Resonance Raman and Electron Paramagnetic Resonance Studies on Oxidized and Ferricyanide-treated *Clostridium pasteurianum* Ferredoxin

VIBRATIONAL ASSIGNMENTS FROM $^{34}$S SHIFTS AND EVIDENCE FOR CONVERSION OF 4 TO 3 IRON-SULFUR CLUSTERS VIA OXIDATIVE DAMAGE*

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Michael K. Johnson and Thomas G. Spiro
From the Department of Chemistry, Princeton University, Princeton, New Jersey 08544

Leonard E. Mortenson
From the Department of Biological Sciences, Purdue University, Lafayette, Indiana 47907

Resonance Raman spectra are reported for oxidized ferredoxin from *Clostridium pasteurianum* and for protein reconstituted with $^{34}$S$^2-$, using 4579 Å laser excitation. The spectra are of much higher quality than that previously reported, and the $^{34}$S shifts provide assignments of the Fe-S modes. After treatment with ferricyanide, the resonance Raman spectrum closely resembles that of the [3Fe-3S] protein, ferredoxin I from *D. gigas*; the $^{34}$S shifts aid in assignments of the [3Fe-3S] modes. The epr signal associated with the [3Fe-3S] cluster ($g = 2.01$) corresponds maximally to 0.80 spin/molecule. An aerobically addition of excess sulfide to the reduced, ferricyanide-treated protein generates a [4Fe-4S]$^{2+}$ epr spectrum, equivalent in intensity to the [3Fe-3S] signal. The ubiquitous occurrence of a $g = 2.01$ signal in preparations of [4Fe-4S] proteins can be attributed to variable amounts of [3Fe-3S] clusters generated by adventitious oxidation. The ready conversion of [4Fe-4S] to [3Fe-3S] clusters in *C. pasteurianum* raises the possibility that some [3Fe-3S] proteins may actually arise by oxidative damage of [4Fe-4S] proteins during aerobic isolation.

The ferredoxin from the bacterium *Clostridium pasteurianum* was the first to be isolated (1) and has since been the subject of numerous physicochemical studies, including epr (2, 3), Mössbauer (4, 5), cd, mc, md (6, 7), and nmr (8). It is a low $M_r$ (≈6000) iron-sulfur protein, containing 8 iron atoms and 8 labile sulfur atoms. It is believed, on the bases of physicochemical similarities and amino acid sequence homology (9) to have the same structure as that of ferredoxin from *Peptococcus aerogenes* (10, 11), with cubane-type Fe,S$_4$ clusters separated by approximately 12 Å, cysteinyl S completing the approximately tetrahedral coordination about each Fe atom. In the oxidized state each cluster is considered to be slightly distorted from an idealized cubic array toward a compressed tetragonal structure comprising 4 short and 8 long Fe-S bonds; reduction of point group symmetry from $T_d$ to $D_{2h}$. A similar tetragonal distortion and comparable Fe-S bond distances are observed for the isoelectric analogue inorganic cluster [Fe$_8$S$_8$(SCH$_2$Ph)$_4$]$^{2+}$, where more accurate structural data is available (12). Three oxidation levels are available to [4Fe-4S] clusters, each differing by one electron (12, 13). Using the IUPAC nomenclature, these are designated [4Fe-4S]$^{2+}$, [4Fe-4S]$^{3+}$, and [4Fe-4S]$^{1+}$ where the charge represents cluster minus cysteine ligands. The bacterial ferredoxins commonly cycle between the 2+ and 1+ states with relatively low redox potentials, between -350 and -450 mV. Photosynthetic bacteria, notably *Chromatium* contain so-called high potential iron protein, which cycles between the 3+ and 2+ levels at a redox potential of ≈+350 mV. Oxidized HiPIP$^+$ and reduced Fd both exhibit epr signals indicative of an $S = \frac{1}{2}$ Kramers doublet ground state; the former giving an essentially axial epr signal with $g_{ax} > 2$ ($g_L = 2.12, g_g = 2.04$) and the latter a rhombic signal with $g_{ax} < 2$ ($g$ values around 2.06, 1.92, and 1.88). The reduced [4Fe-8S] ferredoxins such as that from *C. pasteurianum* generally exhibit a more complex epr spectrum, centered around $g = 1.96$, which is believed to originate from magnetic coupling between the unpaired spins of two identical [4Fe-4S] clusters (14). That both Fd$_{ox}$ and HiPIP$_{ox}$, corresponding to the 2+ state of the cluster, are predominantly diamagnetic with $S = 0$ ground states, has been demonstrated by Mössbauer (15), low temperature mc (7), and magnetic susceptibility (16) studies. However, oxidized *C. pasteurianum* Fd in common with all other bacterial ferredoxins invariably has a weak, almost isotropic epr signal centered around $g = 2.01$. Since this signal has $g_{ax} > 2$ and is greatly enhanced by treatment with ferricyanide, it has been attributed to a small proportion of the clusters in the 3+ cluster oxidation level (17). Thus the ferricyanide or "superoxidized" form of *C. pasteurianum* Fd was considered isoelectric with HiPIP$_{ox}$. However, more recently low temperature mc experiments (18) have provided convincing evidence to suggest that ferricyanide treatment of *C. pasteurianum* Fd results in partial breakdown of the [4Fe-4S] centers to give [3Fe-3S] centers. This result has important consequences in terms of the physiologcal significance of the recently discovered [3Fe-3S] clusters in iron-sulfur proteins. In view of the confusion which has arisen in the literature over the assignment of an almost isotropic $g = 2.01$ epr signal from iron-sulfur proteins and the inability of Mössbauer spectroscopy to clearly differentiate between HiPIP$_{ox}$ centers and oxidized [3Fe-3S] centers (19).

