Analysis of the high-spin states of the $2[4\text{Fe-4Se}]^+$ ferredoxin from *Clostridium pasteurianum* by Mössbauer spectroscopy

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A Mössbauer study of the three spin states of the reduced selenium-substituted $2[4\text{Fe-4Se}]^+$ ferredoxin from *Clostridium pasteurianum* was carried out at $T = 1.6$ K, in perpendicular and parallel applied magnetic fields of up to 10 T. In low (0.1 T) applied fields, the spectra of the classical $S = \frac{1}{2}$ state exhibit magnetic hyperfine patterns arising from two pairs of iron atoms with opposite hyperfine fields. The $S = \frac{7}{2}$ state is in the slow relaxation limit at $T \leq 4$ K in the absence of applied field. The corresponding Mössbauer spectrum can be understood in terms of anti-parallel coupling between one high-spin Fe^{3+} ion ($H_{\text{hf}} = +21.2$ T) and three high-spin Fe^{2+} ions ($H_{\text{hf},xy} = -25.5$ T, $H_{\text{hf},z} = -28.5$ T). For the $S = \frac{3}{2}$ spin state, the use of high (8–10 T) applied fields was necessary to ensure the validity of the high-field approximation and overcome the intercluster spin–spin interaction. The spectral data obtained under such conditions allowed the determination of the hyperfine field (-4.2 T) and a tentative estimation of the zero-field splitting ($D \approx 3 \text{ cm}^{-1}$).

INTRODUCTION

The reduced Se-substituted $2[4\text{Fe-4Se}]$ Cp Fd has been found to display $S = \frac{1}{2}$, $S = \frac{3}{2}$ and $S = \frac{7}{2}$ spin states (Moulis *et al.*, 1984). This unusual occurrence has been shown, by comparing the e.p.r. spectra of several Se-substituted ferredoxins, to result from specific interactions, not yet fully understood, between the polypeptide chain of clostridial ferredoxins and the $[4\text{Fe-4Se}]^+$ clusters (Gaillard *et al.*, 1986).

From the shape and temperature-dependence of its e.p.r. signal, the $S = \frac{1}{2}$ spin state of reduced Se-substituted Cp Fd has been inferred to be very similar to its counterpart of the reduced native ferredoxin (Moulis *et al.*, 1984). The $S = \frac{7}{2}$ spin multiplet has been characterized in detail, as all of its four Kramer's doublets give rise to e.p.r. or Mössbauer components: it has a negative fine-structure parameter, the outer lines of the low-temperature Mössbauer spectra are attributable to the ground $\pm \frac{7}{2}$ doublet, and the $g = 10.11$, 5.17 and 12.76 e.p.r. lines are attributable to the excited $\pm \frac{5}{2}$, $\pm \frac{3}{2}$ and $\pm \frac{1}{2}$ doublets respectively (Gaillard *et al.*, 1986). In contrast, the $S = \frac{3}{2}$ spin state has so far remained uncompletely described: only its $\pm \frac{1}{2}$ ground doublet contributes to the e.p.r. spectra (in the $g = 3$ – 4 region), and its contribution to the Mössbauer spectra could not be analysed because it is superimposed on that of the $S = \frac{1}{2}$ spin state (Moulis *et al.*, 1984).

We report here Mössbauer spectra of the reduced $2[4\text{Fe-4Se}]^+$ Cp Fd, recorded at 1.6 K in perpendicular and parallel magnetic fields of up to 10 T. These data have confirmed our previous results on the $S = \frac{1}{2}$ and $S = \frac{7}{2}$ spin states, and have allowed us to simulate their Mössbauer spectra and, by subtracting the latter from the experimental data, to obtain Mössbauer spectra of the $S = \frac{3}{2}$ spin state.

