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Spectroscopic Evidence for a Reduced Fe_2S_2 Cluster with a $S = {}^9/_2$ Ground State in Mutant Forms of *Clostridium pasteurianum* 2Fe Ferredoxin

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Iron−sulfur clusters containing a $Fe_2(\mu_2\text{-}S)_2$ core ($[Fe_2S_2]$ clusters) are one of the most ubiquitous and versatile electron carriers in nature with midpoint potentials spanning at least 800 mV.[1] As such they are integral components of the mitochondrial and photosynthetic electron transport chains and numerous other redox enzymes. In addition $[Fe_2S_2]$ units can be viewed as the building block for more complex higher nuclearity biological clusters, e.g., cubane-type $[Fe_3S_4]$ and $[Fe_4S_4]$ clusters and double-cubane $[Fe_8S_8]$ clusters. The magnetic properties of synthetic and biological $[Fe_2S_2]^{2+,+}$ clusters are dominated by strong antiferromagnetic exchange interaction between high-spin Fe sites (Fe^{3+}, $S = {}^5/_2$; Fe^{2+}, $S = 2$), which results in an $S = 0$ ground state for the Fe^{3+}/Fe^{3+} oxidized cluster and an $S = {}^1/_2$ ground state for the localized valence Fe^{3+}/Fe^{2+} reduced cluster.[2] However, a valence-delocalized $[Fe_2S_2]^+$ fragment with a $S = {}^9/_2$ ground state that results from double exchange interaction as opposed to ferromagnetic exchange interaction has been invoked to explain the magnetic and spectroscopic properties of several higher nuclearity clusters, e.g., $[Fe_3S_4]^0$, $[Fe_4S_4]^{3+,2+,+}$, and $[Fe_8S_8]^{2+,+}$.[3] In this work we report on EPR and variable temperature magnetic circular dichroism (VTMCD) studies of the Cys60Ser mutant of *Clostridium pasteurianum* 2Fe ferredoxin (*Cp* 2Fe Fd). The results provide the first example of a synthetic or biological $[Fe_2S_2]^+$ cluster with a $S = {}^9/_2$ ground state.

The UV−visible absorption (oxidized), EPR (reduced), and resonance Raman (oxidized) properties of the Cys60Ser mutant are perturbed compared to the wild-type Fd, but are characteristic of the presence of a $[Fe_2S_2]^{2+,+}$ cluster, albeit with one cysteinate replaced with a serinate ligand.[4] Additional support for approximately one $[Fe_2S_2]^{2+,+}$ cluster per monomer comes from Fe and S^{2-} analyses,[5] electrospray-ionization mass spectrometry studies,[6] and the observation that reduction is at least 90% reversible as judged by optical absorption studies of the as prepared, dithionite-reduced and anaerobically reoxidized samples, in the absence and presence of 50% (v/v) glycerol. The first evidence that some of the reduced $[Fe_2S_2]^+$ clusters in this mutant Fd have ground state properties distinct from those of the EPR-characterized $S = {}^1/_2$ species, $g = 2.01, 1.92, 1.88$,[4] came from VTMCD studies, see Figure 1. The temperature-dependence behavior (1.68−20 K) of the positive MCD bands centered at 700 and 470 nm is quite distinct from that of other bands in the spectrum and can only be rationalized in terms of electronic transitions from a ground state with $S > {}^1/_2$. This is most clearly apparent in the MCD magnetization data collected at 700 nm, see Figure 2. The observation that the MCD intensity as a function of $\beta B/2kT$ approaches magnetic saturation much more steeply than expected for a $S = {}^1/_2$ system and the

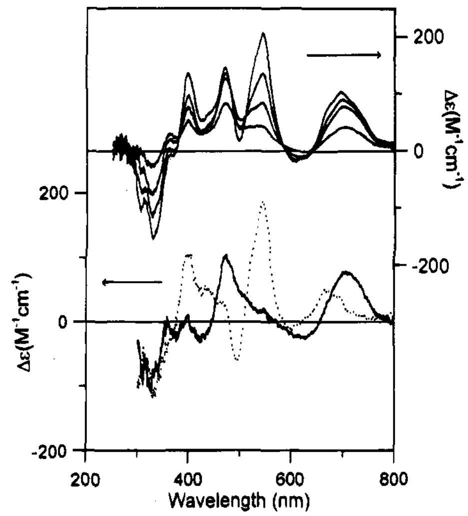

Figure 1. VTMCD spectra of the reduced Cys60Ser mutant of *Cp* 2Fe Fd. The sample, 0.13 mM in $[Fe_2S_2]$ clusters, was in 100 mM Tris/HCl buffer, pH 8.0, with 10 mM sodium dithionite and contained 50% (v/v) glycerol. Upper panel: VTMCD spectra recorded at a magnetic field of 4.5 T, at 1.68, 4.22, 9.2, 24.0 K; the intensity of all transitions increases with decreasing temperature. Lower panel: Deconvolution of $S = {}^1/_2$ (dotted line) and $S = {}^9/_2$ (solid line) contributions to the 1.68 K MCD. The form of the MCD spectrum from the $S = {}^1/_2$ component was determined by the 1.68 K minus 4.22 K difference spectrum. MCD magnetization studies were used to assess the relative contributions of the $S = {}^1/_2$ and $S = {}^9/_2$ components at different wavelengths, see text and Figure 2 for an example.