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1 The abbreviations used are: HiPIP, high potential iron protein; Fd, ferredoxin; RR, resonance Raman; epr, electron paramagnetic resonance; mc, magnetic circular dichroism; cd, circular dichroism; exafs, extended x-ray absorption fine structure.
Resonance Raman spectroscopy has long been the method of choice for monitoring structural information for hemoproteins in solution (20). However, the published resonance Raman data for iron-sulfur proteins is sparse, because of relatively weak resonance enhancement, and the technical problems involved in preventing sample damage via laser irradiation. Thus although preliminary RR data for rubredoxin (21) (2Fe-S) (22--24) and (4Fe-S) (25) containing proteins (including C. pasteurianum Fd (25)) have been reported, the data are of marginal quality and only very tentative assignments have been possible. Advancements in both instrumentation and sampling techniques have improved the situation, as illustrated in a recent study of the ferredoxins from Desulfovibrio gigas in which RR spectroscopy was shown to be capable of distinguishing clearly between both (3Fe-3S) and (4Fe-4S) centers (26).

In the present work, we report improved RR data for oxidized C. pasteurianum including assignments based on 34S isotopic substitution. RR experiments on superoxided C. pasteurianum Fd has been investigated by RR, optical, and quantitative epr spectroscopy. The results confirm the version of the (4Fe-4S) clusters in C. pasteurianum via ferricyanide-oxidation. Furthermore, the use of C. pasteurianum Fd reconstituted with 34S2- in superoxidation experiments has facilitated assignment of the RR spectrum of the (3Fe-3S) center.

**EXPERIMENTAL PROCEDURES**

The isolation of C. pasteurianum Fd was based on the method described by Mortenson et al. (1). All the concentrations were determined using a molar extinction coefficient for the oxidized protein, εmax = 30,800 M-1 cm-1 (27). Ferricyanide oxidation was achieved by addition of the stated excess of K3Fe(CN)6 to a solution of ferredoxin in 0.05 M Tris-HCl, pH 7.5, followed by incubation for the stated time period at 5°C. Where the resulting samples were purified before use, the solution was passed down a Sephadex G-25 column equilibrated with 0.05 M Tris-HCl, pH 7.5. A bright blue band, presumed to be Prussian blue (K3FeFe(CN)6), adheres at the top of column suggesting some Fe has been removed from the ferredoxin. The brown protein was precipitated from the resultant solution by addition of (NH4)2SO4 up to 80% saturation at 0°C. After centrifugation, the protein was redissolved in 0.05 M Tris-HCl, pH 7.5. For RR experiments the protein solutions were made up to be 0.5 M Na2S04 to facilitate optical alignment and wave number calibration. All reductions were performed anaerobically by the addition of a small excess of sodium dithionate. The formation of apoprotein and subsequent reconstitution using isotopically labeled 34S2- were performed using the methods described by Rabinowitz (28). To ensure complete removal of all labile S, the precipitation using trichloroacetic acid, centrifugation, and redissolution of apoprotein in the appropriate buffer was repeated three times. Elemental 34S (95% enrichment) was obtained from Oakridge National Laboratories, TN and Na2S04 was prepared by reaction of elemental Na with 34S in liquid ammonia. Absorption spectra were recorded using a Cary 118 spectrophotometer. In all cases absorption and in some instances epr spectra were recorded both before and after RR experiments. These checks gave no indication of any damage to laser irradiation.

