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Hydrogen-1 Nuclear Magnetic Resonance of Selenium-Substituted Clostridial Ferredoxins†

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^{1}H NMR spectra of Se-substituted 2(4Fe-4Se) clostridial ferredoxins were recorded at 250 MHz and compared to those of their native counterparts. Upon S* → Se* substitution, the spectra of the oxidized ferredoxins underwent only minor modifications, consisting mainly of slight (ca. 1–2 ppm) increases in the chemical shifts of the hyperfine-shifted proton resonances. In contrast, the spectra of the reduced ferredoxins were strikingly dependent upon the inorganic chalcogenide present in the active sites; whereas the hyperfine-shifted proton resonances of the reduced native proteins were restricted to the 0–60 ppm region, those of the reduced Se-substituted proteins were observed over the much wider −40 to +160 ppm range. The bulk magnetic susceptibility of the latter ferredoxins (ca. 6 μ_B) was larger than that (ca. 4 μ_B) of $(4Fe\text{-}4S)^+$ clusters assuming $S = {}^1/_2$ spin states at low temperature. The proton resonances of the reduced Se-substituted clostridial ferredoxins could be divided into two subsets differing markedly by the temperature dependencies of their chemical shifts. In one of these subsets, the temperature dependencies of the chemical shifts were lower than 0.2 ppm/K, whereas in the other one they were on the average three times higher (1 ppm/K in some cases). From these observations it is inferred that two different spin-state ladders occur in the active sites of $2(4Fe\text{-}4Se)^+$ ferredoxins and that these proteins retain at room temperature some of the unusual magnetic properties previously evidenced at cryogenic temperatures by EPR and Mössbauer spectroscopy.

Introduction

The inorganic sulfur atoms of the two (4Fe-4S) clusters present in *Clostridium pasteurianum* ferredoxin (CpFd) can be selectively replaced by selenium[1] without significantly altering either the biochemical properties of the protein[2] or the structure of its oxidized active sites.[3,4] However, the EPR and Mössbauer spectra of the reduced Se-substituted Fd display unusual features[3] which have been attributed to $S = {}^3/_2$ and $S = {}^7/_2$ spin states of the two $(4Fe\text{-}4Se)^+$ clusters in Cp Fd[3] and in other clostridial ferredoxins.[5] These high spin states, which occur in addition to the usual $S = {}^1/_2$ spin state of $(4Fe\text{-}4S)^+$ clusters,[6,7] have been shown to arise from specific interactions between the clostridial Fd polypeptide chain and the reduced $(4Fe\text{-}4Se)^+$ clusters.[5]

However, all the above results have been obtained with frozen samples, and the occurrence of unusual electronic and magnetic properties in $2(4Fe\text{-}4Se)^+$ Fd remains to be established at functionally relevant temperatures. Valuable information on the latter point may be expected from ^{1}H NMR spectroscopy, which is particularly suited to the investigation of systems in liquid aqueous solutions, and which can use the protons neighboring metal centers as probes of their magnetic state. ^{1}H NMR studies of (4Fe-4S) proteins[8,9] have allowed the characterization of numerous magnetically shifted resonances of C_α and C_β protons of cysteine residues coordinated to the cluster. The shifts experienced by the protons coupled to the $(4Fe\text{-}4S)^{2+}$ clusters, which display a diamagnetic ground state, were largely increased upon reduction to the $(4Fe\text{-}4S)^+$ or oxidation to the $(4Fe\text{-}4S)^{3+}$ paramagnetic ($S = {}^1/_2$) states.[8] ^{1}H NMR studies of model compounds have shown that the proton shifts are mainly contact in origin.[10,11]

We here report ^{1}H NMR spectra of 2(4Fe-4Se) Cp Fd and *Clostridium acidi-urici* Fd in both of their redox states. The spectra of the oxidized Se-substituted proteins are very similar to those of their native counterparts. In the reduced proteins, however, the presence of selenium results in the occurrence of very large chemical shifts for some protons and unusually high values of bulk magnetic susceptibility. Thus, the reduced Se-substituted clostridial ferredoxins retain unusual magnetic properties at temperatures that are physiologically relevant.

Materials and Methods

The ferredoxins from *C. pasteurianum* and from *C. acidi-urici* were purified and their Se-substituted counterparts were prepared as previously described.[2,5] All experiments were performed under an argon atmosphere. Solvent exchange was carried out by repeated concentration and dilution with deuterated solvent in an Amicon filtration device fitted with a YM5 membrane. The deuterated solvent (50 mM potassium phosphate) was prepared by dissolving 8.2 g of K_2HPO_4 and 0.36 g of KH_2PO_4 in 1 L of D_2O (99.8%, from the Commissariat à l'Energie Atomique, Saclay, France). When dissolved in H_2O, these amounts of phosphate yield solutions having a pH of 8.0, and therefore the pD of the deuterated solvent was not measured. The ferredoxin samples were reduced by adding small increments of a 0.1 M dithionite solution prepared with the deuterated solvent. The extent of reduction was monitored spectrophotometrically,[2] and checked after recording the NMR spectra.