MATERIALS AND METHODS

The purification of Cp Fd from *Clostridium pasteurianum* (A.T.C.C. 6013) and the preparation of its Se-substituted analogue were carried out as previously reported (Moulis & Meyer, 1982). The Mössbauer spectra were recorded at various temperatures in a cryostat equipped with a superconducting magnet. Parallel magnetic fields of up to 10 T, or transverse magnetic fields of up to 5 T, were applied to the sample. The emitter (a 100 mCi ^{57}Co source in a rhodium matrix) was maintained at 4 K. All isomeric shifts are given relative to a metallic iron absorber at $T = 300$ K, which was used for calibration.

COMPUTER ANALYSIS

In the presence of an applied magnetic field (H_{app}), the spin Hamiltonian for a spin state S may be written:

$$\mathcal{H} = \beta \cdot \vec{S} \cdot \vec{g} \cdot \vec{H}_{\text{app}} + \{D[S_z^2 - \frac{1}{3}S(S+1)] + E(S_x^2 - S_y^2)\} + \sum_i \vec{S} \cdot \vec{A}(i) \cdot \vec{I}_i + g_n \cdot \beta_n \cdot \vec{H}_{\text{app}} \cdot \vec{I} + \mathcal{H}_Q \quad (1)$$

where the first term is the electronic Zeeman interaction, the second is the zero-field splitting, the third is the hyperfine interaction between the cluster spin S and the nuclear spin I , the fourth is the nuclear Zeeman interaction and the last term is the nuclear quadrupole interaction described by $\eta = (V_{xx} - V_{yy})/V_{zz}$, the asymmetry parameter, and $\Delta E = (e \cdot Q \cdot V_{zz}/2)\sqrt{1 + \eta^2/3}$, the quadrupole splitting; i sums over the inequivalent iron sites of the cluster. We then make approximations and assumptions in order to reduce the above general Hamiltonian to a readily soluble form representative of the active centre.

Abbreviations used: Cp Fd, *Clostridium pasteurianum* ferredoxin; H_{app} , applied field; H_{eff} , effective field; H_{hf} , hyperfine field.

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The quantity $\vec{S} \cdot \vec{A}$ could be treated as a hyperfine magnetic field $\vec{H}_{\text{hf}} = -\vec{A} \langle \vec{S} \rangle / g_n \beta_n$, where the electron spin enters as an expectation value because its magnitude and its direction are determined by the applied magnetic field $\vec{H}_{\text{app}}$. In that case, the nucleus senses an effective field $\vec{H}_{\text{eff}}$, such that:

$$\vec{H}_{\text{eff}} = \vec{H}_{\text{app}} + \vec{H}_{\text{hf}} \quad (2)$$

Analysis of the Mössbauer spectra of a paramagnetic cluster subjected to $\vec{H}_{\text{app}}$ will provide the magnitude of H_{hf} and its direction with respect to the principal axis of the electric-field-gradient tensor, which is the co-ordinate system for the calculation. The values of $\vec{H}_{\text{hf}}$ ($\vec{H}_{\text{hf},x}$, $\vec{H}_{\text{hf},y}$ and $H_{\text{hf},z}$) when $\vec{H}_{\text{app}}$ lies along the gradient axes (x , y and z) have been evaluated.

The intensities of the Mössbauer absorption peaks depend on the angle between $\vec{H}_{\text{eff}}$ and the γ beam. In the case of a Kramer's doublet with an isotropic $\vec{g}$ -tensor (e.p.r.-active), the Zeeman splitting gives rise to four lines in a longitudinal field ($\Delta m = 0$ transitions quenched), and six lines in a transverse field. In contrast, in the case of an e.p.r.-silent Kramer's doublet with an extremely anisotropic $\vec{g}$ -tensor (e.g. $g_x = 0.01$, $g_y = 0.01$ and $g_z = 14.32$; see below), $\vec{H}_{\text{hf}}$ is fixed relative to the molecular frame, and the Mössbauer spectra are independent of the direction of the applied field (Huynh & Kent, 1983).

