Mutated Forms of the [2Fe-2S] Ferredoxin from *Clostridium pasteurianum* with Noncysteinyl Ligands to the Iron−Sulfur Cluster

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ABSTRACT: The [2Fe-2S] ferredoxin from *Clostridium pasteurianum* is unique among ferredoxins, both by its sequence and by the distribution of its cysteine residues (in positions 11, 14, 24, 56, 60). Thus, no homologous sequences are available to infer, by comparison, the identity of the ligands of the iron−sulfur cluster. Therefore, in order to obtain information on the latter point, a combination of site-directed mutagenesis and UV−vis, EPR, and resonance Raman spectroscopy has been implemented. All of the cysteine residues have individually been replaced by serine and two of them by alanine. Cysteine 14 could be replaced by either serine or alanine without any modification of the spectroscopic properties of the protein and was therefore dismissed as a ligand of the [2Fe-2S] cluster. The C56S, and C60S-mutated proteins were both found to display UV−vis, EPR, and resonance Raman spectra consistent with serine-coordinated [2Fe-2S] clusters. The C11S-mutated protein was considerably less stable than the wild type ferredoxin. This observation, together with the hypsochromic shifts of UV−visible absorption features upon cysteine 11 ⟹ serine mutation, suggested cysteine 11 to be a ligand of the [2Fe-2S] cluster. Cysteine 24 could be replaced by either serine or alanine without decreasing the stability of the protein and without dramatically changing its spectroscopic properties. Thus, either cysteine 24 is not a ligand of the [2Fe-2S] cluster or it is replaced by another ligand in the C24A mutated protein. A [2Fe-2S] cluster was also assembled in the C14A/C24A doubly mutated protein, i.e., in a polypeptide chain containing only three cysteine residues. Thus, at least in the latter case, an unknown noncysteinyl ligand has to be involved in the coordination of the iron−sulfur cluster. The possible occurrence of noncysteinyl ligation in the wild type protein as well is discussed.

Ferredoxins (Fds[1]), which are small electron-transferring iron−sulfur proteins, have now been purified in the hundreds from an extremely wide range of organisms (Matsubara & Saeki, 1992). These proteins have been grouped in families according to their function or active-site structure (Cammack, 1992; Matsubara & Saeki, 1992). An alternative classification, based on amino acid sequence and, when such data are available, on crystal structures, suggests that Fds belong to several phylogenetically unrelated families (Meyer, 1988). The best known among the latter are the low potential Fds containing one or two $[4Fe\text{-}4S]^{2+/+}$ or $[3Fe\text{-}4S]^{+/0}$ clusters, the high potential $[4Fe\text{-}4S]^{3+/2+}$ Fds (HiPIPs), and the plant-type $[2Fe\text{-}2S]^{2+/+}$ Fds (Meyer, 1988; Matsubara & Saeki, 1992).

A unique [2Fe-2S] Fd has been isolated from the nitrogen-fixing saccharolytic anaerobe *Clostridium pasteurianum* (Hardy et al., 1965). Although the function of this protein has remained unknown, a significant body of data is now available. This includes biochemical properties (Cardenas et al., 1976; Meyer et al., 1984), spectroscopic features of the iron−sulfur chromophore (Cardenas et al., 1976; Meyer et al., 1984; 1986b; 1992; Fu et al., 1992), and the amino acid sequence (Meyer et al., 1986a), which has recently been confirmed by the sequence of the encoding gene (Meyer, 1993). The primary structure of the [2Fe-2S] Fd from *Clostridium pasteurianum* (Cp) is not related to any other protein sequence (Meyer, 1988). This uniqueness is underscored by the very unusual distribution of the five cysteines (in positions 11, 14, 24, 56, and 60) along the sequence of 102 residues (Meyer et al., 1986a). Thus, important pieces of information such as the folding of the polypeptide chain around the iron−sulfur chromophore, or the identity of the ligands of the latter, cannot be deduced from comparisons with homologous proteins, and will become directly accessible only when the three dimensional structure of the [2Fe-2S] CpFd is available. Useful data relevant to these questions can nevertheless be obtained through complementary biochemical and spectroscopic studies of the protein and appropriately modified forms thereof.