Samples for RR were contained in sealed spinning NMR tubes, cooled with cold N2 gas, and scattering was collected at an angle of 135° to the incident beam. The spectrometer consists of a Spex 1401 double monochrometer fitted with cooled RCA 31034 photomultiplier. Spectra were recorded digitally using photon counting electronics. The spectra were collected using a Digital minicomputer, smoothed with a Fourier transform filter. Lines from either a Spectra Physics 171 Kr+ or 170 Ar+ laser were used for excitation. The band positions cited in the text are accurate to ±1 cm-1.

epr spectra were measured on a Varian E-9, X-band epr spectrometer fitted with a Varian Oxford Instruments X-Band Q-band epr spectrometer. Double integration and subsequent quantitation of epr spectra were performed using the methods outlined by Wyand (29) and Aasa and Vännägård (30), respectively. 1 mm CuEDTA was used as the standard. To increase the accuracy of the quantitations, epr of both sample and standard under identical conditions were obtained in the same epr tube and the helium flow rate was not adjusted between the two measurements. For all signals, checks were made of the power dependence, to ensure no signal saturation was occurring.

**RESULTS AND DISCUSSION**

Fdα RR Spectrum—Fig. 1 shows the RR spectrum C. pasteurianum Fdα, and of the protein reconstituted using 34S2-, obtained with 4579 Å Ar+ laser excitation. This wave length appears to give maximal enhancement of the RR bands, all of which are found between 290 and 400 cm-1. Below 200 cm-1 the spectrum is obscured by the rising Rayleigh wing, the rising background above 400 cm-1 is due to scattering from the glass nmr sample tube. No RR bands at higher frequencies have been detected so far. The spectrum is of much better quality than that reported previously (25), and the 34S shifts permit more reliable assignments. There are four bands that exhibit 34S downshifts: 247 (--4), 271 (--5), 335 (--5), and 394 (--6) cm-1, and therefore involve motions of the bridging sulfur atoms (Sbr). They are attributable to the four expected Fe-Sbr modes in idealized Td symmetry, A1, E, 2T2. Suggested assignments, based in part on IR spectra of analog complexes (31) and preliminary normal coordinate analysis,2 are given in Table I. Two bands do not show 34S shifts: 356 and 285 cm-1. A band at 356 cm-1 in the IR spectrum of the analog complex [Fe3S4(SCH2Ph)4]2- shifts down upon [SCH2Ph substitution (31) and is therefore attributable to stretching of the terminal Fe-S bonds. In Td symmetry, there should be two terminal Fe-S (cys) modes, A1 and T2, but they may be coincident, since the terminal bonds are not connected to common atoms, and therefore have only potential, not kinematic interactions. Assignment of the weak band at 295 cm-1 is uncertain at the present time.

To the extent that the spectrum can be assigned on the basis of Td symmetry, it provides no support for significant distortion of the clusters associated with unequal Fe-S bond lengths. Symmetry lowering should produce splittings of the T2 and E modes. Slight distortion might escape detection, especially since the nontotally symmetric modes are weak. Splitting of 6 cm-1 or more should have been detectable however. The quantitative aspects of this problem are currently being investigated2 and will be addressed elsewhere.

Superoxidized Fd RR Spectrum—The RR spectrum of ferricyanide-treated C. pasteurianum Fd is shown in Fig. 2. It is different from that of C. pasteurianum Fdα, or of HiPIP (25, 31). However, it shows a remarkable similarity to that of D. gigas Fd II, shown in the same figure, which is known to contain exclusively (3Fe-3S) clusters (32, 33). Aside from a slight upshift of the D. gigas 260 cm-1 band, to 264 cm-1, the frequencies and relative intensities of the RR bands are the same for the two proteins. Together with the mcd evidence (18), this result leaves no doubt that ferricyanide treatment leaves C. pasteurianum Fd with a [3Fe-3S] cluster.