The total array of absorption energies and intensities for a polycrystalline (frozen solution) sample was obtained by summing up 36 simulated spectra corresponding to 36 applied field directions distributed over an octant of sphere. The spectra have been fitted with a sum of Lorentzians by least-squares analysis by using a Prime computer. We have assumed that, at a given temperature, the relative concentrations of inequivalent iron sites are directly proportional to the proportions of the areas of their respective Mössbauer spectra.

RESULTS AND DISCUSSION

The $S = \frac{1}{2}$ spin state

Previous experiments (Thompson *et al.*, 1974; Gersonde *et al.*, 1974; Moulis *et al.*, 1984) on the native $2[4\text{Fe-4S}]^+$ Cp Fd have shown that without an external field the Mössbauer spectrum at $T = 1.6\text{ K}$ is a quadrupole doublet; the application of a small magnetic field results in the appearance of a quite well-resolved magnetic hyperfine pattern. This has been rationalized by considering the $2[4\text{Fe-4S}]^+$ clusters of a protein molecule as a system of two paramagnetic ions separated by nearly 1.2 nm (12 Å) and undergoing spin-spin interactions (Afanas'ev *et al.*, 1980). In this model, the spin-lattice and the spin-spin relaxation times of the system were assumed to be long compared with the Larmor precession time because of the low-temperature conditions and because of the relatively long intermolecular distances [approx. 5.0 nm (50 Å)] at the concentrations used respectively. The spins of the two clusters were assumed to have identical principal g -values but non-parallel g -tensor

Fig. 1. Mössbauer spectra of $2[4\text{Fe-4Se}]^+$ Cp Fd recorded at $T = 1.6\text{ K}$

The protein concentration was 8 mg/ml. Spectrum A, without external field; spectrum B, in a longitudinal applied field ($H_{\text{app}} = 0.1\text{ T}$); spectrum C, in a longitudinal applied field ($H_{\text{app}} = 3\text{ T}$); spectrum D, in a transverse applied field ($H_{\text{app}} = 3\text{ T}$); spectrum C', in a longitudinal applied field ($H_{\text{app}} = 5\text{ T}$); spectrum D', in a transverse applied field ($H_{\text{app}} = 5\text{ T}$).

directions, which induces a difference in their g -values for a given orientation of the magnetic field. The Mössbauer spectra of the $2[4\text{Fe-4S}]^+$ Cp Fd in low magnetic fields ($0 \leq H_{\text{app}} \leq 3\text{ T}$) were rationalized with this model (Afanas'ev *et al.*, 1980). External fields as high as 2 T were needed to overcome the intercluster interaction. From comparisons of previously published Mössbauer spectra of $2[4\text{Fe-4S}]^+$ Cp Fd (see, for example, Fig. 7 in Moulis *et al.*, 1984) with the corresponding ones of $2[4\text{Fe-4Se}]^+$ Cp Fd (Fig. 1), it can be inferred that the $S = \frac{1}{2}$ states of

Fig. 2. Mössbauer spectra of 2[4Fe-4Se]⁺ Cp Fd recorded at $T = 1.6$ K in longitudinal applied fields of 3 T, 5 T and 10 T

The protein concentration was 8 mg/ml. The experimental spectra are represented by crosses (+). Simulations of the $S = \frac{1}{2}$ spin state (major component) and of the two sites of the $S = \frac{7}{2}$ spin state (minor components) were generated from eqns. (1) and (2) (see the Computer analysis section) by using the parameters of Table 1 for $S = \frac{1}{2}$ (central part) and $S = \frac{7}{2}$ (outer lines).

the two proteins display very similar behaviours. In particular, we have seen for the 2[4Fe-4S] Cp Fd that, with a longitudinal field $H_{app.} > 3$ T, the $\Delta m = 0$ transitions are not observed, indicating that the H_{hf} has been aligned by the applied field. The same conclusion can be drawn for the $S = \frac{1}{2}$ spin state of 2[4Fe-4Se]⁺ Cp Fd, which has a nearly isotropic g -tensor. Therefore the differences between the central parts of spectra C and D in Fig. 1 are essentially due to the activation of the $\Delta m = 0$ transitions of the $S = \frac{1}{2}$ spin state in a transverse applied field.

