyperfine Parameters at 4.2 K for Fd$_{1/2}$ and Fd$_{9/2}$[12,13]

		ΔE_Q (mm/s)	δ (mm/s)	A_{iso} (MHz)
Fd$_{1/2}$	Fe^{2+}	3.2	0.73	20.0
	Fe^{3+}	1.0	0.32	−48.3
Fd$_{9/2}$	Fe$^{2.5+}$Fe$^{2.5+}$	1.8	0.50	−9.2
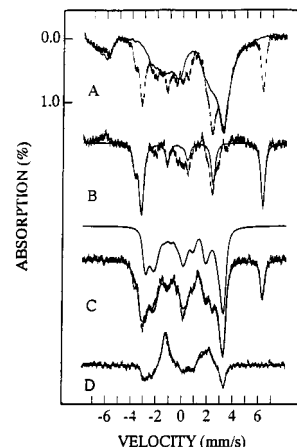
Figure 1. Mössbauer spectra (4.2 K) of the dithionite-reduced Cys56Ser mutant (30% glycerol). (A) Spectrum recorded in zero field. The solid line is the experimental zero-field spectrum of an [Fe$_2$S$_2$]$^{1+}$ Rieske protein[11] scaled to 72% of the area of the mutant ferredoxin. (B) Spectrum of Fd$_{9/2}$ obtained by taking the difference of the spectra shown in part A. The solid line is a spectral simulation based on the spin Hamiltonian $\mathcal{H} = D\{S_z^2 - S(S + 1)/3 + (E/D)(S_x^2 - S_y^2)\} + 2\beta H \cdot S + \sum_{i=1,2}\{S \cdot A_i \cdot I_i - g_n\beta_nH \cdot I_i + \mathcal{H}_Q(i)\}$ for $S = {}^9/_2$ ($-D \geq 1.5$ cm^{-1} (this work); $E/D = 0.13$ (ref 6)). (C) Spectrum recorded in a parallel field of 0.05 T (hash marks). The solid lines are spectral simulations for Fd$_{1/2}$ (shown above the data) and for the sum of Fd$_{1/2}$ (72%) and Fd$_{9/2}$ (28%). For Fd$_{1/2}$, the above Hamiltonian was used for $S = {}^1/_2$, with the other parameters as given in ref 13. (D) Difference spectrum obtained by subtracting a spectrum recorded in a transverse field of 0.05 T (not shown) from the spectrum of part C. The solid line is a theoretical difference spectrum for Fd$_{1/2}$.

intensity pattern for each site of the [Fe$_2$S$_2$]$^+$ cluster. For $\beta H \ll |D|$, the intensities of these lines do not depend on the orientation of H relative to the γ-radiation. Two methods have been used to quantitate the contributions of Fd$_{1/2}$ and Fd$_{9/2}$. The first one is based on the fact that the spectra of Fd$_{1/2}$ depend on the direction of H relative to the γ-rays,[3] in contrast to those of Fd$_{9/2}$. Thus, the difference of the spectra collected in parallel and transverse field will contain only the Fd$_{1/2}$ contribution, which can be used to quantitate the amount of Fd$_{1/2}$. In a second approach, we used the fact that the low-energy feature of the *zero-field* spectrum of Fd$_{1/2}$[11] does not overlap with the Fd$_{9/2}$ spectrum. Therefore, this feature can be used to determine the amount of Fd$_{1/2}$ in a sample. Both methods gave the same result.

Figure 1 shows 4.2 K Mössbauer spectra of the Cys56Ser mutant. The spectrum of Fd$_{9/2}$, which is the difference spectrum given in Figure 1B, exhibits a *single* six-line pattern (the two

(9) The sample was produced by reducing the ferredoxin with 3-fold molar excess sodium dithionite; 8% of the ferredoxin was observed as Fd$_{9/2}$. Addition of glycerol to 30% (v/v) (sample of Figure 1) and 50% (v/v) yielded 28% and 8%, respectively, of Fd$_{9/2}$. Information on glycerol concentrations has been communicated to us by B. R. Crouse and M. K. Johnson. The amount of Fd$_{9/2}$ attainable may depend on factors such as incubation time after glycerol addition, sequence for reduction and glycerol addition, freezing rate, or protein concentration. Moreover, different batches of glycerol-free samples contained variable amounts of Fd$_{9/2}$ ranging from 0 to 8%. These effects are presently being studied.
(10) Mössbauer results for Fd$_{9/2}$ show that $-D \geq 1.5$ cm^{-1}. This value is larger than the reported[6] $D = -1$ cm^{-1}.
(11) The zero-field spectra of [Fe$_2$S$_2$]: ferredoxins are remarkably similar. We have used here the Rieske protein from *Pseudomonas mendocina*: Pikus, J. D.; Studts, J. M.; Achim, C.; Kauffmann, K. E.; Münck, E.; Steffan, R.; McClay, K.; Fox, B. G. *Biochemistry* **1996,** *35,* 9106.

