High-Multiplicity Spin States of 2[4Fe-4Se]$^+$ Clostridial Ferredoxins

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ABSTRACT: The electron paramagnetic resonance (EPR) spectra of the reduced selenium-substituted 2-[4Fe-4Se]$^+$ ferredoxins from three bacteria of the *Clostridium* genus display low-field signals at $g = 5.17$, $g = 10.11$, and $g = 12.76$. The positions, shapes, and temperature dependencies of these signals have allowed their assignments to the three excited states of an $S = {}^7/_2$ spin multiplet, the fundamental state of which is observed as unusual features in low-temperature ($T \leq 20$ K) Mössbauer spectra. The $S = {}^7/_2$ spin state is present in 2[4Fe-4Se]$^+$ clostridial ferredoxins together with the classical $S = {}^1/_2$ state and with a $S = {}^3/_2$ state, the fundamental doublet of which is observed as a broad signal in the $g = 3$–4 region. The relative intensities of the EPR signals corresponding to these spin states depend on the species of *Clostridium* that the ferredoxin is extracted from. In contrast with clostridial ferredoxins, the reduced selenium-substituted ferredoxin from *Bacillus stearothermophilus*, which differs significantly from the clostridial proteins by its primary structure and by its containing only one tetranuclear cluster, displays only the $S = {}^1/_2$ state. Thus, the high-multiplicity spin states arise from a specific interaction between the clostridial ferredoxin polypeptide chain and the reduced [4Fe-4Se]$^+$ clusters.

The reduced tetranuclear iron–sulfur clusters [4Fe-4S]$^+$ display in most cases an $S = {}^1/_2$ ground spin state, in proteins (Cammack et al., 1977) as well as in synthetic analogues (Lane et al., 1977). The occurrence of spin states of higher multiplicity has been evidenced in synthetic clusters distorted by solid-state effects (Laskowski et al., 1978; Collison & Mabbs, 1982), as well as in certain proteins (Zimmerman et al., 1978; Vollmer et al., 1983; Huynh et al., 1984; Lindahl et al., 1985). In the latter cases, the high spin states may be functionally significant, although this remains to be established.

We have recently observed unusual features in EPR[1] and Mössbauer spectra of the reduced Se-substituted ferredoxin (Fd) from *Clostridium pasteurianum* and interpreted these phenomena as arising from the occurrence of $S = {}^3/_2$ and $S = {}^7/_2$ spin states, in addition to the classical $S = {}^1/_2$ state (Moulis et al., 1984a). We here report the observation, in the spectra of 2[4Fe-4Se]$^+$ Cp Fd, of additional EPR signals that confirm our previous conclusions and that allow the determination of the spin-Hamiltonian parameters describing the $S = {}^7/_2$ spin state. Several other [4Fe-4Se] ferredoxins have also been prepared, and EPR spectra of their reduced state have been recorded in order to elucidate the role of the polypeptide chain in the generation of high-multiplicity spin states.

Materials and Methods

Clostridium pasteurianum (ATCC 6013) Fd was purified and Se-substituted as previously reported (Moulis & Meyer, 1982). *Bacillus stearothermophilus* (ATCC 29609) was grown at 55 °C and its Fd isolated as described (Mullinger et al., 1975), except that the preparative polyacrylamide gel electrophoresis was replaced by a precipitation in 90% saturated ammonium sulfate and subsequent chromatography on Ultrogel AcA 202 (LKB). The Fd was obtained with an A_{388}:A_{280} ratio of 0.62. *Clostridium acidi-urici* (ATCC 7906) Fd was purified (Hong & Rabinowitz, 1970) from cells grown on uric acid (Rabinowitz, 1963). Two of the three successive anion-exchange chromatographies (Hong & Rabinowitz, 1970) were suppressed, and the protein was instead filtered on Ultrogel AcA 202 after ammonium sulfate fractionation between 65 and 90% saturation. The A_{388}:A_{280} ratio of this Fd was 0.78, i.e., nearly equal to that of crystalline material (Hong & Rabinowitz, 1970). The purification of *Clostridium thermosaccharolyticum* (ATCC 7956) Fd was based on the procedure published for several ferredoxins from thermophilic clostridia (Devanathan et al., 1969). The Fd solution, as obtained after DEAE-cellulose chromatography and Ultrogel AcA 202 gel filtration of the crude extract, was concentrated on a YM5 (Amicon) membrane in 1 mM phosphate buffer at pH 7 and passed over a HA-Ultrogel (LKB) column equilibrated with the same buffer. The major contaminant, nucleic acids, binds to the gel, whereas Fd is collected with the pass through. A final filtration over CPG 75-Å (Serva) silica beads yields Fd with a purity index (A_{388}:A_{280}) of 0.80, whereas the previously reported value was 0.78 (Devanathan et al., 1969).