The contribution of the $S = \frac{1}{2}$ spin state to the spectra of Fig. 2 has been analysed in the same way as for the native 2[4Fe-4S]⁺ Cp Fd (Dickson *et al.*, 1976): it has been simulated assuming the presence of two pairs of equivalent iron atoms, one with $A > 0$ and one with $A < 0$. The $\tilde{g}$ and $\tilde{A}$ tensors have been supposed to have the same principal axis system as that of the electric field gradient. The hyperfine field components, as listed in Table 1, are nearly isotropic for one couple of iron, and anisotropic for the other one. These parameters are very similar to the parameters that we obtained for the native 2[4Fe-4S]⁺ Cp Fd (listed in Table 1). The contribution of this spin state accounts for 44% of the total iron sites, a value that is consistent with previous determinations by integration of e.p.r. spectra (Moullis *et al.*, 1984).

The $S = \frac{7}{2}$ spin state

The Mössbauer absorption pattern corresponding to the e.p.r.-silent ground state of the $\frac{7}{2}$ multiplet consists mainly of the outer lines of the spectrum A in Fig. 1 (Moullis *et al.*, 1984). This magnetically split spectrum can be detected below 13 K and is independent of temperature below 4 K. The latter observation indicates a long electronic spin-relaxation time compared with the nuclear Larmor precession time. The iron atoms involved in the $S = \frac{7}{2}$ spin state account for 24% of the total absorption. The spectral pattern arises from the contribution of two antiferromagnetically coupled magnetic species of 3:1 relative intensities. The Mössbauer spectra recorded in various applied fields are shown in Figs. 1 and 2. In Fig. 1 the comparison of spectra C and D at $H_{app.} = 3$ T indicates that the change in the field direction does not modify the intensity of the external lines relative to the total absorption ($I_C/I_D = 1$). This means that the angle θ between $\vec{H}_{eff.}$ and the γ beam is not affected by the direction of the applied field and

Table 1. Hyperfine parameters of 2[4Fe-4Se]⁺ Cp Fd at $T = 1.6$ K

The isomer shift values are relative to iron metal at $T = 300$ K. Γ is the half-width at half height.

Spin state	δ (mm/s)	ΔE_q (mm/s)	Γ (mm/s)	η	$H_{hf,x}$ (T)	$H_{hf,y}$ (T)	$H_{hf,z}$ (T)	% of total iron
$S = \frac{1}{2}$	0.53	1.50	0.22	0.5	+9.5	+4.5	+2.7	22
	0.48	1.14	0.22	0.5	-11.5	-11.5	-12.0	22
$S = \frac{7}{2}$	0.67	1.67	0.20	0.30	-25.5	-25.5	-28.5	18
	0.39	1.17	0.14	0.75	+21.2	+21.2	+21.2	6
$S = \frac{3}{2}$	0.56	1.00	0.24†	0.5	-4.2‡	-4.2‡	-4.2‡	32
$S = \frac{1}{2}^*$	0.55	1.70	0.27	0.5	+9.0	+4.0	+2.7	50
	0.50	1.05	0.33	0.5	-11.2	-11.2	-8.5	50

* For 2[4Fe-4S]⁺ Cp Fd.

† Measured at 35 K

‡ Values of the hyperfine field H_{hf} when $H_{app.} = 10$ T.

confirms the previously inferred (Gaillard *et al.*, 1986) anisotropic g -values of this state ($g_x = 0.01$, $g_y = 0.01$ and $g_z = 14.32$).