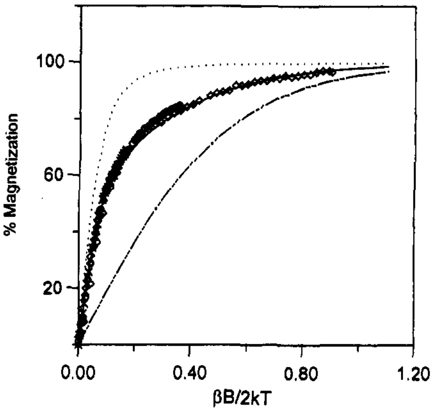

Figure 2. MCD magnetization data for the reduced Cys60Ser mutant of *Cp* 2Fe Fd collected at 700 nm. Data collected at fields between 0 and 4.5 T at 1.68 K (◇), 4.22 K (○), and 9.2 K (×). Sample is described in Figure 1. The broken line is theoretical magnetization data for an isolated $S = {}^1/_2$ doublet with $g_{\parallel} = 2.01$ and $g_{\perp} = 1.90$, and the dotted line is theoretical magnetization for the $M_s = \pm{}^9/_2$ doublet of a zero-field split $S = {}^9/_2$ ground state, $g_{\parallel} = 18.0$ and $g_{\perp} = 0.0$. The solid line is the best fit to the 1.68 K magnetization data, i.e., 60% $S = {}^9/_2$ and 40% $S = {}^1/_2$.

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(1) For recent reviews of the properties and diversity of biological $[Fe_2S_2]$ clusters, see: Johnson, M. K. In *Encyclopedia of Inorganic Chemistry*; King, R. B., Ed.; Wiley: Chichester, U.K., 1994; Vol. 4, pp 1896−1915. Cammack, R. *Adv. Inorg. Chem.* **1992**, *38*, 281.

(2) (a) Palmer, G. In *Iron-Sulfur Proteins*; Lovenberg, W., Ed.; Academic Press: New York, 1973; Vol. 2, pp 285−325. (b) Beardwood, P.; Gibson, J. F. *J. Chem. Soc., Dalton Trans.* **1983**, 737. (c) Bertrand, P.; Guigliarelli, B.; Gayda, J.-P.; Beardwood, P.; Gibson, J. F. *Biochim. Biophys. Acta* **1985**, *831*, 261.

(3) (a) Papaefthymiou, V.; Girerd, J.-J.; Moura, I.; Moura, J. J. G.; Münck, E. *J. Am. Chem. Soc.* **1987**, *109*, 4703. (b) Hagen, W. R. *Adv. Inorg. Chem.* **1992**, *38*, 165. (c) Noodleman, L.; Case, D. A. *Adv. Inorg. Chem.* **1992**, *38*, 423. (d) Mouesca, J.-M.; Noodleman, L.; Case, D. A. *Inorg. Chem.* **1994**, *33*, 4819.

(4) (a) Fujinaga, J.; Gaillard, J.; Meyer, J. *Biochem. Biophys. Res. Commun.* **1993**, *194*, 104. (b) Meyer, J.; Fujinaga, J.; Gaillard, J.; Lutz, M. *Biochemistry* **1994**, *33*, 13642.

(5) Colorimetric analyses of three samples for non-heme Fe, S^{2-}, and protein (Lowry assay) indicated 1.6 ± 0.3 mol of Fe and 1.7 ± 0.3 mol of S^{2-} per apoprotein monomer ($M_r = 11\,412$).

(6) Petillot, Y.; Golinelli, M.-P.; Forest, E.; Meyer, J. *Biochem. Biophys. Res. Commun.* **1995**, *210*, 686.