^{1}H NMR measurements were carried out on a Bruker WM-250 spectrometer. Samples were transferred anaerobically into 5-mm tubes (Wilmad Glass Co) stoppered with rubber septa. Typical spectra resulted from the sum of ca. 5000 transients, which represented an acquisition time of ca. 30 min. Chemical shifts were referenced to the internal standard sodium 3-(trimethylsilyl)tetradeuteriopropionate (TSTP). Magnetic susceptibilities were measured as described previously.[12] The standard pulse sequence was used for the determination of T_1 relaxation times.

Results

Spectra of Oxidized Proteins. ^{1}H NMR spectra of the oxidized native and Se-substituted Cp Fd are shown in Figure 1. They display very similar patterns of broad signals extending down to almost 20 ppm. All these resonances are shifted to lower field upon increasing the temperature (not shown) and probably undergo interactions of contact origin.[8] In view of the general resemblance of the spectra shown in Figure 1 with that of native Cau Fd, the assignments proposed for the latter protein[9] have been indicated in Table I and may be used as a guide for discussing

† Abbreviations used throughout this paper: Cau, *Clostridium acidi-urici*; Cp, *Clostridium pasteurianum*; EPR, electron paramagnetic resonance; Fd, ferredoxin; NMR, nuclear magnetic resonance; S* and Se*, bridging inorganic chalcogenide atoms of ferredoxin active sites; TSTP, sodium 3-(trimethylsilyl)tetradeuteriopropionate.

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(1) Meyer, J.; Moulis, J.-M. *Biochem. Biophys. Res. Commun.* **1981**, *103*, 667.
(2) Moulis, J.-M.; Meyer, J. *Biochemistry* **1982**, *21*, 4762.
(3) Moulis, J.-M.; Auric, P.; Gaillard, J.; Meyer, J. *J. Biol. Chem.* **1984**, *259*, 11396.
(4) Moulis, J.-M.; Meyer, J.; Lutz, M. *Biochemistry* **1984**, *23*, 6605.
(5) Gaillard, J.; Moulis, J.-M.; Auric, P.; Meyer, J. *Biochemistry* **1986**, *25*, 464.
(6) Cammack, R.; Dickson, D. P. E.; Johnson, C. E. In *Iron-Sulfur Proteins*; Lovenberg, W., Ed.; Academic: New York, 1977; Vol. 3, Chapter 8.
(7) Lane, R. W.; Wedd, A. G.; Gillum, W. O.; Laskowski, E. J.; Holm, R. H.; Frankel, R. B.; Papaefthymiou, G. C. *J. Am. Chem. Soc.* **1977**, *99*, 2350.
(8) Phillips, W. D.; Poe, M. In *Iron-Sulfur Proteins*; Lovenberg, W., Ed.; Academic: New York, 1977; Vol. 2, Chapter 7.
(9) Packer, E. L.; Sweeney, W. V.; Rabinowitz, J. C.; Sternlicht, H.; Shaw, E. N. *J. Biol. Chem.* **1977**, *252*, 2245.
(10) Holm, R. H.; Phillips, W. D.; Averill, B. A.; Mayerle, J. J.; Herskovitz, T. *J. Am. Chem. Soc.* **1974**, *96*, 2109.
(11) Reynolds, J. G.; Laskowski, E. J.; Holm, R. H. *J. Am. Chem. Soc.* **1978**, *100*, 5315.
(12) Phillips, W. D.; Poe, M. *Methods Enzymol.* **1972**, *24B*, 304.

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Table I. Hyperfine-Shifted ^{1}H NMR Signals of Oxidized $(4Fe\text{-}4X)^{2+}$ (X = S, Se) Ferredoxins[a]

C. acidi-urici[b] (native)	*C. pasteurianum*[c] (native)	*C. pasteurianum*[d] (Se substituted)	*B. polymyxa*[e] (native)	*B. stearothermophilus*[f] (native)
16.4 (β)	17.2 (β)	18.6 (β)		
15.6 (β)	16.1 (β)	18.4 (β)		
15.4 (β)	15.7 (β)	17.4 (β)		
15.1 (β)	14.8 (β)	16.7 (β)		
13.5 (β)	13.5 (β)	16.2 (β)	15.4 (β)	15.5 (β)
12.3 (β)	12.3 (β)	14 (β)		
11.5 (β)	11.9 (β)	13.3 (β)	13.6 (β)	13.5 (β)
11 (β)	11.1 (β)	11.7 (β)		
10 (α)	10 (α)	11.7		
9.6 (α)	9.9 (α)	11.1 (α)		
9.4	9.4	10.9	10.9	10.8
9.4	9.1	10.5 (α)	10.6	
9.2 (β)	8.9	9.2	9.9	
9		9.1	9.8	
8.2 (β)		8.9		
8 (β)		8.7	8.8	