low-energy lines overlap at -3 mm/s). Thus, the two Fe sites of $Fd_{9/2}$ contribute nearly identical spectra proving that the system is *delocalized*. The analysis shows that 28% of the iron in this sample belongs to $Fd_{9/2}$. The evidence for the site equivalence is supported by the high-temperature data. At 150 K, the spectrum of $Fd_{9/2}$ (not shown) consists of *one* quadrupole doublet with $\Delta E_Q \approx 1.8$ mm/s and $\delta \approx 0.46$ mm/s. As shown in Table 1, the 4.2 K isomer shift of the delocalized pair in $Fd_{9/2}$, $\delta_{av} = 0.50$ mm/s,[12] is close to the average of the values for the ferric and ferrous sites in the $Fd_{1/2}$. We used the isotropic parts of the $Fd_{1/2}$ magnetic hyperfine coupling tensors, A_{iso}, to estimate the A value for the delocalized pair; this yields the value -9.1 MHz,[13] in excellent agreement with the experimental result, -9.2 MHz.

Spin state and electron delocalization in mixed-valence compounds with paramagnetic sites depend on an interplay of HDvV exchange (J), double exchange (B), and vibronic coupling (χ).[7] In the absence of vibronic coupling, the dependence of the energy of a binuclear system on B and J can be expressed as $E = E_0 - JS(S + 1) \pm B(S + \frac{1}{2})$. The ground state has $S = \frac{9}{2}$ for $|J/B| \leq \frac{1}{9}$. Figure 2A shows that for a broad J/B range the ground state has intermediate spin, $\frac{3}{2} \leq S \leq \frac{7}{2}$. This range narrows in the presence of vibronic coupling up to a single point at the localization limit of the $S = \frac{9}{2}$ state ($10|B|/\chi = 1$).[14] The narrowing diminishes the likelihood of finding an intermediate-spin ground state, and it reduces the size of the variation in J needed for converting the ground state from $S = \frac{1}{2}_{loc}$ into $\frac{9}{2}_{deloc}$ (Figure 2B). Parameters for $[Fe_2S_2]^+$ clusters in plant-type ferredoxins have been estimated as $|B| \approx 1000$ cm^{-1},[15a] $J \approx -250$ cm^{-1} ($-2JS_1 \cdot S_2$), and $\chi \approx 7000$ cm^{-1}.[16] For these values, theory predicts a *localized* doublet ground state, $S = \frac{1}{2}_{loc}$, in accord with the state observed for $Fd_{1/2}$, while the $S = \frac{9}{2}$ excited state is delocalized. We have estimated the parameters needed for stabilizing $S = \frac{9}{2}_{deloc}$[17] as the ground state

(12) Simulations suggest that the two sites of the $S = \frac{9}{2}$ species are slightly inequivalent; $\Delta E_Q(1) = \Delta E_Q(2) = 1.80$ mm/s, $\delta(1) = 0.46(4)$ mm/s, $\delta(2) = 0.54(4)$ mm/s, $A(1) = -9.4(2)$ MHz, and $A(2) = -9.1(2)$ MHz. Since these differences are barely larger than the uncertainties, we have quoted the average values in Table 1. Moreover, the ground doublet of the $Fd_{9/2}$ state is sensitive only to A_z; the x- and y-components of the $\mathbf{A}$ tensors are undetermined.

(13) The fits to $Fd_{1/2}$ gave $A(Fe^{3+}) = -(50,51,44)$ MHz, $\Delta E_Q(Fe^{3+}) = +1.0$ mm/s, $\eta(Fe^{3+}) = 0.5$ and $A(Fe^{2+}) = +(6,26,28)$ MHz, $\Delta E_Q(Fe^{2+}) = -3.2$ mm/s, $\eta(Fe^{2+}) = -1.8$. Averaging the components to obtain A_{iso} and correcting by the spin-coupling factors[3] $7/3$ and $-4/3$ yields for the intrinsic a values $a(Fe^{3+}) = -20.7$ MHz and $a(Fe^{2+}) = -15$ MHz. For the delocalized $S = \frac{9}{2}$ system, we then obtain $A^{9/2} = [(5/9)a(Fe^{3+}) + (4/9)a(Fe^{2+})]/2 \approx -9.1$ MHz.