For this purpose, the gene encoding the [2Fe-2S] CpFd has been overexpressed in *Escherichia coli* (Fujinaga & Meyer, 1993). Variants of this Fd with cysteine residues mutated into serine were prepared, and the first evidence of

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[1] Abbreviations: Fd(s), ferredoxin(s); Cp, *Clostridium pasteurianum*; PCR, polymerase chain reaction; C11S, mutated ferredoxin in which cysteine 11 has been replaced by serine; C14A/C24A, doubly mutated ferredoxin in which both cysteine 14 and cysteine 24 have been replaced by alanine; WT, wild type.

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Table 1: Mutagenic Oligonucleotides[a]

oligonucleotide	sequence	complementary to
O4	5′TAATACGACTCACTATA 3′	noncoding strand
O9	5′ TTGGTAACTGTCAGACC 3′	coding strand
C11S	5′ CCATTAAGTCTACAACTAGTAGAAACGAAGATGTG 3′	coding strand
C14S	5′ CCATTAAGTCTAGAACTAGTACAAACGAAGATGTG 3′	coding strand
C14A	5′ CCATTAAGTCTAGCACTAGTACAAACGAAGATG 3′	coding strand
C24S	5′ GGAATTTTTGGAGTAACTAAAACCTTGCTGC 3′	coding strand
C24A	5′ GGAATTTTTGGAGTAAGCAAAACCTTGCTGC 3′	coding strand
C56S/C60S	5′ TAATACAGGTT(GC)CTTTGGTATAT(GC)CAGTCAAGGCC 3′	noncoding strand

[a] Mutated bases are underlined.

serine-ligated [2Fe-2S] clusters in an isolated iron−sulfur protein was brought forth (Fujinaga et al., 1993). We have now individually mutated all five cysteine residues of the [2Fe-2S] CpFd into either serine or alanine and report here spectroscopic data on these molecular variants. The identity of the ligands of the [2Fe-2S] chromophore, as well as possible structural frameworks of the latter, are discussed on the basis of these results.

MATERIALS AND METHODS

All common DNA manipulations were as described (Ausubel et al., 1988; Meyer & Gagnon, 1991; Fujinaga & Meyer, 1993). Restriction enzymes were purchased from Eurogentec, T4 DNA ligase and DNase I from Boehringer Mannheim, RNase A from Sigma, Taq DNA polymerase from Appligene, bacterial alkaline phosphatase from Amersham, and competent DH5α *E. coli* cells from Gibco-BRL. Oligonucleotides were synthesized by phosphoramidite chemistry on a 381A Applied Biosystems synthesizer.

Site-directed mutagenesis was performed by a modification (Kammann et al., 1989) of a method (Higuchi et al., 1988) which uses two successive rounds of polymerase chain reaction (PCR) to create a mutation and amplify a DNA fragment surrounding it. The DNA on which mutations were introduced was the pTCP2F plasmid (Fujinaga & Meyer, 1993), where a sequence encoding the [2Fe-2S] ferredoxin from *Clostridium pasteurianum* was cloned between the *Nde*I (5'end) and *Hin*dIII (3'end) restriction sites of the pT7-7 expression vector (Tabor, 1990). The oligonucleotides used as primers for the mutagenesis are listed in Table 1, and the preparations of the mutated plasmids are described in Table 2. The products of the second rounds of PCR were precipitated with ethanol, cut with *Nde*I and *Hin*dIII, and electrophoresed through a 2% low melting agarose gel (Nusieve, FMC). The DNA fragments having the size expected for the ferredoxin encoding sequence were excised and ligated into the pT7-7 vector cleaved with the same enzymes and dephosphorylated. The ligation mixtures were used to transform *E. coli* DH5α cells. Clones were first scanned by restriction analysis and partial sequencing. In case they contained the desired mutations, the ferredoxin gene was completely sequenced (Sanger et al., 1977) to ensure the absence of additional unwanted mutations.