In spectra C' and D' at $H_{\text{app.}} = 5$ T the intensities of the outer lines relative to the total absorption are different ($I_{C'}/I_{D'} = 1.7$). We therefrom conclude that the internal field has been oriented by a large applied field, but not completely. If $\vec{H}_{\text{eff.}}$ had exactly the same direction as $\vec{H}_{\text{app.}}$, then $I_{C'}/I_{D'}$ would be equal to 2. When the applied field is increased from 5 T to 10 T, the intensity of the Mössbauer lines corresponding to the $S = \frac{7}{2}$ spin state (24% of the total iron absorption) remains nearly constant and the line-width does not change significantly, which confirms that we are dealing with the lowest level of the $\frac{7}{2}$ multiplet (Moulis *et al.*, 1984; Gaillard *et al.*, 1986). Since the contribution of this spin state is small relative to the total absorption, and only the external spectral components are well resolved, we have kept the number of unknowns to a minimum. The best fits of the less-intense contribution, a ferric-type site, have been obtained with an isotropic hyperfine field of +21.2 T. The more-intense contribution could be fitted, in a first approach, by assuming three equivalent iron sites and nearly isotropic hyperfine interactions ($H_{\text{hf},x} = H_{\text{hf},y} = -25.5$ T, $H_{\text{hf},z} = -28.5$ T). The hyperfine magnetic interactions are easily deduced from spectra recorded in large applied fields ($H_{\text{app.}} > 5$ T). A small asymmetry parameter ($\eta = 0.3$) accounts for the observed differences in widths of the outer lines at -4 mm/s and $+5$ mm/s Doppler velocities. The Mössbauer parameters are listed in Table 1. All the spectra corresponding to this spin state have been fitted assuming no spin-spin interaction, as suggested by previous e.p.r. results (Moulis *et al.*, 1984) and by the constant evolution of the Mössbauer spectra from $H_{\text{app.}} = 0$ to $H_{\text{app.}} = 10$ T.

As indicated by the Mössbauer results, the less-intense site is dominantly of 'ferric' character, and the more-intense one of 'ferrous' type. Taking into account this relatively localized picture, we have applied a vectorial model to the $S = \frac{7}{2}$ spin state, as previously done by Gibson *et al.* (1966) in the case of the [2Fe-2S] centres and by Münck & Kent (1986) in the case of three-iron centres. We have taken the mean hyperfine coupling values given by Münck & Kent (1986) for isolated atoms: $\langle A \rangle_{\text{Fe}^{3+}} = -22.5$ MHz and $\langle A \rangle_{\text{Fe}^{2+}} = -26.8$ MHz. Whereas the mean value is a good approximation for Fe^{3+} , it may be a poor one in the case of Fe^{2+} . It may nevertheless be valid, in view of the observation that the 'ferrous'-type site of the $S = \frac{7}{2}$ spin state has a quite isotropic hyperfine coupling. We have then considered the three iron atoms of 'ferrous' type as ferromagnetically coupled ions giving an $S' = 6$ spin state. The latter has further been antiferromagnetically coupled to the $S = \frac{7}{2}$ spin of the 'ferric' iron, thus giving a resultant $S = \frac{5}{2}$ spin. For such a system, the predicted values of hyperfine coupling are: $\langle A' \rangle_{\text{Fe}^{3+}} = +12.6$ MHz and $\langle A' \rangle_{\text{Fe}^{2+}} = -13.8$ MHz and the experimentally determined values are $\langle A_{\text{exp.}} \rangle_{\text{Fe}^{3+}} = +8.1$ MHz and $\langle A_{\text{exp.}} \rangle_{\text{Fe}^{2+}} = -10.4$ MHz. Considering the approximations made in the determination of the experimental values and the simplicity of the model, we can consider the agreement as being fairly good. The description of the $S = \frac{7}{2}$ spin in terms of localized valence states is thus reasonable.