[a] Chemical shifts are given in ppm relative to the TSTP reference. Unless otherwise specified, the relative positioning, in this table, of the resonances of the various proteins should not be taken as inferring any correlation between their assignments. [b] Reference 9. (α) and (β) indicate assignments of resonances to C_α and C_β protons, respectively, of the cysteine residues. [c] Reference 13. The assignments of protons resonances are deduced from those of *C. acidi-urici* Fd by homology. [d] This work. The assignments of proton resonances are only tentative (see text). [e] Reference 14. [f] Reference 15.

Table II. Hyperfine-Shifted ^{1}H NMR Signals of Reduced $(4Fe\text{-}4X)^{+}$ (X = S, Se) Ferredoxins[a]

C. pasteurianum[b] (native)	*C. acidi-urici*[c] (native)	*C. pasteurianum*[d] (Se substituted)	*C. acidi-urici*[d] (Se substituted)	*B. polymyxa*[e] (native)	*B. stearothermophilus*[f] (native)
60.3	56	159.9[h]	145.5[h]	43.2 (2)	48
57.2[g]	56[g]	107.2[h]	106.2[h]	36.8[g]	46
54.8	41	106.4[h]	97.9[h]	34.5	37
41	40	87.6	90.3	33.2[g]	35[g]
38.5[g]	37.3 (2)[g]	86.4	80.1	22.7[g]	31[g]
33.5 (2)[g]	37.3	76.6[h]	79	20	21
28	31.8[g]	71.2	74[h]	17.3[g]	18[g]
26.5	28.2	64.7[g]	63.8	15.5	17[g]
25.8[g]	26.4[g]	62.5[h]	54.4[h]	14.5[g]	16
25.4[g]	25[g]	57.1	38[g]	13.6[g]	13[g]
22	24	36.8	33.7		
20[g]	19[g]	31	29.8		
17.2[g]	18.2[g]	19.7	10.9		
15.8[g]	15.9 (2)[g]	11.4			
14.5	13.6[g]	10.6	−1.5		
11.5[g]		−4.7[h]	−1.6		
		−8.9 (2)	−3.5 (2)		
		−40.8[h]	−36.7[h]		
		−43.5[h]	−38.2[h]		

[a] Chemical shifts are given in ppm relative to the TSTP reference. Unless otherwise specified, the relative positioning, in this table, of the resonances of the various proteins should not be taken as inferring any correlation between their assignments. Numbers in parentheses indicate the numbers of peaks observed at that frequency. [b] Reference 13. T = 295 K. [c] Reference 8. T = 295 K. [d] This work. T = 295 K. [e] Reference 14. T = 303 K. [f] Reference 15. T = 300 K. [g] Peaks whose shifts increase with increasing temperature. [h] Peaks whose shift variation with temperature is higher than 0.2 ppm/K.

the spectra of Cp ferredoxins: according to these assignments, most of the shifted lines arise from C_β cysteinyl protons. Some C_α cysteinyl or very slowly exchangeable protons may occur in the 8–10 ppm range. The peaks observed between 9.5 and 17.2 ppm in the spectrum of native Cp Fd are shifted to the 10.5–18.6 ppm range upon S* → Se* substitution. Among the latter, the 11.06 and 10.52 ppm peaks display a somewhat narrower line width than the others and they can therefore confidently be attributed to C_α cysteinyl protons. Thus, the C_α and C_β cysteinyl protons giving rise to these resonances experience slightly stronger magnetic interactions in the Se-substituted protein than in the native protein.

Spectra of Reduced Proteins. The spectrum of reduced Cp Fd (Figure 2) is identical with the one published previously[13] and

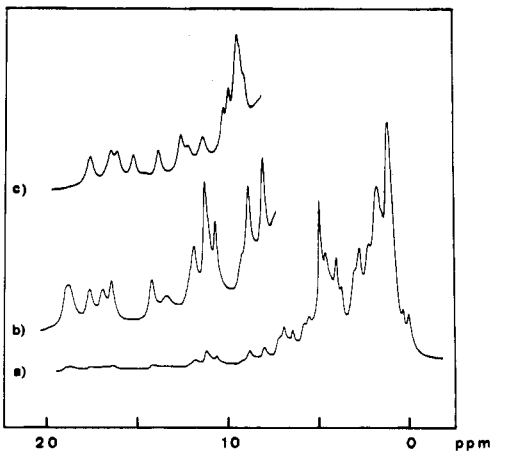

Figure 1. 250-MHz ^{1}H NMR spectra of oxidized *C. pasteurianum* ferredoxins at 295 K and pH 8.0: (a) Se-substituted ferredoxin; (b) Se-substituted ferredoxin with an 8-fold expansion of the vertical scale; (c) Native ferredoxin.