The mutated plasmids were used to transform *E. coli* K38 (HfrC λ) cells harboring the pGP1-2 plasmid. The latter carries the T7 RNA polymerase gene under the control of the heat-inducible λp_L promoter and the gene encoding the temperature sensitive repressor cI857. In this double plasmid system, cultures grown at 30 °C are submitted to a temperature jump at 41 °C, which induces the gene for T7 RNA polymerase. The latter enzyme then becomes responsible for most of the transcription, which results in overproduction of the target protein (Tabor, 1990). *E. coli* K38/pGP1-2 harboring the wild type or mutated pTCP2F plasmids was grown at 30 °C in 0.5 or 1 L batches of Luria Broth supplemented with 75 mg/L of ampicillin and 40 mg/L of kanamycin. When A_{590} reached 1, the temperature was raised to 41 °C for 1 h, and the incubation was subsequently carried on overnight at 30 °C. The cells were centrifuged (5000*g*, 25 min), resuspended in 60 mL of 0.1 M NaCl, 0.02 M Tris−HCl, pH 7.5 (buffer A), and disrupted by sonication, and the cell debris was removed by centrifugation (35000*g*, 20 min). All subsequent steps were performed with argon-sparged buffers and vials. The cell extract was loaded on a 10 × 1.5 cm anion exchange (DE52, Whatman) column equilibrated with buffer A. The ferredoxin was clearly visible as a reddish brown band at the top of the column. After being washed with four volumes of buffer A and four volumes of 0.2 M NaCl, 0.02 M Tris−HCl, pH 7.5, the ferredoxin-containing fraction was eluted with 0.4 M NaCl, 0.02 M Tris−HCl, pH 7.5, and subsequently treated with DNase I and RNase A (0.1 mg/mL each) for 2 h at 37 °C. Precipitated material was removed by centrifugation (10000*g*, 6 min), and the supernatant was concentrated in an Amicon cell fitted with a PM 10 membrane and chromatographed on a 100 × 2.5 cm Sephadex G75 (Pharmacia) column equilibrated with 0.2 M NaCl, 0.02 M Tris−HCl, pH 7.5. The last step, which yielded pure Fd (Fujinaga & Meyer,

Table 2: Generation of Mutated Genes[a]

mutation	implemented plasmid[b]	primers for first round of PCR	primer for second round of PCR
C11S	pTCP2F(WT)	O4 and C11S	O9
C14S	pTCP2F(WT)	O4 and C14S	O9
C14A	pTCP2F(WT)	O4 and C14A	O9
C24S	pTCP2F(WT)	O4 and C24S	O9
C24A	pTCP2F(WT)	O4 and C24A	O9
C14S/C24A	pTCP2F(C24A)	O4 and C14S	O9
C14A/C24S	pTCP2F(C24S)	O4 and C14A	O9
C14A/C24A	pTCP2F(C24A)	O4 and C14A	O9
C56S	pTCP2F(WT)	O9 and C56S/C60S	O4
C60S	pTCP2F(WT)	O0 and C56S/C60S	O4

[a] The first rounds of PCR were performed as follows: plasmid pTCP2F (10 ng) was denatured for 5 min at 95 °C in the presence of 0.3 μM of each primer in a final volume of 100 μL containing 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris−HCl, pH 8.3, and 0.2 mM of each dNTP. Taq DNA polymerase (2.5 U) was then added, the reaction volume was covered with mineral oil, and 15 reaction cycles (1 min at 94 °C, 1 min at 50 °C, 1 min at 72 °C,) were run, followed by a 7 min elongation at 72 °C. The second rounds of PCR were as above, except that 10 μL of the first PCR was used as one of the primers. [b] The pTCP2F plasmid has been described previously (Fujinaga & Meyer, 1993; see also Methods). The sequence of the Fd gene present in the plasmid is in parentheses.