The $S = \frac{3}{2}$ spin state

The contributions of the $S = \frac{3}{2}$ spin state are plotted in Fig. 3. They have been obtained by subtracting the contributions of the $S = \frac{1}{2}$ and $S = \frac{7}{2}$ spin states (44% and 24% of the total absorption respectively) from the spectra of Fig. 2, with the assumption that at $H_{\text{app.}} > 3$ T the intercluster interactions involving the $S = \frac{1}{2}$ spin state are overcome. To analyse these data, which are obtained with relatively large uncertainties, we have first supposed that all the iron sites are equivalent. The spectra of the $S = \frac{3}{2}$ spin state, as recorded at $T = 1.6$ K, either without external field or with $H_{\text{app.}} = 1.5$ T, are quadrupole doublets. When $H_{\text{app.}} = 3$ T, the spectrum can be fitted with $H_{\text{hf}} = 0$ and broad lines ($\Gamma = 0.32$ mm/s).

These results can tentatively be interpreted by taking into account previous e.p.r. observations showing that the ground doublet of the $S = \frac{3}{2}$ spin state has a shorter relaxation time than the $S = \frac{1}{2}$ spin state at 6 K (Moulis *et al.*, 1984). The latter state has been observed in an intermediate relaxation rate in Mössbauer experiments carried out at $T = 4.2$ K and $H_{\text{app.}} = 4$ T. Therefore the $S = \frac{3}{2}$ ground state might have a high relaxation frequency at $T = 1.6$ K and $H \leq 3$ T, which would imply low-lying excited states. As the zero-field splitting of the $S = \frac{3}{2}$ multiplet is unknown, the external field required to polarize it remains undetermined. Moreover, this spin state is involved in intercluster spin-spin interactions, of which the nature and magnitude are unknown. Because of these indeterminacies, the spectra recorded in $H_{\text{app.}} \leq 3$ T can be interpreted as arising either from fast-relaxing paramagnetic species, or from a slowly relaxing coupled spin system with a very small hyperfine field. In either case, the isomer shift and the quadrupole splitting can be estimated (Table 1) and were verified to be adequate for the stimulation of the spectrum recorded at $T = 20$ K without applied field, which consists of a well-resolved doublet characteristic of a fast-relaxing paramagnetic species.

When very high (8–10 T) magnetic fields are applied, all intercluster interactions are most probably overcome, and the high-field approximation should be valid. The Mössbauer spectrum recorded with $H_{\text{app.}} = 10$ T (Fig. 3) has been simulated with a negative isotropic hyperfine tensor ($H_{\text{hf}} = -4.2$ T). The resolved, but still broad, lines may reflect some inequivalence among the iron sites. The simulation is improved if the spectrum of the $S = \frac{3}{2}$ spin state is assumed to include a small diamagnetic component amounting to approx. 6% of the total absorption of $2[4\text{Fe-4Se}]^+ \text{ Cp Fd}$. This small diamagnetic contribution (Fig. 3, $H_{\text{app.}} = 10$ T) may be due to a small residual amount of non-reduced $2[4\text{Fe-4Se}]^{2+} \text{ Cp Fd}$, and may explain at least in part the broad lines of the quadrupole doublet observed in Fig. 1, spectrum A: according to the model proposed by Afanes'ev *et al.* (1980), the spin-spin interaction between the two reduced $S = \frac{1}{2}$ clusters in a ferredoxin molecule is responsible for the non-magnetic Mössbauer pattern at $H_{\text{app.}} = 0$. If one of the clusters is diamagnetic, because of an incomplete reduction of the ferredoxin sample, the other one ($S = \frac{1}{2}$ or $S = \frac{3}{2}$) will produce a magnetically split spectrum.