(13) Phillips, W. D. In *NMR of Paramagnetic Molecules*; La Mar, G. N., Horrocks, Jr., W. D., Holm, R. H., Eds.; Academic: New York, 1973; Chapter 11.
(14) Phillips, W. D.; Mc Donald, C. C.; Stombaugh, N. A.; Orme-Johnson, W. H. *Proc. Natl. Acad. Sci. U.S.A.* **1974**, *71*, 140.
(15) Nagayama, K.; Ozaki, Y.; Kyogoku, Y.; Hase, T.; Matsubara, H. *J. Biochem. (Tokyo)* **1983**, *94*, 893.

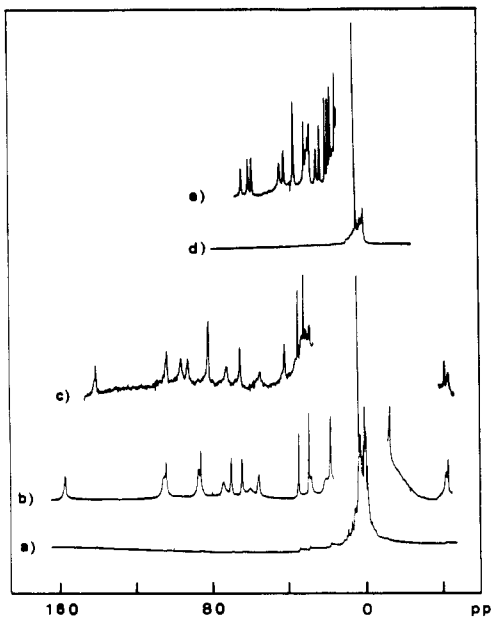

Figure 2. 250-MHz ^{1}H-NMR spectra of reduced clostridial ferredoxins at 295K and pH 8.0: (a) Se-substituted *C. pasteurianum* ferredoxin; (b) Se-substituted *C. pasteurianum* ferredoxin with a 16-fold expansion of the vertical scale; (c) Se-substituted *C. acidi-urici* ferredoxin; (d) Native *C. pasteurianum* ferredoxin; (e) native *C. pasteurianum* ferredoxin with a 16-fold expansion of the vertical scale.

shows that large chemical shifts are experienced by the protons neighboring the paramagnetic cluster (Table II). The same number of shifted resonances is observed in spectra of *C. acidi-urici* Fd, with, however, differences of up to 14 ppm between the shifts of some presumably homologous peaks.[8]

The 250-MHz ^{1}H NMR spectrum of the reduced Se-substituted Cp Fd differs markedly from that of the reduced native protein (Figure 2); resonances occur over a considerably more extended field range, from −40 to 160 ppm. In addition, all the positive resonances are displaced to higher fields and all the negative ones to lower fields, with increasing temperature, except for the 65 ppm one, which has a constant shift magnitude in the investigated temperature range (Figure 3). Altogether at least 20 peaks are observed outside of the 0–10 ppm range, each of them having an integrated intensity amounting to one proton. These resonances can be split in two groups following the temperature dependencies of their chemical shifts (Figure 2, Table II): some of them have slopes lower than 0.2 ppm/K, which is the highest slope observed in the case of the reduced native Fd, whereas the other ones display slopes that may be as high as 1 ppm/K and are in average three times higher than those of the first group. The resonances that are most temperature dependent occur in the highest and lowest field regions, with the few following exceptions: the shift of the −4.7 ppm peak is highly temperature dependent, whereas the low-field 87.6 and 86.4 ppm peaks display weak temperature dependencies (Table II). Both groups include similar numbers of resonances (eight to ten), and within each group the temperature dependencies increase with increasing chemical shift magnitude.