Table 3: UV-Vis Absorption Bands (nm) of the Wild Type and Mutated [2Fe-2S] Ferredoxins from *C. pasteurianum*[a]

						A450/A280 ratio
wild type	336	420 (sh)	464	550	700 (sh)	0.50
C11S	330 (sh)	418	460 (sh)			0.10
C14S	336	420 (sh)	464	550	700 (sh)	0.48
C14A	336	420 (sh)	464	550	700 (sh)	0.48
C24S	332	418	458	548	700 (sh)	0.48
C24A	332	420	456	544	700 (sh)	0.47
C14A/C24A	332	442		560 (sh)		0.37
C56S	328	410 (sh)	446	530 (sh)	620 (sh)	0.40
C60S	328	410 (sh)	444	530 (sh)	620 (sh)	0.33

[a] Abbreviation: sh, shoulder.

1993), consisted of an anion exchange HPLC chromatography on a PL-SAX (Polymer Labs) column developed with a 0.1−1 M NaCl gradient in Tris−HCl 0.01 M, pH 7.5.

UV-visible spectra were recorded on a Hewlett-Packard 8452 diode array spectrophotometer.

EPR spectra were recorded with a X-band Varian E-109 spectrometer equipped with a liquid helium transfer system (Oxford Instruments ESR 900) at a klystron frequency of 9.226 GHz and a temperature of 12 K with a microwave power of 0.01 mW. All proteins (ca. 0.3 mM in NaCl 0.1 M, Tris−Cl 10 mM, pH 7.5) were reduced with dithionite (3 mM final concentration).

Low-temperature (15−20 K) resonance Raman spectra were excited at 457.9 nm and recorded as previously described (Lutz, 1977; Lutz et al., 1983; Moulis et al., 1984).

RESULTS

The results of the mutations were first monitored by running preliminary purifications from 0.5 L cultures. In all cases investigated, a colored fraction bound to the top of the DE52 column indicated the presence of iron−sulfur chromophores in the mutated proteins. Furthermore, additional indications were given by the nature and the intensity of the color. All mutations on cysteines 14 and 24 yielded brownish red fractions indistinguishable from the wild type. In contrast, the mutations bearing on cysteines 11, 56, and 60 resulted in colors having a more brownish cast. The intensity of the color was similar in all cases, except for the C11S mutant, the low yield of which was predictable from the notably less colored DE52 column. Indeed, the C11S Fd was obtained in yields of 0.2−0.4 mg of pure protein per liter of culture, whereas 1 to 2 mg/L were obtained for the wild type or for the other mutants. The mutated proteins were purified to homogeneity from 5 to 10 L of pooled cultures (see Methods) and characterized by UV−vis, EPR, and resonance Raman spectroscopy.

UV−Vis Absorption Spectroscopy. The UV−vis absorption properties of all the mutant Fds are collected in Table 3 and compared with those of the wild type. The absorption spectrum of the C11S Fd (Figure 1) was characterized by a low (0.1) A_{450}/A_{280} absorption ratio (Table 3), which indicated a relatively low content of chromophore. The purification steps which, for the wild type protein and for the other mutants, yielded pure ferredoxin (Fujinaga & Meyer, 1993; see Methods), were unsuccessful in increasing the A_{450}/A_{280} ratio of the C11S Fd. It may be inferred from this observation that the chromophore of this mutated Fd is unstable and decomposes as the protein is processed. This instability would suggest that cysteine 11 is a ligand of the [2Fe-2S] cluster. It should also be noted that the spectrum

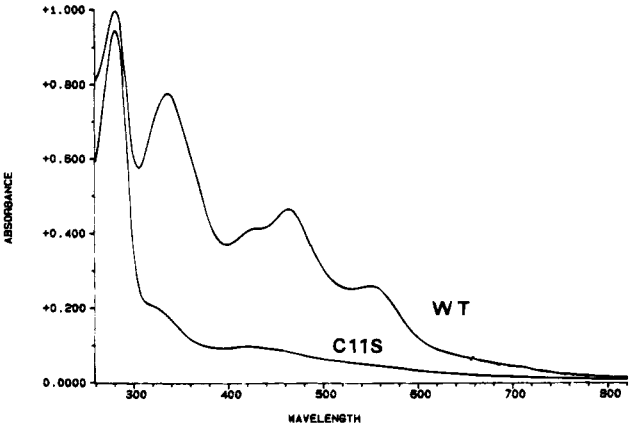

FIGURE 1: UV−vis absorption spectra of wild type and C11S-mutated [2Fe-2S] *C. pasteurianum* ferredoxins. The protein concentrations were 5 mg/mL for the wild type and 8 mg/mL for the C11S mutant. The optical pathlength was 1 mm.