The conversion to apoferredoxins and the preparation of proteins containing [4Fe-4Se] clusters were carried out as for Cp Fd (Moulis & Meyer, 1982). The reactions, however, were more difficult for Bst Fd and Cth Fd than for Cp Fd. This probably arises from the higher intrinsic stability of the thermophilic proteins. All the experiments, including the preparations of apoproteins, were performed under an argon atmosphere, and the ferredoxins were reduced by addition of appropriate volumes of a 0.1 M dithionite solution buffered at pH 8.0.

EPR spectra were obtained with an X-band Varian E-109 spectrometer coupled to a Hewlett-Packard 9826 calculator. An Oxford Instruments ESR 900 flow cryostat was used to

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[1] Abbreviations: Fd, ferredoxin; Cp, *Clostridium pasteurianum*; Bst, *Bacillus stearothermophilus*; Cth, *Clostridium thermosaccharolyticum*; Cau, *Clostridium acidi-urici*; DMF, dimethylformamide; Me_2SO, dimethyl sulfoxide; PRPP, 5-phospho-α-D-ribosyl pyrophosphate; EPR, electron paramagnetic resonance; S* and Se*, bridging chalcogenide atoms of the active sites of Fd; T, tesla; g_{av}, average of the main values of the g tensor.

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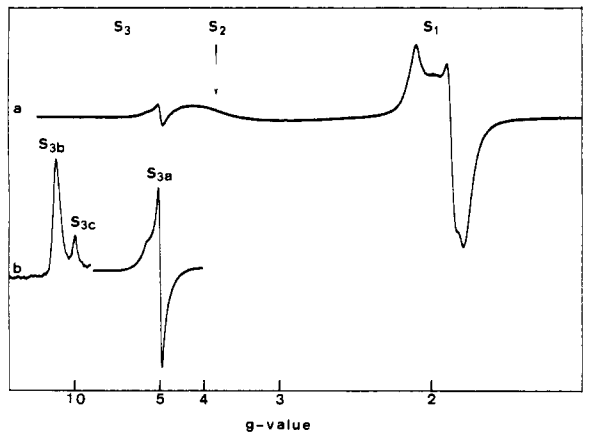

FIGURE 1: EPR spectra of 2[4Fe-4Se]$^+$ *C. pasteurianum* ferredoxin (24 mg/mL): (trace a) spectrum at 6 K, microwave power 0.05 mW; (trace b) low-field part of 15 K spectrum, microwave power 2 mW. Signals S_{3b} and S_{3c} have been recorded with a 100-fold higher gain than signal S_{3a}. Other EPR conditions: modulation amplitude 0.8 mT, 100-kHz field modulation, and klystron frequency 9.25 GHz.

adjust the EPR sample temperature (Moulis et al., 1984a). The determination of the g values was carried out by simultaneously monitoring the microwave frequency (EIP 548A microwave frequency counter) and the magnetic field (Varian NMR gaussmeter). 1,1-Diphenyl-2-picrylhydrazyl radical (g = 2.0036) was used as a standard.