0002-7863/95/1517-9612$09.00/0

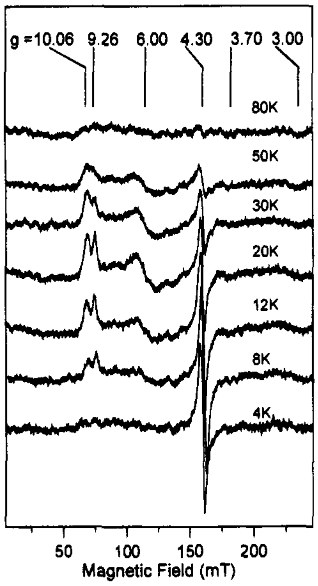

Figure 3. X-band EPR spectra in the low-field region for the reduced Cys60Ser mutant of *Cp* 2Fe Fd. The sample is as described in Figure 1. Conditions of measurement: microwave power, 100 mW; microwave frequency, 9.58 GHz; modulation amplitude, 0.64 mT; temperatures as indicated.

non-coincidence of the data obtained at different fixed temperatures ("nesting") are both indicative of a $S > {}^{1}/_{2}$ ground state.[7] The observed magnetization data at 1.68 K are well simulated as a composite of the theoretical data computed for a $S = {}^{1}/_{2}$ ground state and the "$M_s = \pm{}^{9}/_{2}$" doublet of a $S = {}^{9}/_{2}$ ground state (predicted to be the lowest doublet based on the EPR analysis presented below). Since the MCD intensity of the $S = {}^{9}/_{2}$ species is predicted to remain constant over the temperature range 1.68−4.2 K at a magnetic field of 4.5 T (see Figure 2, dotted line), the 4.2 K MCD spectra can be deconvoluted into separate $S = {}^{1}/_{2}$ and ${}^{9}/_{2}$ components via temperature difference techniques with magnetization studies determining the relative contribution at discrete wavelengths, see Figure 1.

The VTMCD results lead to careful inspection of the EPR spectra of the corresponding samples in the low-field region, see Figure 3. In addition to the previously reported $S = {}^{1}/_{2}$ resonance, $g = 2.01, 1.92, 1.88$ (not shown), which accounted for 0.4 spins/[Fe_2S_2] cluster for the samples used in this work, numerous low-field resonances were observed at temperatures between 4 and 80 K: positive absorption-shaped features at $g = 10.06$ and 9.26, derivative-shaped features centered at $g = 6.00$ and 3.00, a negative absorption-shaped band at $g = 3.70$, and an intense derivative-shaped feature centered at $g = 4.30$ that is attributed to a minor component of adventitiously bound high-spin Fe^{3+} ion. Provided the $g = 4.3$ component is excluded, these resonances and their temperature-dependent behavior can all be rationalized in terms of the conventional $S = {}^{9}/_{2}$ spin Hamiltonian[3b] with $g_0 = 2.04$, $D < 0$, and $E/D = 0.16$ (D and E are the axial and rhombic zero-field splitting parameters, respectively), see Table 1.[8] Plots of the log of the ratio of the intensity of the $g = 10.06$ and 9.26 components versus $1/T$ (not shown) are linear, and the slope enables an estimate of the axial zero-field splitting parameter, $D = -1.1$ cm^{-1}.

Taken together, the EPR and VTMCD results indicate that the [Fe_2S_2]$^+$ cluster in the reduced Cys60Ser mutant of *Cp* 2Fe Fd exists as a mixture of species with $S = {}^{1}/_{2}$ and ${}^{9}/_{2}$ ground states. Very similar VTMCD and EPR results have also been obtained for the Cys56Ser mutant, i.e., $S = {}^{1}/_{2}$ species, $g = 2.01, 1.92, 1.88$,[4] accounting for 0.3 spin/[Fe_2S_2] cluster, and $S = {}^{9}/_{2}$ species, characterized by $g_0 = 2.03$, $D = -1.0$ cm^{-1}, $E/D = 0.13$ (data not shown). For the $S = {}^{1}/_{2}$ species in both mutants, the increased g-value anisotropy compared to wild-type ($g = 2.00, 1.95, 1.92$) and the similarity in the VTMCD spectra in the S → Fe^{3+} charge transfer region (350−550 nm) compared to wild-type indicate a localized valence Fe^{3+}/Fe^{2+} cluster with serinate ligation at the Fe^{2+} site.[9,10] The VTMCD spectrum of the $S = {}^{9}/_{2}$ species in both mutants is characterized by an intense positive band at 700 nm, see Figure 1, that is tentatively attributed to the valence-delocalized intervalence band.[11] What is striking is that an intense positive MCD band is observed at a similar wavelength (700−800 nm) for paramagnetic higher nuclearity Fe−S clusters that are considered to contain a $S = {}^{9}/_{2}$ valence-delocalized [Fe_2S_2]$^+$ fragment, i.e., [Fe_3S_4]^0, [Fe_4S_4]$^+$, [Fe_8S_8]$^{2+}$, and a range of [MFe_3S_4]$^{2+,+}$ clusters, where M = Zn, Cd, Co, Ni, Tl, Cu.[12] While Mössbauer studies will be required for direct assessment of the extent of valence localization, the spectroscopic data presented here and the available theoretical data suggest that antiferromagnetic exchange interaction within a localized valence [Fe_2S_2]$^+$ cluster (serinate coordination at the Fe^{2+} site) is responsible for the $S = {}^{1}/_{2}$ species and that double exchange within a valence-delocalized [Fe_2S_2]$^+$ cluster is responsible for the $S = {}^{9}/_{2}$ ground state.