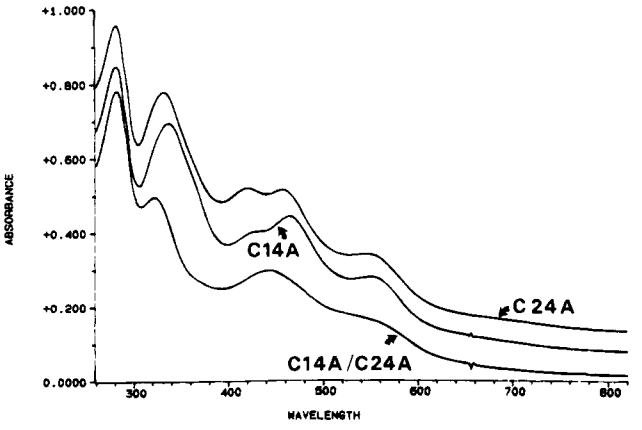

FIGURE 2: UV−vis absorption spectra of mutated [2Fe-2S] *C. pasteurianum* ferredoxins. For the sake of clarity, offsets of +0.06 and +0.12 absorbance units have been applied to the spectra of the C14A and C24A mutated proteins, respectively. The protein concentrations were 4 mg/mL. The optical path length was 1 mm.

of the C11S Fd, though rather featureless, appears to be altogether shifted to shorter wavelengths, as compared to that of the wild type protein (Figure 1).

The UV−vis absorption spectra of the C14S (not shown) and C14A (Figure 2) Fds were found to be identical with that of the wild type protein (Table 3). This, together with additional evidence shown below, rules out cysteine 14 as a ligand of the [2Fe-2S] cluster.

The UV−vis absorption spectra of the C24S (Fujinaga et al., 1993) and of the C24A (Figure 2) Fds are practically identical with each other and display A_{450}/A_{280} ratios similar to that of the wild type Fd (Table 3). These data show that cysteine 24 is not indispensable for the assembly of the

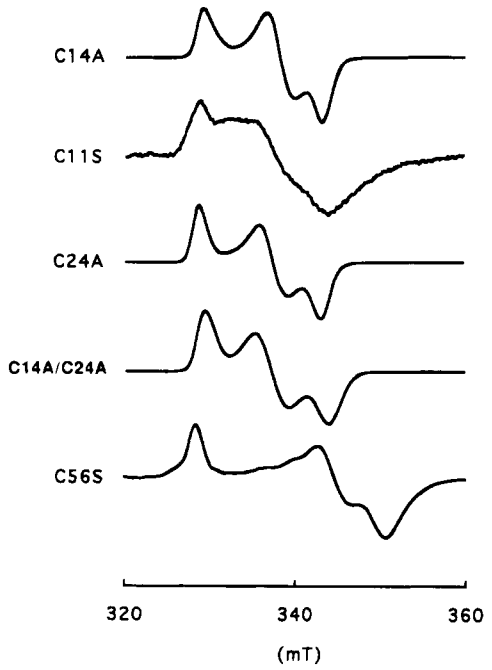

FIGURE 3: EPR spectra of wild type and mutated [2Fe-2S] *C. pasteurianum* ferredoxins. The spectra were recorded as described in the Methods. The protein concentrations were 3 mg/mL, except for the C11S Fd (10 mg/mL).