An estimation of the T_1 relaxation times has been made: they vary from 2 ms for the broadest, most shifted lines, to 8–10 ms for the narrowest ones at ca. 20 ppm. These values are consistent with protons experiencing contact shifts of variable amplitude due

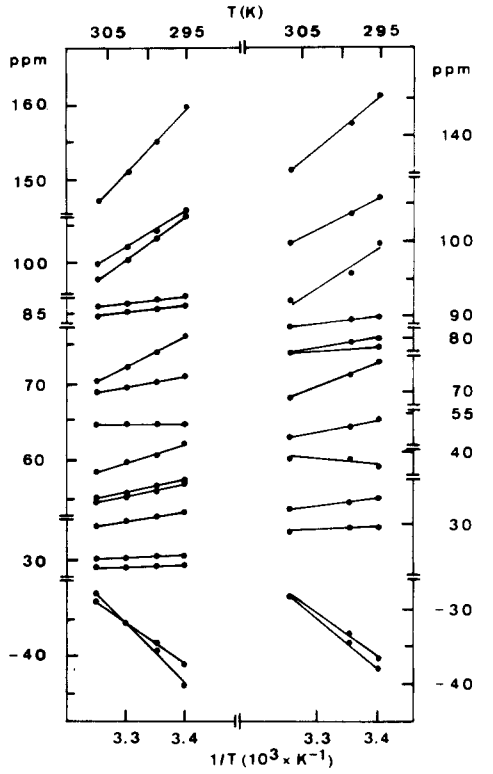

Figure 3. Curie plots for reduced Se-substituted clostridial ferredoxins: Left, *C. pasteurianum*; right, *C. acidi-urici*.

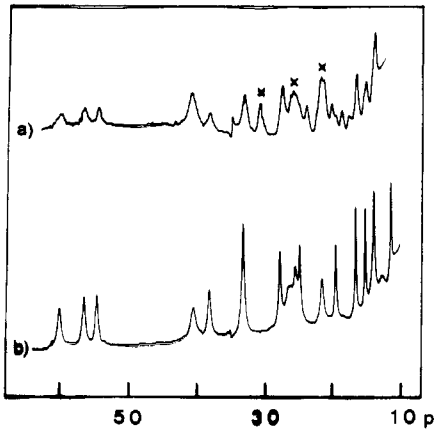

Figure 4. 250-MHz ^{1}H-NMR spectra of native *C. pasteurianum* ferredoxin: (a) 50% reduced, with peaks arising from averaged resonances labeled ×; (b) 100% reduced.

to their relative positions with respect to the spin density distribution.

The spectrum of reduced Se-substituted Cau Fd is very similar to that of Cp Fd (Figure 2). In view of the homology of their temperature dependencies, a close correlation seems likely for most of the peaks in the two spectra (Table II). This resemblance reveals very similar spin density distributions in the two proteins, as previously inferred from comparative EPR investigations[5] and similar patterns of proton arrangements around the (4Fe-4Se) clusters.

Partially Reduced Proteins. In previous NMR studies of clostridial ferredoxins, spectra of protein samples in intermediate reduction states were found to display resonances additional to those observed in the fully reduced or in the fully oxidized state. They were interpreted as arising from the occurrence of rapid (on the NMR time scale) electron transfer between reduced and oxidized clusters.[8] This is shown in Figures 4 and 5 for both the native and the Se-substituted Cp Fd. At 40–60% reduction, the resonances of the intermediate state reach their maximum intensity, while those of the fully reduced and of the fully oxidized

(16) Poe, M.; Phillips, W. D.; Mc Donald, C. C.; Lovenberg, W. *Proc. Natl. Acad. Sci. U.S.A.* **1970**, *65*, 797.
(17) Poe, M.; Phillips, W. D.; Mc Donald, C. C.; Orme-Johnson, W. H. *Biochem. Biophys. Res. Commun.* **1971**, *42*, 705.
(18) Bobrik, M. A.; Laskowski, E. J.; Johnson, R. W.; Gillum, W. O.; Berg, J. M.; Hodgson, K. O.; Holm, R. H. *Inorg. Chem.* **1978**, *17*, 1402.
(19) Laskowski, E. J.; Reynolds, J. G.; Frankel, R. B.; Foner, S.; Papaefthymiou, G. C.; Holm, R. H. *J. Am. Chem. Soc.* **1978**, *101*, 6562.
(20) Moura, J. J. G.; Xavier, A. V.; Bruschi, M.; LeGall, J. *Biochim. Biophys. Acta* **1977**, *459*, 278.