Partial assignment of the [3Fe-3S] RR spectrum can be achieved by treating the 34S reconstituted protein with ferricyanide. The RR spectrum of the resulting proteins shows three bands downshifted: 264 (--4), 346 (--8), and 365 (--3) cm-1, and therefore attributable to bridging sulfur modes. The [3Fe-3S] cluster in Azotobacter vinelandii Fd I has an approximately equilateral triangle of Fe atoms, with S atoms bridging the edges (34). In the idealized D3h symmetry (the actual 6-membered ring is substantially puckered), there are three Raman active bridging modes: A1 and 2E', and an A2 mode which is inactive. (Actually it has an antisymmetric
Resonance Raman and EPR Studies on C. pasteurianum Ferredoxin

**FIG. 1.** Resonance Raman spectra of oxidized *C. pasteurianum* Fd (a) as isolated and (b) after reconstitution of apoprotein using isotopically labeled $^{34}$S$. Protein concentration was 0.65 mM, pH 7.5, 0.05 M Tris-HCl buffer containing 0.5 M Na$_2$SO$_4$. Both spectra were obtained using 4579 Å Ar$^+$ laser excitation (100 mW) and 6 cm$^{-1}$ spectral bandwidth.

**TABLE I**

Assignment of the RR spectrum of oxidized *C. pasteurianum* Fd

<table>
<thead>
<tr>
<th>Assignment</th>
<th>Frequency</th>
</tr>
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<tbody>
<tr>
<td>Fe-S$_{t}$ (T$_2$)</td>
<td>394 (6)*</td>
</tr>
<tr>
<td>Fe-S(cys) (A$_1$, T$_2$)</td>
<td>336 (6)</td>
</tr>
<tr>
<td>Fe-S$_{a}(A_3)$</td>
<td>318 (5)</td>
</tr>
<tr>
<td>Fe-S$_{a}(E)$</td>
<td>271 (5)</td>
</tr>
<tr>
<td>Fe-S$_{a}(T_3)$</td>
<td>247 (4)</td>
</tr>
</tbody>
</table>

*The figures in parentheses indicate downshifts on $^{34}$S$_{t}$ substitution.*

Raman tensor and would be activated in resonance, via a vibronic mechanism (35) but this is unlikely). The strong 346 cm$^{-1}$ band is no doubt the $A_1$ mode, while the weak flanking bands at 264 and 365 cm$^{-1}$ are assignable to the $E'$ modes. Again, there is no evidence for symmetry lowering beyond the 6 cm$^{-1}$ spectral resolution.

The band at 393 cm$^{-1}$ shifts by no more than 1 cm$^{-1}$ upon $^{34}$S bridge substitution, and is therefore a candidate for a terminal Fe-S(cys) mode. The frequency is quite high for a Fe-S(cys) mode. In the *C. pasteurianum* Fd [4Fe-4S] spectrum, the frequency is 356 cm$^{-1}$, while in rubredoxin, which has four Fe-S(cys) bonds, the frequencies range from 312 to 371 cm$^{-1}$ (31). The [3Fe-3S] cluster of *A. vinelandii* Fd I has

**FIG. 2.** Resonance Raman spectra of oxidized FdII from *D. gigas* (a), superoxidized *C. pasteurianum* Fd (b), and a superoxidized sample of *C. pasteurianum* Fd after reconstitution of apoprotein using isotopically labeled $^{34}$S$. Spectra were recorded using 4579 Å Ar$^+$ laser excitation and 6 cm$^{-1}$ spectral band width. The *D. gigas* FdII was $\approx 1$ mm in terms of the monomeric unit, pH 7.6, 0.01 M Tris-HCl buffer containing 0.5 M Na$_2$SO$_4$. Both superoxidized samples were prepared by incubation for 15 h at 5 °C in the presence of a 15-fold excess of potassium ferricyanide and subsequently purified as described under "Experimental Procedures." Based on the quantitation of their epr signals each sample was $\approx 0.7$ mm in [3Fe-3S] clusters, pH 7.5, 0.05 M Tris-HCl buffer containing 0.5 M Na$_2$SO$_4$. 