Results and Discussion

Characterization of an $S = {}^7/_2$ Spin State in 2[4Fe-4Se]$^+$ Cp Fd. Reduced selenium-substituted Cp Fd displays a very complex EPR spectrum, with features appearing over a wide range of magnetic field (Figure 1). The rhombic signal of g_{av} = 1.98 has been attributed to an $S = {}^1/_2$ spin state, and the broad lines in the g = 4 region (signal S_2) have been attributed to the fundamental $\pm{}^1/_2$ doublet of an $S = {}^3/_2$ species (Moulis et al., 1984a). At lower field, an isotropic signal at g = 5.17 has been tentatively assigned, together with the most unusual lines of the Mössbauer spectra, to sublevels of an $S = {}^7/_2$ spin state (Moulis et al., 1984a).

Subsequently, two peaks of very low intensity at g = 12.76 and g = 10.11 (Figure 1) have been detected. Their shapes are clearly indicative of low-field components of rhombic EPR signals. The product of the amplitude of these peaks by the temperature increases with increasing temperature, a behavior shared by the g = 5.17 line. It can therefrom be concluded that the low-field components ($g > 5$, Figure 1) arise from transitions associated with excited levels.

A spin Hamiltonian suitable for the description of high-multiplicity spin systems may be written as

$$\mathcal{H} = \vec{\beta}S\tilde{g}\vec{H} + D[S_z^2 - (1/3)S(S+1)] + E(S_x^2 - S_y^2)$$

where the D term is generally the largest for the high-spin iron systems present in iron–sulfur proteins. Half-integer spin states afford a zero field splitting into $(2S+1)/2$ Kramers doublets, which may contribute to the EPR spectrum.

In this framework, the upper $\pm{}^3/_2$ doublet of the $S = {}^3/_2$ spin state, the fundamental doublet of which gives the S_2 signal (Moulis et al., 1984a), is not a likely candidate for the newly detected excited levels, since its g values are expected at 0.45, 0.5, and 6 (Maltempo, 1979), taking $\lambda = E/D = 0.08$ (Moulis et al., 1984a). With a fine structure parameter $[\lambda] = [E/D]$ of 0.12, the three g values of the $\pm{}^3/_2$ doublet belonging to the $S = {}^7/_2$ system are expected at about g = 5 (Nicklin et al., 1973), which is in agreement with the observation of a nearly isotropic EPR signal at $g \simeq 5.17$ (Figure 1). The

Table I: Energy Diagram and Corresponding g Values of the $S = {}^7/_2$ Multiplet in a Strong Ligand Field[a]

Kramers doublets	energy (in [D] units)	g values (g_x, g_y, g_z)	
		calcd	obsd
$\pm{}^1/_2$	12.49	2.4, 12.88, 1.12	12.76
$\pm{}^3/_2$	9.71	5.18, 5.21, 5.08	5.17, 5.17, 5.17
$\pm{}^5/_2$	5.92	0.6, 10.08, 0.54	10.11
$\pm{}^7/_2$	0	0.01, 0.01, 14.32	

[a] The energy and g values have been calculated as described in the text for $[\lambda] = [E/D] = 0.12$, $g_0 = 2.05$, and $D < 0$. The energy of the ground state has been arbitrarily set at the origin of the scale.

energy levels of the $S = {}^7/_2$ system can be calculated in [D] units, and the perturbation applied to these sublevels by the Zeeman interaction leads to a set of effective g values associated with the different Kramers doublets (Nicklin et al., 1973). Taking an isotropic g_0 = 2.05 value, it appears that the two peaks at g = 12.76 and g = 10.11 are consistent with g values expected for EPR transitions arising from the $\pm{}^1/_2$ and $\pm{}^5/_2$ Kramers doublets, respectively, of the $S = {}^7/_2$ multiplet (Table I). Since such signals have been associated with excited doublets (see above), the D parameter is negative and the $\pm{}^7/_2$ doublet constitutes the ground state of the multiplet, as represented in Table I. The latter doublet is EPR silent due to its very anisotropic g tensor (Table I), but it can be detected in the 4.2K Mössbauer spectra as a component with fully developed paramagnetic hyperfine interactions in the absence of an applied field (Moulis et al., 1984a). The intensity of these lines is insensitive to the orientation of the applied magnetic field (up to H = 3 T), which further confirms the large anisotropy of the corresponding $\tilde{g}$ tensor.