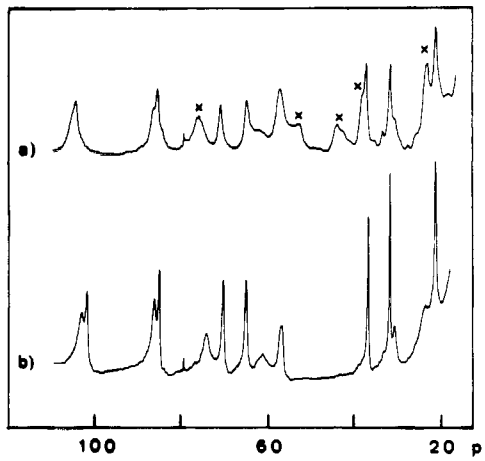

Figure 5. 250-MHz ^{1}H-NMR spectra of Se-substituted *C. pasteurianum* ferredoxin: (a) 60% reduced, with peaks arising from averaged resonances labeled ×; (b) 100% reduced.

protein are weak. As reduction proceeds, the peaks of the fully reduced protein strengthen and narrow, but those of the intermediate state disappear only above 90% reduction. We therefore believe that the spectra shown in Figure 2, which display no coalesced lines arising from rapid intercluster electron exchange, are the true spectra of the completely reduced proteins. A lower limit of 10^3 s^{-1} for the rate of the intercluster electron exchange had previously been estimated.[8] As the proton resonances of the Se-substituted Cp Fd undergo up to 3-fold larger shifts upon reduction than those of the native protein (Table II), the lower limit for the rate of electron exchange should probably be increased by a factor of 3.

Magnetic Susceptibility Measurements. The frequency shifts undergone by a reference proton in various ferredoxin solutions were measured and used to calculate the effective magnetic moments of these ferredoxins (Table III). For comparison, we have also gathered in Table III the previously published effective magnetic moments of several proteins and synthetic analogues in various oxidation states, as measured at room temperature. The somewhat lower value obtained here for native oxidized Cp Fd may be due to the large relative uncertainty on the measurement of the weak frequency shift or to the more complete removal of adventitious iron in our sample. Otherwise the magnetic moments of all the oxidized clusters are nearly equal.

Among the reduced clusters, those which display low-temperature EPR spectra characteristic of $S = {}^1/_2$ spin states only, have room-temperature effective magnetic moments in the 3.2–4.4-μ_B range. Higher values (ca. 5 μ_B; see Table III) have been measured with synthetic clusters distorted by solid-state effects and displaying low-field EPR features.[11] The reduced Se-substituted Cp Fd has an effective magnetic moment higher than 6 μ_B (Table III), which provides further evidence for the occurrence of higher spin states in this protein than in $(4Fe\text{-}4S)^+$ ferredoxins.

Discussion

The $(4Fe\text{-}4S)^{2+}$ clusters possess a diamagnetic $S = 0$ ground state arising from antiferromagnetic coupling of the iron atoms.[6] As the temperature is raised, higher energy paramagnetic states are populated in both the native and the Se-substituted Fd, as revealed by the low-field positions and the temperature dependencies of the resonances of the protons neighboring the active site. The greater magnitude of the shifts in spectra of Se-substituted Fd results at least in part from an easier accessibility of the excited states, as shown by the magnetic susceptibility values measured at room temperature (Table III). Similar increases of the contact shifts upon S* → Se* substitution have been observed in the case of synthetic analogues.[21-23] Despite these slight differences in chemical shifts, the spectra of Figure 1 are similar to each other, which confirms that the oxidized (4Fe-4S) and (4Fe-4Se) clusters in Cp Fd differ very little in structure and properties, as previously inferred from other investigations.[1-4]

The contact-shifted proton resonances of Se-substituted Cp Fd undergo large frequency shifts upon reduction of the active sites from the $(4Fe\text{-}4Se)^{2+}$ to the $(4Fe\text{-}4Se)^+$ state. These shifts are considerably greater than those observed upon a one-electron-redox transition for any other (4Fe-4X) cluster, since the ca. 60 ppm chemical shift range (from 0 to +60 ppm) of native reduced ferredoxins is expanded to ca. 200 ppm (from −40 ppm to +160 ppm) in the case of the reduced Se-substituted Cp Fd. These spectral differences do not result simply from a change in spin density distribution due to the replacement of S* by Se* in the $S = {}^1/_2$ $(4Fe\text{-}4X)^+$ (X = S, Se) structure. Indeed, in the case of synthetic analogues, variations of proton resonance shifts upon reduction are similar for (4Fe-4S) and for (4Fe-4Se) clusters.[11,22] The large shifts experienced by the proton resonances of reduced 2(4Fe-4Se) ferredoxins are therefore best rationalized by assuming the occurrence, in these proteins, of high-spin states. The latter hypothesis is supported by the large magnetic susceptibility of $2(4Fe\text{-}4Se)^+$ Cp Fd (Table III) and by our previous low-temperature EPR and Mössbauer investigations.[3,5]