Table 1. Calculated and Observed Effective g Values for $S = {}^{9}/_{2}$ species in the Reduced Cys60Ser Mutant of *Cp* 2Fe Fd[a]

doublet	g_x	g_y	g_z
$\lvert\pm{}^{1}/_{2}\rangle$	0.39	17.62	0.73
$\lvert\pm{}^{3}/_{2}\rangle$	3.67 (3.70)	10.06 (10.06)	6.02 (6.00)
$\lvert\pm{}^{5}/_{2}\rangle$	9.26 (9.26)	2.81 (3.00)	3.25 (3.00)
$\lvert\pm{}^{7}/_{2}\rangle$	14.09	0.17	0.20
$\lvert\pm{}^{9}/_{2}\rangle$	18.32	0.00	0.00

[a] Theoretical values were calculated in the weak-field limit, $D \gg g\beta B$, using a conventional $S = {}^{9}/_{2}$ spin Hamiltonian with $E/D = 0.16$ and $g_0 = 2.04$ using the RHOMBO program supplied by Dr. Wilfred Hagen. Observed values are in parentheses.[8]

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(7) (a) Thomson, A. J.; Johnson, M. K. *Biochem. J.* **1980**, *191*, 411. (b) Bennett, D. E.; Johnson, M. K. *Biochim. Biophys. Acta* **1987**, *911*, 71.

(8) Resonances within the $M_s = \pm{}^{1}/_{2}, \pm{}^{7}/_{2}, \pm{}^{9}/_{2}$ doublets are not observed. Only the low-field g value would be observable since the other two components have $g < 1$. However, the transition probabilities for the low-field components of the resonances within low-lying $M_s = \pm{}^{7}/_{2}$ and $\pm{}^{9}/_{2}$ doublets are vanishingly small, since they are proportional to the sum of the squares of the other two g values. The low-field component of the resonance with the highest energy $M_s = \pm{}^{1}/_{2}$ doublet has a slightly higher transition probability, but is likely to be broadened by relaxation effects at the temperatures required for significant population of this doublet.

(9) (a) Werth, M. T.; Cecchini, G.; Manodori, A.; Ackrell, B. A. C.; Schröder, I.; Gunsalus, R. P.; Johnson, M. K. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 8965. (b) Cheng, H.; Xia, B.; Reed, G. H.; Markley, J. L. *Biochemistry* **1994**, *33*, 3155.

(10) Fu, W.; Drozdzewski, P. M.; Davies, M. D.; Sligar, S. G.; Johnson, M. K. *J. Biol. Chem.* **1992**, *267*, 15502.

(11) In closely related mixed-valence compounds, the intervalence band is red-shifted in valence-delocalized compared to valence-localized species (Hupp, J. T.; Dong, Y. *J. Am. Chem. Soc.* **1993**, *115*, 6428). The intervalence band for valence-localized [Fe_2S_2]$^+$ clusters has been assigned to an absorption band and a positive VTMCD feature in the range 500−570 nm, see footnote 10.

(12) (a) Johnson, M. K.; Robinson, A. E.; Thomson, A. J. In *Iron-Sulfur Proteins*; Spiro, T. G., Ed.; Wiley: New York, 1982; pp 367−406. (b) Butt, J. N.; Armstrong, F. A.; Breton, J.; George, S. J.; Thomson, A. J.; Hatchikian, E. C. *J. Am. Chem. Soc.* **1991**, *113*, 6663. (c) Conover, R. C. Ph.D. Thesis, University of Georgia, 1993. (d) Fu, W.; Telser, J.; Hoffman, B. M.; Smith, E. T.; Adams, M. W. W.; Finnegan, M. G.; Conover, R. C.; Johnson, M. K. *J. Am. Chem. Soc.* **1994**, *116*, 5722. (e) Butt, J. N.; Niles, J.; Armstrong, F. A.; Breton, J.; Thomson, A. J. *J. Struct. Biol.* **1994**, *1*, 427. (f) Finnegan, M. G.; Conover, R. C.; Park, J.-B.; Zhou, Z. H.; Adams, M. W. W.; Johnson, M. K. *Inorg. Chem.*, in press.