At sufficiently high temperature, 18K for instance, the excited doublets of the $S = {}^7/_2$ multiplet are significantly populated, and since their respective energies are functions of [D], the latter value can be evaluated by measuring the intensity ratios of their associated EPR signals. The relative intensities of the $\pm{}^1/_2$ and $\pm{}^5/_2$ doublets can be obtained from the areas of the g = 12.76 and g = 10.11 peaks, respectively, by using the calculated high-field g values listed in Table I (Aasa & Vänngård, 1975), as no measured values are available. This procedure yields $D = -2.1 \pm 0.7$ cm^{-1}. The uncertainty on the determination of [D] is due to the weakness of the g = 10.11 peak, which precludes an accurate measurement of its area. Nevertheless, the energy gap between the ground $\pm{}^7/_2$ doublet and the first excited $\pm{}^5/_2$ doublet, calculated for $[D] = 2$ cm^{-1}, is consistent both with the constant absorption, below 4.2 K, of the Mössbauer features associated with the $\pm{}^7/_2$ doublet and with the decrease of this signal when the temperature increases from 4.2 to 8 K (Moulis et al., 1984a).

The occurrence of an $S = {}^7/_2$ spin state in the active sites of reduced Se-substituted Cp Fd is thus supported by a substantial amount of EPR and Mössbauer data. While such a high multiplicity spin system must arise from some kind of structural distortion, the latter is certainly not a significant structural rearrangement of the cubane clusters: indeed, the [4Fe-4Se] clusters of oxidized Cp Fd have been found to assume the same symmetry as the native clusters (Moulis et al., 1984b), and no degradation of the selenium-containing prosthetic groups has been evidenced upon reduction, neither in biochemical (Moulis & Meyer, 1982) nor in spectroscopic (Moulis et al., 1984a) investigations. It can therefore be concluded that the occurrence of the $S = {}^3/_2$ and $S = {}^7/_2$ (Moulis et al., 1984a; this work) species in reduced selenium-substituted Cp Fd is a property of the [4Fe-4Se]$^+$ clusters in this particular environment. In order to further define the

Table II: Principal g Values of the $S_1 = {}^1/_2$ Spin State of Various Bacterial Ferredoxins[a]

bacterium	[4Fe-4S]$^+$				[4Fe-4Se]$^+$			
	g_x	g_y	g_z	g_{av}	g_x	g_y	g_z	g_{av}
B. stearothermophilus	1.892	1.929	2.063	1.963	1.888	1.94	2.079	1.971
C. pasteurianum	1.883	1.925	2.056	1.956	1.888	1.94	2.103	1.979
C. acidi-urici	1.905	1.939	2.077	1.975	1.884	1.941	2.104	1.979
C. thermosaccharolyticum	1.898	1.934	2.063	1.966	1.894	1.944	2.082	1.975

[a] The principal g values have been determined with fully reduced samples of Bst Fd and partially reduced samples of Fd containing two tetranuclear centers. The average g value has been calculated from $g_{av}^2 = (1/3)(g_x^2 + g_y^2 + g_z^2)$.