(14) Energy splittings between spin states S with $(2S + 1)|B|/\chi \leq 1$ (the $<$ sign corresponds to localized states) are described by an effective HDvV Hamiltonian with coupling constant $J_{eff} = J + B^2/\chi$.[7]

(15) (a) Gamelin, D. R.; Bominaar, E. L.; Kirk, M. L.; Wieghardt, K.; Solomon, E. I. *J. Am. Chem. Soc.* In press. Gamelin, D. R.; Bominaar, E. L.; Mathonière, C.; Kirk, M. L.; Wieghardt, K.; Girerd, J.-J.; Solomon, E. I. *Inorg. Chem.* **1996**, *35*, 4323. (b) Ding, X.-Q.; Bominaar, E. L.; Bill, E.; Winkler, H.; Trautwein, A. X.; Drüeke, S.; Chaudhuri, P.; Wieghardt, K. *J. Chem. Phys.* **1990**, *92*, 178.

(16) χ represents the reorganization energy and is assumed to contain equal contributions for inner-sphere (χ_{in}) and outer-sphere (χ_{out}) reorganization energies.[7,15a]
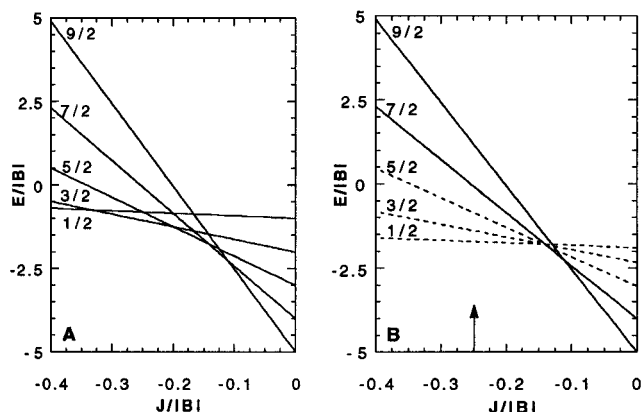
Figure 2. Spin level diagrams evaluated by taking energies at potential-well minima. The levels are labeled by system spin S. Solid and broken lines represent delocalized and localized states, respectively. Parameters used: $\chi = 0$ (A, all states delocalized) and $\chi = 7|B|$ (B). Energies at the arrow are obtained for the parameter values for $S = \frac{1}{2}$ $[Fe_2S_2]^+$ clusters given in text.

by analyzing the effect of varying one parameter at a time while keeping the remaining ones fixed at the above values. The procedure gives either $|B| \geq 2250$ cm^{-1} for $-J \geq 250$ cm^{-1} or $-J < 111$ cm^{-1} for $|B| \geq 1000$ cm^{-1}, while variations of χ fail to stabilize the ground state $\frac{9}{2}_{deloc}$.[18] B values as large as 2250 cm^{-1} have as yet not been reported for Fe dimers. $[Fe_2(OH)_3-(tmtacn)_2]^{2+}$, the only extensively studied *delocalized* $Fe^{2.5+}$-$Fe^{2.5+}$ complex[15,19] has $|B| = 1350$ cm^{-1}; this complex contains face-sharing octahedral Fe sites, an arrangement favorable for double exchange. Thus, it seems not very likely that $Fd_{9/2}$ has $|B| \geq 2250$ cm^{-1}.[20]

In summary, our analysis indicates that the values for J and possibly for B in $Fd_{9/2}$ must significantly differ from those in plant-type ferredoxins. Exploring the intrinsic electronic determinants and extrinsic factors that govern the spin state variability in the Cys56Ser mutant is the subject of ongoing investigations.

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(17) The delocalization for $S = \frac{9}{2}$ requires $|B| \geq \chi/10 = 700$ cm^{-1}, which implies strong double exchange in $Fd_{9/2}$.

(18) Modifications in ligation may cause the change in the values for J and B. It is also possible that the observed $Fd_{1/2}/Fd_{9/2}$ mixture reflects a situation where the system is close to the crossing point of Figure 2B and the quantity J/B is distributed. Then, changes in the reorganization energy as a consequence of variation in the glycerol content could alter the composition of the mixture.

(19) Recent studies indicate valence delocalization in another $Fe^{2+}Fe^{3+}$ complex with octahedral N/O coordination at each site: Haase, W.; Fleischauer, P.; Werner, R.; Behlendorf, M.; Ensling, J.; Dutta, S. K.; Nag, K. *J. Inorg. Biochem.* **1995**, *59*, 285.

(20) For the Cys60Ser mutant, Crouse et al.[6] have tentatively attributed an MCD band at 700 nm to a valence-delocalized intervalence transition which would correspond to $|B| = 1430$ cm^{-1}.