The $S = \frac{3}{2}$ spectrum recorded with $H_{\text{app.}} = 5$ T displays a splitting that is not equal to that expected from the applied field (i.e. $H_{\text{hf}} \neq 0$). In such a high applied field, the coupling between the clusters should be released, and

Fig. 3. Mössbauer spectra of the $S = \frac{3}{2}$ spin state of the $2[4\text{Fe-4Se}]^+$ Cp Fd at $T = 1.6\text{ K}$ in longitudinal applied fields (3 T, 5 T, 8 T and 10 T)

The spectra were obtained by subtracting the contributions of the $S = \frac{1}{2}$ and $S = \frac{7}{2}$ spin systems from the experimental data (Fig. 2). The continuous lines are theoretical simulations of the $S = \frac{3}{2}$ spin state made by using the parameters of Table 1. In the $H = 10\text{ T}$ spectrum, the fit was improved by adding a diamagnetic contribution (continuous line, minor component) to the simulation of the $S = \frac{3}{2}$ spin state (continuous line, major component) (see the text).

spin-lattice relaxation should be responsible for the shape of the spectrum. One may alternatively consider that this pattern is due to the contributions of different levels of the $S = \frac{3}{2}$ spin multiplet in the slow relaxation

limit. With the latter hypothesis, and from the field-dependence of the Mössbauer spectra, it may be inferred that the two lower levels of the multiplet are equally populated when $H_{\text{app.}} \approx 6\text{ T}$, and thus the zero-field splitting may be estimated as $D \gtrsim +3\text{ cm}^{-1}$. As deduced above from the high-field Mössbauer spectra (Fig. 3), the iron atoms involved in the $S = \frac{3}{2}$ spin state experience a hyperfine field of -4.2 T .

The occurrence of spin states higher than $S = \frac{1}{2}$ in $[4\text{Fe-4S}]^+$ clusters had long been restricted to synthetic analogues distorted by solid-state effects (Laskowski *et al.*, 1979; Collison & Mabbs, 1982) and to the thionine-oxidized MoFe protein of nitrogenase (Zimmermann *et al.*, 1978). The biochemical interest of the $S = \frac{3}{2}$ and $S = \frac{7}{2}$ spin states found in reduced Se-substituted clostridial ferredoxins (Moulis *et al.*, 1984; Gaillard *et al.*, 1986; the present work) has been enhanced considerably by the more-recent discoveries of high-spin $[4\text{Fe-4S}]^+$ clusters in several native proteins (Vollmer *et al.*, 1983; Huynh *et al.*, 1984; Lindahl *et al.*, 1985). In particular, some similarities and differences between $2[4\text{Fe-4Se}]^+$ Cp Fd and nitrogenase Fe protein (Av2) (Lindahl *et al.*, 1985) are worthy of note: in both cases $S = \frac{1}{2}$ and $S = \frac{3}{2}$ spin states are coexistent. Whereas for Av2 protein conditions could be worked out for the interconversion of these two spin states (Lindahl *et al.*, 1985), we have been unable to obtain a similar result for Se-substituted ferredoxins. We have nevertheless observed that the relative abundances of the $S = \frac{1}{2}$ and $S = \frac{3}{2}$ spin states were dependent on the species of *Clostridium* from which the ferredoxin was extracted. The $S = \frac{3}{2}$ spin states of Av2 protein and of Se-substituted Cp Fd differ by their g -values, by the sign of their zero-field splittings, but in both proteins the iron atoms experience similar hyperfine fields.

In most cases investigated to date, the high-spin states, when present, coexist with the classical $S = \frac{1}{2}$ spin state. It is therefore essential to discriminate the contributions of the various spin states to the experimental spectra. In the e.p.r. spectra of Se-substituted Cp Fd, the signals of the $S = \frac{1}{2}$, $S = \frac{3}{2}$ and $S = \frac{7}{2}$ spin states were differentiated by their g -values and temperature-dependencies (Moulis *et al.*, 1984; Gaillard *et al.*, 1986). In the present paper, we have isolated the contributions of the three spin states to the Mössbauer spectra of $2[4\text{Fe-4Se}]^+$ Cp Fd. The combined e.p.r. and Mössbauer data have afforded an almost complete description of the $S = \frac{1}{2}$, $S = \frac{3}{2}$ and $S = \frac{7}{2}$ spin states. Similar approaches are expected to be fruitful in the investigation of unusual spin states found in native proteins.

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