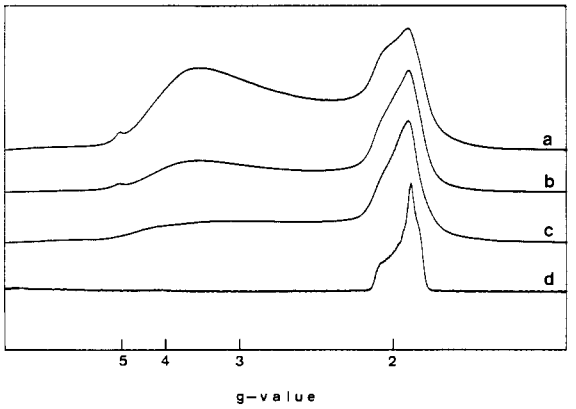

FIGURE 2: EPR absorptions of several reduced Se-substituted ferredoxins: (trace a) 2[4Fe-4Se]$^+$ *C. aridi-urici* ferredoxin, 3.3 mg/mL; (trace b) 2[4Fe-4Se]$^+$ *C. pasteurianum* ferredoxin, 24 mg/mL; (trace c) 2[4Fe-4Se]$^+$ *C. thermosaccharolyticum* ferredoxin, 2.5 mg/mL. These spectra were recorded at 6 K and are given as integrals of $d\chi''/dH$ to emphasize signal S_2. EPR conditions are as in Figure 1a. (Trace d) [4Fe-4Se]$^+$ *B. stearothermophilus* ferredoxin, 0.8 mg/mL, at 13 K. In this case, the signal was detected at twice the modulation frequency of 50 kHz. Other EPR conditions are as in Figure 1 except microwave power is 0.5 mW. The heights of the prominent features just below $g = 2$ have been arbitrarily equalized.

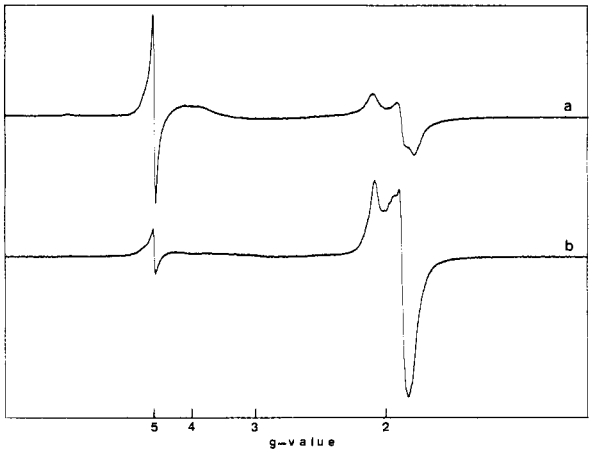

FIGURE 3: EPR spectra of reduced Se-substituted clostridial ferredoxins, at 15 K: (a) *C. acidi-urici* Fd, 3.3 mg/mL; (b) *C. thermosaccharolyticum* Fd, 2.5 mg/mL. The ordinate is a linear function of $d\chi''/dH$. EPR conditions are as in Figure 1 except microwave power is 0.5 mW.

role of the polypeptide chain, we have recorded EPR spectra of other reduced ferredoxins containing tetranuclear iron–selenium clusters.

Influence of the Polypeptide Chain on the Occurrence of $S > {}^3/_2$ Spin States in [4Fe-4Se]$^+$ Clusters. The EPR spectrum of reduced Se-substituted *B. stearothermophilus* Fd consists of a single rhombic signal in the $g = 2$ region (Figure 2d). Thus, the [4Fe-4Se]$^+$ cluster of the latter protein displays no other spin state than the classical $S = {}^1/_2$ system. Bst Fd is larger (M_r 9100) than Cp Fd (M_r 6200), contains only one cubane cluster, and has a primary structure differing significantly from that of clostridial ferredoxins (Hase et al., 1976). The EPR spectrum of [4Fe-4Se]$^+$ Bst Fd is slightly shifted to lower field ($g_{av} = 1.971$) and displays a higher rhombicity than that of the native protein ($g_{av} = 1.963$, Table II).