a pair of cysteine sulfur atoms bound to each of two Fe atoms, while the third Fe has one cysteine S and one O atom from glutamate or a water molecule, according to the current x-ray analysis (34). Pairs of terminal Fe-S(cys) bonds should give rise to two modes with significant frequency separation due to the kinematic coupling. If the 393 cm\(^{-1}\) band is one of these modes, it is possible that the other underlies the 363 cm\(^{-1}\) band. If the ferricyanide-treated \textit{C. pasteurianum} Fd has any O atom ligands, they should give rise to Fe-O vibrations, which are expected in the 400-500 cm\(^{-1}\) region. These would most likely escape detection, since they would probably not be enhanced significantly in resonance with the S \rightarrow Fe charge transfer transitions which are responsible for the visible absorption bands. The presence of O atom ligands should, however, have some influence on the cluster spectrum, via both kinematic and electronic effects. The close similarity of the ferricyanide-treated \textit{C. pasteurianum} Fd, and \textit{D. gigas} Fd II spectra, suggest that the terminal ligands, as well as the clusters, are the same. For \textit{D. gigas} Fd II, exafs spectra appear to be consistent with exclusively sulfur ligation.\(^3\)

\textbf{epr Studies on Ferricyanide-treated \textit{C. pasteurianum} Fd---}\n
Fig. 3 shows the epr spectra of \textit{C. pasteurianum} Fd samples, before and after reduction with dithionite, after treatment for varying lengths of time with various amounts of ferricyanide. In the absence of ferricyanide, a freshly prepared sample of the oxidized protein gives a very weak \(g = 2.01\) signal, accounting for \(\approx 0.02\) spin/molecule. This is in agreement with previous epr studies (17). The size of this weak signal varied with the sample history. It increased by a factor of 3 upon prolonged (6 h) exposure to air, and by a factor of 6 upon reduction of a 10-fold excess of sodium dithionite and subsequent aerobic reoxidation. Reduction of \textit{C. pasteurianum} Fd produced the expected complex epr spectrum, which has been interpreted as being due to magnetically interacting \(S = \frac{1}{2}\), [4Fe-4S] clusters (14).

Ferricyanide treatment leads to a marked increase in the \(g = 2.01\) signal, and a corresponding diminution in the epr spectrum obtained on reduction. There appear to be two overlapping signals both centered around \(g = 2.01\), indicating a mixture of slightly different species. After incubation with a 15-fold excess of ferricyanide, at \(5^\circ C\) for 15 h, the \(g = 2.01\) signal corresponds to 0.80 spin/molecule while a negligible signal is observed on reduction. At lower temperatures, 10 K, and higher microwave powers, 20 mW, a small rhombic signal, \(g = 2.05\) and 1.88 was evident for the reduced species but this always accounted for \(<0.04\) spin/molecule. This signal is attributable to unreacted [4Fe-4S]\(^{3+}\) centers and is similar to that observed for reduced \textit{D. gigas} Fd II (36). The changes observed in the optical spectrum of \textit{C. pasteurianum} Fd on treatment with ferricyanide are analogous to those reported by Sweeney et al. (17) for \textit{C. acidiluvis} Fd. The absorbance ratio \(A_{280}/A_{260}\) is found to be a good monitor of the degree of [4Fe-4S] to [3Fe-3S] conversion, varying from 0.79 for the enzyme as isolated to 0.64 for the sample with a fully developed \(g = 2.01\) signal.

It appears, therefore, that ferricyanide treatment results in the loss of both [4Fe-4S] centers in \textit{C. pasteurianum} Fd, and the creation of approximately one [3Fe-3S] center. Evidently one of the [4Fe-4S] clusters has been disrupted completely while the other has lost one Fe, and presumably one S atom, to become a [3Fe-3S] cluster. An interesting question concerns the identity of the terminal ligands. A [3Fe-3S] cluster, if it follows the structural principles deduced by Ghosh et al. for \textit{A. vinelandii} Fd I (34), must have six terminal ligands, while [4Fe-4S] clusters have only four. \textit{C. pasteurianum} Fd has only the eight cysteine residues that act as ligands to the two [4Fe-4S] clusters in the native protein (9). If the ferricyanide-generated [3Fe-3S] cluster has six cysteine residues, then they must be contributed by both 4Fe-4S sites, presumably via a substantial conformation change. It cannot be excluded however, that some of the [3Fe-3S] ligands are O (or N) atoms of protein side chains, or water. As noted above this seems unlikely on the basis of the RR spectrum, but the evidence is not conclusive. In this connection, it is significant that recent mcd and epr experiments (37) indicate that [3Fe-3S] clusters also form in ferricyanide-treated ferredoxin from \textit{Bacillus steatorrhophilus}, which contains only one [4Fe-4S] cluster, and four cysteine residues (38), per molecule. Unless the
protein aggregates, a requirement for six ligands [3Fe-3S] cluster would mean that at least two of them are not cysteine in this case.