The spin states occurring at room temperature, where the NMR spectra were recorded, cannot be easily determined, mainly because of the thermal population of spin states of higher energy than those observed at cryogenic temperatures. Evidence for the thermal population of high-spin states has been given above for the oxidized $(4Fe\text{-}4X)^{2+}$ clusters and is also available in the case of the reduced $(4Fe\text{-}4X)^+$ clusters; indeed, those among the latter that assume a $S = {}^1/_2$ spin state at low temperature show room-temperature

Table III. Magnetic Susceptibility of (4Fe-4X) (X = S, Se) Clusters in Proteins and in Synthetic Analogues

	concn, μM	Δf,[a] Hz	μ_{eff}, μ_B	T, K	source
		$(4Fe\text{-}4X)^{2+}$			
		X = S			
C. pasteurianum Fd	740	1	1.3	298	this work
C. pasteurianum Fd			2.1	298	16
C. acidi-urici Fd			2.4	298	17
B. polymyxa Fd			1.8	295	14
$(Me_4N)_2[Fe_4S_4(SPh)_4]$			2.2	299	18
$(Et_4N)_2[Fe_4S_4(SCH_2Ph)_4]$			2.1	299	19
		X = Se			
C. pasteurianum Fd	684	2.7	2.1	298	this work
$(Me_4N)_2[Fe_4Se_4(SPh)_4]$			2.5	299	18
		$\|4Fe\text{-}4X\|^+$			
		X = S			
C. pasteurianum Fd	600	9	4.2	298	this work
C. pasteurianum Fd	1000	17	4.4	298	this work
D. gigas FdI			4		20
B. polymyxa Fd			3.2–3.4	288–303	14
$(Et_4N)_3[Fe_4S_4(SPh)_4]$	solution		3.8	304	11
$(Et_3MeN)_3[Fe_4S_4(SPh)_4]$	solid		4.6	299	19
$(Et_4N)_3[Fe_4S_4(SCH_2Ph)_4]$	solution		3.7	294	11
$(Et_4N)_3[Fe_4S_4(SCH_2Ph)_4]$	solid		5.1	299	19
		X = Se			
C. pasteurianum Fd	620	22	6.4	298	this work
	1800	62	6.3	298	this work

[a] Change in the proton resonance frequency of the TSTP reference caused by the paramagnetic protein (see Materials and Methods).

(21) Christou, G.; Ridge, B.; Rydon, H. N. *J. Chem. Soc., Dalton Trans.* **1978**, 1423.

(22) Reynolds, J. G.; Coyle, C. L.; Holm, R. H. *J. Am. Chem. Soc.* **1980**, *102*, 4350.

(23) Reynolds, J. G.; Holm, R. H. *Inorg. Chem.* **1981**, *20*, 1873.

magnetic susceptibilities indicative of spin states significantly higher than $^1/_2$[19] (Table III). The room temperature magnetic susceptibility and the chemical shifts of the protons of the $(4Fe\text{-}4Se)^+$ clusters of reduced Se-substituted Cp Fd are larger than those of native reduced Cp Fd (Figure 2, Table III). These observations may be correlated with the occurrence of high-multiplicity spin states at low temperature.

At cryogenic temperatures, the reduced Se-substituted clostridial ferredoxins display three spin states, namely $S = {}^1/_2$, $S = {}^3/_2$ and $S = {}^7/_2$[3,5], and one may wonder whether a similar spin-state heterogeneity occurs at functionally relevant temperatures. The ^{1}H NMR spectra of the reduced Fd from *Bacillus polymyxa*[14] and *Bacillus stearothermophilus*,[15] which contain a single $(4Fe\text{-}4S)^+$ cluster, display eight to ten manetically shifted proton resonances, out of the 0–10 ppm range. The spectra of the reduced clostridial ferredoxins involve ca. twice as many (17–20) shifted proton resonances as those of the ferredoxins containing a single cluster[8] (Table II). It may therefrom be inferred that the two clusters in clostridial Fd differ from each other with respect to the chemical shifts of their neighboring protons. The reduced Se-substituted Cp Fd displays no more shifted proton resonances than its native counterpart (Table II), which suggests the presence of only two different types of $(4Fe\text{-}4Se)^+$ clusters in the former protein; the occurrence of three sets of spin states would imply that ca. eight to ten proton resonances have escaped observation. This seems unlikely, since no additional proton resonances have been observed in the 170–330 ppm range. Proton resonances occurring at lower field would probably be difficult to detect, due to line broadening. They would however be associated with clusters having high magnetic susceptibility, and therefore the presence of very low field resonances would result in a bulk magnetic susceptibility value even higher than the measured one (6.4 μ_B; see Table III). The ^{1}H NMR data thus show that two different spin-state ladders occur at room temperature. The relationship between these two spin ladders and the three low-temperature spin states remains to be established. In any case, the presence, in the spectra of $2[4Fe\text{-}4Se]^+$ ferredoxins, of two sets of proton resonances differing strongly by their chemical shifts and by the temperature dependencies of these shifts indicate that the $(4Fe\text{-}4Se)^+$ clusters bound to clostridial Fd polypeptide chains possess unusual magnetic properties not only at low temperature but also at room temperature. The evidence that high-spin states of $(4Fe\text{-}4X)^+$ clusters are not simply a freezing artifact, at least in the presently investigated case, clearly point to the functional relevance of the high-spin states found in some native $(4Fe\text{-}4S)^+$ proteins.[24]