For further comparison with Cp Fd, the S* → Se* substitution has been carried out on two other clostridial ferredoxins. *C. acidi-urici* Fd is very homologous to Cp Fd: there are only 14 unconserved amino acid residues, 8 of which do not affect the hydropathic character of the 55 amino acid polypeptide chain (Yasunobu & Tanaka, 1973). The EPR spectrum of Se-substituted Cau Fd displays the characteristic features of the $S = {}^3/_2$ and $S = {}^7/_2$ spin states, in addition to the rhombic signal around $g = 2$ (Figure 3a). The less homologous Fd from *C. thermosaccharolyticum* differs from Cp Fd in 22 positions, and most of these substitutions tend to increase the hydrophilicity of the polypeptide chain (Yasunobu & Tanaka, 1973). Se-substituted Cth Fd displays three spin states at the reduced level (Figure 3b). However, the EPR signal at $g = 5.17$, attributed to the $\pm{}^3/_2$ doublet of the $S = {}^7/_2$ multiplet (see above), is not as well developed at 6 K as that of Cau Fd (Figure 3a) or Cp Fd (Figure 1). This discrepancy might result either from a lower concentration of the $S = {}^7/_2$ species (as determined by the Mössbauer absorption of its fundamental $\pm{}^7/_2$ doublet in the case of Cp Fd) in 2[4Fe-4Se]$^+$ Cth Fd or from a larger value of $[D]$, which could result in the observation of the $g = 5.17$ signal at higher temperatures for Cth Fd than for the other clostridial ferredoxins. It should be possible to select one of the two alternatives by studying the temperature dependencies of the $g = 12.76$ and $g = 10.11$ peaks, and thereby measuring $[D]$ (see above). However, the relatively low concentration of the 2[4Fe-4Se]$^+$ Cth Fd sample has forbidden any reliable determination of this parameter. In the case of 2[4Fe-4Se]$^+$ Cau Fd, the value of $[D]$ has been found to be identical with that of Cp Fd, taking the same uncertainty into account.

The $S = {}^1/_2$ spin states of both the native and the Se-substituted ferredoxins are best observed on partially reduced samples, in which the intercluster interaction is minimized. S* → Se* substitution in all of the clostridial ferredoxins investigated here results in a shift of the S_1 signal to lower field, as observed in the case of Bst Fd (Table II). This phenomenon has previously been evidenced in [2Fe-2S] proteins (Tsibris et al., 1968; Orme-Johnson et al., 1968; Fee & Palmer, 1971; Bertrand & Gayda, 1980), as well as in binuclear (Beardwood & Gibson, 1983) and tetranuclear (Bobrik et al., 1978) synthetic analogues. It probably reflects the quantitatively different contribution of the Fe–Se bonds, compared to the Fe–S bonds, to the electronic structure of such heteromultinuclear clusters. The low-field shift of signal S_1 is generally accompanied by a slight increase in rhombicity (Table II), which can be attributed to the stronger strains probably exerted by the polypeptide chain on the larger [4Fe-4Se]$^+$ clusters than on the smaller [4Fe-4S]$^+$ clusters (Bobrik et al., 1978). However, there seems to be no simple correlation between the increase in rhombicity (Table II) and the occurrence of high-multiplicity spin states (Figure 2).

The major discrepancy among the EPR spectra of Se-substituted clostridial Fds concerns the relative intensities of the S_1 and S_2 signals arising from the $S = {}^1/_2$ and $S = {}^3/_2$ species, respectively. Signal S_2 is better observed on the integrated spectra (Figure 2), and the procedure used to quantitate the species corresponding to S_1 and S_2 in Cp Fd (Moulis et al., 1984a) allows the determination of their intensity ratio. The $I(S_2)/I(S_1)$ ratio is approximately equal to 2, 1, and 0.5 for the 2[4Fe-4Se]$^+$ Fd from Cau, Cp, and Cth, respectively. Thus, whereas all the three examined clostridial Fds develop three spin states for the [4Fe-4Se]$^+$ clusters, the relative intensities of these spin states depend on the polypeptide chain involved.