When a sample of ferricyanide-treated C. pasteurianum Fd with a fully developed $g = 2.01$ signal is purified (see “Experimental Procedures”) anaerobically reduced with sodium dithionite and subsequently treated with a large excess (~100-fold) of sodium sulfide, the very weak epr signal attributed to residual, magnetically uncoupled [4Fe-4S] $^*$ center is greatly enhanced. Quantitation indicates that this signal corresponds to essentially the same number of spins as the $g = 2.01$ signal prior to reduction. This result suggests that the [3Fe-3S] clusters is capable of regenerating a [4Fe-4S] cluster upon treatment with sulfide. Since no iron was added, the protein is therefore required to contain some adventitiously bound iron. This raises the possibility of facile interconversion of [3Fe-3S] and [4Fe-4S] clusters which may have considerable physiological significance. It is presently unknown whether the observed reaction is a simple addition of sulfide and iron to the [3Fe-3S] cluster, with associated terminal ligand rearrangement, or a more profound reassembly of an iron-sulfur core since C. pasteurianum Fd is capable of being reconstituted from the apoprotein by addition of iron and sulfide ions (28). These preliminary results require further investigations to establish more clearly the mechanism for regeneration of the [4Fe-4S] center.

Implications for Other Proteins—It is of general significance that a protein so clearly constructed to accommodate [4Fe-4S] clusters as C. pasteurianum Fd, can nevertheless stabilize a [3Fe-3S] cluster upon oxidative treatment. There is much current interest in the occurrence and function of the newly discovered [3Fe-3S] centers. In addition to A. vinelandii Fd I (39) and D. gigas Fd II (32), Mossbauer has been instrumental in characterizing [3Fe-3S] centers in mitochondrial aconitase (19, 39), glutamate synthase from A. vinelandii (25), and a bacterial Fd from Thermus thermophilus (40). All of these proteins exhibit an almost isotropic $g = 2.01$ signal as isolated. However, until now the widespread use of this epr signal in identifying [3Fe-3S] centers has been hindered by its appearance in the epr spectra of well characterized [4Fe-4S] containing proteins, such as C. pasteurianum Fd. As a result of the present work and the low temperature mcd studies (18), we conclude that an almost isotropic epr signal centered around $g = 2.01$ is uniquely indicative of a [3Fe-3S] center. This signal is readily observable at temperatures below 35 K, being very intense compared to other iron-sulfur protein epr signals due to its narrow band width, $\approx 30$ G. A survey of the literature shows this signal to have been observed for bacterial ferredoxins from Rhodospirillum rubrum (41), Corynebacterium autotrophicum (42), Mycobacterium flavum (43), Spirillum lipoferum (43), Azobacter coccococcus (44), and Methanosarcina barkeri (44), mammalian and bacterial succinate dehydrogenases (45-47) in a nitrate reductase from Micrococcus denitrificans (48), and in D. gigas hydrogenase (49). Clearly [3Fe-3S] clusters are ubiquitous species.

In view of the ready conversion of C. pasteurianum Fd to a form containing only [3Fe-3S] clusters, the question must be asked whether proteins which are now known to contain [3Fe-3S] clusters may have acquired them via oxidative damage during protein isolation. In this connection, it should be noted that most isolations are performed aerobically from organisms which function anaerobically. It may be worth reinvestigating the known [3Fe-3S] proteins using rigorously anaerobic isolation procedures to ensure that the [3Fe-3S] cluster is really a native structure.

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References
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Resonance Raman and electron paramagnetic resonance studies on oxidized and ferricyanide-treated Clostridium pasteurianum ferredoxin. Vibrational assignments from 34S shifts and evidence for conversion of 4 to 3 iron-sulfur clusters via oxidative damage. Vibrational assignments from 34S shifts and evidence for conversion of 4 to 3 iron-sulfur clusters via oxidative damage.

M K Johnson, T G Spiro and L E Mortenson


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