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(24) Lindahl, P. A.; Day, E. P.; Kent, T. A.; Orme-Johnson, W. H.; Münck, E. *J. Biol. Chem.* **1985**, *260*, 11160.

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Low-Temperature Oxidation of [4Fe-4S] Analogues. Generation of an Fe/S Cluster Spectroscopically Similar to the 3-Fe Clusters in the 3-Fe Ferredoxins

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The oxidation of the cubane cluster compound $[Fe_4S_4(SR)_4]^{2-}$ can be directed to yield a 3-Fe cluster (i.e. a Fe/S cluster spectroscopically similar to the 3-Fe centers in the 3-Fe ferredoxins) by the choice of DMF/water as reaction medium, $K_3[Fe(CN)_6]$ as oxidant, and a low reaction temperature. The resulting compound at 4.2 and 40 K yields Mössbauer spectra that are typical for a 3-Fe cluster. At 40 K a quadrupole splitting of 0.58 mm/s and an isomer shift of 0.31 mm/s are observed. Its X-band ESR signal at $g = 2.01$ has a width of 2.8 mT and displays the same shape as that of 3-Fe proteins, including the remarkably broad wing at the high-field side of the spectrum. The influences of the reaction medium, the reagents, and the initial concentrations are discussed.

Introduction

Since 1980 it has been well established that *Azotobacter vinelandii* Fd (ferredoxin) I *crystals* contain a 3-Fe cluster[3] with an $Fe_3S_3L_6$ ring structure[4] (where L = RS^- or RO^-; Figure 1a). A second type of 3-Fe cluster with an $Fe_3S_4L_3$ cap structure (Figure 1b) has been postulated for Aconitase on the basis of EXAFS measurements.[5] This has been supported by X-ray diffraction measurements.[6] Another type of 3-Fe cluster has recently been found in denatured Aconitase and proved to have a linear structure[7] (Figure 1c). Of these three structures the cap type seems to be the most common.[8,9] It probably also is the structure of the 3-Fe cluster in *A. vinelandii* Fd I *solutions*.[9,10] The native 3-Fe structures typically display axial $S = {}^1/_2$ ESR spectra slightly above $g = 2$ with a width of about 3 mT. When first observed, these resonances were confused with the HP (high-potential protein) signal. The linear 3-Fe cluster is readily distinguished by its $S = {}^5/_2$ ESR signal around $g = 4.3$ and $g = 9.6$. Until recently it was thought that 3-Fe clusters might only

(1) Eindhoven University of Technology.
(2) Gray Freshwater Biological Institute.
(3) Emptage, M. H.; Kent, T. A.; Huynh, B.-H.; Rawlings, J.; Orme-Johnson, W. H.; Münck, E. *J. Biol. Chem.* **1980**, *255*, 1793.
(4) Stout, C. D.; Ghosh, D.; Pattabhi, V.; Robbins, A. H. *J. Biol. Chem.* **1980**, *255*, 1797.
(5) Beinert, H.; Emptage, M. H.; Dreyer, J.-L.; Scott, R. A.; Hahn, J. E.; Hodgson, K. O.; Thomson, A. J. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 393.
(6) Robbins, A. H.; Stout, C. D. *J. Biol. Chem.* **1985**, *260*, 2328.
(7) Kennedy, M. C.; Kent, T. A.; Emptage, M.; Merkle, H.; Beinert, H.; Münck, E. *J. Biol. Chem.* **1984**, *259*, 14463.
(8) Antonio, M. R.; Averill, B. A.; Moura, I.; Moura, J. J. G.; Orme-Johnson, W. H.; Teo, B.-K.; Xavier, A. V. *J. Biol. Chem.* **1982**, *257*, 6646.
(9) Johnson, M. K.; Czernuszewicz, R. S.; Spiro, T. G.; Fee, J. A.; Sweeney, W. V. *J. Am. Chem. Soc.* **1983**, *105*, 6671.
(10) Scott, R. A.; Penner-Hahn, J. E.; Hodgson, K. O.; Beinert, H.; Stout, C. D. *Springer Proc. Phys.* **1984**, *2* (*EXAFS Near Edge Struct. 3*), 105–110.

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