Large differences in the EPR spectra of analogue compounds have been observed, depending on the physical state of the sample (Laskowski et al., 1978; Beardwood & Gibson, 1983). In order to evaluate the effect of such parameters on reduced Se-substituted Cp Fd, the latter protein has been frozen in media containing up to 60% (v/v) DMF or Me_2SO or 30% (v/v) glycerol. No changes in the EPR spectra have been evidenced in these experiments, nor in others involving high salt concentrations or the presence of chaotropic agents such as 8 M urea. The EPR spectrum of reduced Se-substituted Cp Fd is also insensitive to the nature of the buffer, in the pH range where the protein is stable. The lack of dependence of the EPR spectra upon such physicochemical factors shows that the active site of the ferredoxins are protected by the protein matrix against the physical strains exerted by the solvent or the chemical reactivity of some solutes.

It must therefrom be inferred that the differences observed among the EPR spectra (Figure 2) are due to differences in the polypeptidic environments of the clusters, which may be derived from the known amino acid sequences of the clostridial ferredoxins (Yasunobu & Tanaka, 1973) and from the tridimensional structure of 2[4Fe-4S]$^{2+}$ *Peptococcus aerogenes* Fd (Adman et al., 1973). In the latter protein, the [4Fe-4S] clusters are surrounded by hydrophobic amino acids, which are highly conserved among clostridial ferredoxins, except for two aromatic residues (Tyr-2 and Tyr-28) situated at ca. 4 Å from the clusters (Carter, 1977). The nonidentity of these aromatic amino acids for Cp Fd (Tyr-2 and Phe-30) could have explained the occurrence of two spin states ($S = {}^1/_2$ and $S = {}^3/_2$) with nearly the same intensity (Moulis et al., 1984a), each corresponding to one of the two [4Fe-4Se]$^+$ clusters, if some interaction took place between the clusters and the aromatic residues. This possibility is clearly ruled out by the spectra obtained from the reduced Se-substituted ferredoxins from Cau (Tyr-2 and Tyr-30) and Cth (His-2 and Tyr-30), which display the same spectroscopic pattern despite the identity of the aromatic amino acids (Cau) or the replacement of one of them by a histidine residue (Cth). This failure to directly relate the EPR differences to interactions between the [4Fe-4Se]$^+$ clusters and aromatic residues confirms the results obtained with modified polypeptide chains (Lode et al., 1976), which ruled out the direct involvement of the aromatic residues in electron transfer to or from the active sites of clostridial ferredoxins. A direct participation of these residues in redox processes had previously been suggested on the basis of X-ray diffraction (Adman et al., 1973) and ^{13}C NMR (Packer et al., 1972) data.

Thus, at this stage, no direct explanation can be derived from the screening of the amino acid residues neighboring the prosthetic groups, with oxidized *Peptococcus aerogenes* Fd as a reference (Adman et al., 1973). It may therefore be anticipated that the unprecedented occurrence of three spin states for the same iron–sulfur (selenium) cluster arises from a specific effect of the clostridial polypeptide chain, considered as a whole, on the presumably larger, relative to the native ones, [4Fe-4Se]$^+$ active sites.

Conclusions

The occurrence, in addition to the classical $S = {}^1/_2$ state, of $S = {}^3/_2$ and $S = {}^7/_2$ spin states in reduced Se-substituted clostridial ferredoxins is now based on a substantial set of EPR and Mössbauer data. In particular, all of the Kramers doublets of the most unusual $S = {}^7/_2$ multiplet have been detected in either the EPR or the Mössbauer spectra, allowing the determination of the zero field splitting parameters.

These results are interesting in relation to the recent findings of $S > {}^1/_2$ spin states in several proteins containing tetranuclear iron–sulfur centers: thionine-oxidized MoFe protein of nitrogenase (Zimmerman et al., 1978), PRPP amidotransferase from *Bacillus subtilis* (Vollmer et al., 1983), CO-treated or cytochrome c_3 treated periplasmic hydrogenase from *Desulfovibrio vulgaris* (Huynh et al., 1984), and Fe protein of nitrogenase (Lindahl et al., 1985). In the case of the Se-substituted clostridial ferredoxins, the spectroscopic studies described here and previously (Moulis et al., 1984a) have allowed a comprehensive analysis of the $S = {}^7/_2$ spin state. Furthermore, these ferredoxins are highly homologous to *Peptococcus aerogenes* Fd, the tridimensional structure of which has been elucidated (Adman et al., 1973). On the basis of these data, it should ultimately be possible to unveil some of the conditions under which [4Fe-4S(e)]$^+$ clusters assume $S > {}^1/_2$ spin states.

Acknowledgments

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Active Site Directed Inactivation of Rat Mammary Gland Fatty Acid Synthase by 3-Chloropropionyl Coenzyme A†

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ABSTRACT: 3-Chloropropionyl coenzyme A (CoA) irreversibly inhibits rat mammary gland fatty acid synthase. Enzyme inactivation proceeds with first-order kinetics. NADPH (150 μM) as well as acetyl-CoA (500 μM) affords protection against inactivation, suggesting that the inhibitor is active site directed. In contrast, malonyl-CoA (500 μM) offers little protection. With chloro[1-^{14}C]propionyl-CoA, stoichiometries of modification that approach one per enzyme protomer (240 kilodaltons) have been measured. When chloropropionyl-[3′-^{32}P]CoA is used for inactivation, modification stoichiometries are less than 10% of the value observed in the ^{14}C labeling experiments, suggesting that acylation of the enzyme occurs. Radioactivity remains associated with the ^{14}C-labeled protein after performic acid oxidation, indicating that another linkage, in addition to the thio ester adduct, is formed during inactivation. Recovery of ([^{14}C]carboxyethyl)cysteine from digests of the inactivated enzyme indicates that alkylation of an active site cysteine occurs. The cysteamine sulfhydryl of the acyl carrier peptide is clearly *not* the site of modification. Loss of overall enzyme activity is tightly linked to decreases in the ketoacyl synthase partial reaction. This observation, coupled with the differential protection measured with acetyl-CoA and malonyl-CoA, suggests that the reagent modifies a residue at the active site involved in condensation. While inactivated enzyme shows good ketoacyl reductase activity when *S*-(acetoacetyl)-*N*-acetylcysteamine is used as a substrate, only poor activity for this partial reaction is measured when acetoacetyl-CoA is the substrate. This implies that the function of the acyl carrier peptide (ACP) is impaired during the inactivation process. Since the ACP of one protomer appears to interact with the active site of the condensing enzyme on the adjacent subunit of the dimeric fatty acid synthase [Wakil, S. J., Stoops, J. K., & Joshi, V. C. (1983) *Annu. Rev. Biochem. 52*, 537-579], enzyme inactivated as a result of sequential thio esterification and alkylation events should contain covalently cross-linked protomers. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) of inactivated enzyme under conditions designed to preserve labile thio ester linkages demonstrates the formation of cross-linked enzyme. Treatment of a paired sample of inactivated enzyme with hydroxylamine prior to SDS-PAGE eliminates all cross-linking, validating this aspect of our model for the mechanism of chloropropionyl-CoA-dependent inhibition.

A variety of approaches have been used to specifically modify amino acids at the active site of fatty acid synthase. Bloch's group (Helmkamp et al., 1968) utilized 3-decynoyl-*N*-acetylcysteamine to inhibit *Escherichia coli* β-hydroxydecanoyl-thioester dehydrase and subsequently demonstrated that a mechanism-based process accounted for the irreversible inactivation (Endo et al., 1970). The highly reactive analogue

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