[2Fe-2S] cluster. On the other hand, the optical absorption spectra of both of these mutant Fds display small bandshifts (5 nm or less) and slight variations in the relative intensities of the bands at 420 and 460 nm. The latter data would suggest some involvement of cysteine 24 in the Fe−S chromophore structure. One of the reasons why a [2Fe-2S] cluster is nevertheless assembled in the C24A Fd might be that another residue, and possibly cysteine 14, takes over the role of cys 24 as a ligand of the chromophore. A somewhat similar situation has been encountered in *Azotobacter vinelandii* FdI (Martin et al., 1990). We therefore prepared the following double mutants: C14S/C24A, C14A/C24S, and C14A/C24A (Table 2). As the properties of these mutated Fds were found to be practically identical with each other, only those of the C14A/C24A double mutant will be described in some detail. The UV−vis absorption spectrum of the latter Fd differs from those of the wild type, C14A, and C24A mutants by its displaying a single maximum, at 442 nm, in the 400−500 nm range (Figure 2) and a lower A450/A280 absorbance ratio (Table 3). These data show that a Fe−S cluster is assembled in the C14A/C24A Fd, although its polypeptide chain contains only three cysteine residues.

The UV−vis absorption spectra of the C56S and C60S Fds have previously been shown to display hypsochromic shifts of ca. 20 nm in the 400−500 nm region, as compared to the spectrum of the wild type protein (Fujinaga et al., 1993; Table 3). This is consistent with the replacement of a sulfur by an oxygen ligand (Cleland & Averill, 1984) and, therefore, suggests that cysteines 56 and 60 are ligands of the [2Fe-2S] cluster in the wild type Fd (Fujinaga et al., 1993).

EPR Spectroscopy. The EPR spectrum of the reduced C11S Fd is broad and displays a low signal to noise ratio (Figure 3), in keeping with the previously noted instability of the chromophore, but its apparent g values differ little from those of the wild type Fd (Table 4). The EPR spectra

Table 4: Apparent EPR g Values of the Wild Type and Mutated [2Fe-2S] Ferredoxins from *C. pasteurianum*

	g_z	g_y	g_x
wild type	2.004	1.948	1.922
C11S	2.003	1.955	1.916
C14S	2.003	1.950	1.921
C14A	2.004	1.950	1.921
C24S	2.005	1.955	1.921
C24A	2.004	1.954	1.923
C14A/C24A	2.001	1.956	1.917
C56S	2.007	1.916	1.883
C60S	2.005	1.923	1.882

of the C14S (not shown) and C14A (Figure 3) Fds are identical with that of the wild type Fd (Table 4). As observed in the case of the UV−vis absorption spectra, the EPR spectra of the C24S (Fujinaga et al., 1993) and of the C24A (Figure 3) Fd are nearly identical with each other (Table 4). They both differ from the spectrum of the wild type Fd by a slight shift to lower field of the g_y value (Table 4). The EPR spectrum of the C14A/C24A double mutant differs from that of the C24A mutant only by small shifts of the g_x and g_z values to higher field (Figure 3; Table 4), which suggests that a [2Fe-2S] cluster is present in the doubly mutated protein. The EPR spectra of the C56S (Figure 3) and C60S Fds were found to differ from that of the wild type protein more than those of any of the other mutants (Table 4). They are characterized by an increased rhombicity of the g tensor (Fujinaga et al., 1993; Table 4). Similar spectral changes have been reported for reduced [2Fe-2S] synthetic analogues upon partial substitution of sulfur terminal ligation by oxygen (Beardwood & Gibson, 1985, 1992).

The saturation behavior of the wild type and of all the mutant Fds has been investigated by varying the microwave power in the 0.005−5 mW range at fixed temperature (in the 10−20 K range) and was found to be identical in all samples (not shown) within the accuracy of the measurement. This affords further evidence that [2Fe-2S] clusters are present in all of these proteins.

Resonance Raman Spectroscopy. The resonance Raman spectrum, excited at 457.9 nm, of the wild type [2Fe-2S] CpFd expressed in *E. coli* is shown in Figure 4. This spectrum is identical with the spectrum of the protein purified from *C. pasteurianum* (Meyer et al., 1984, 1986b, 1992), not only in the region (200−450 cm^{-1}) where Fe−S stretching modes are expected (Figure 4; Table 5), but also in higher frequency regions (550−800 cm^{-1}, not shown) where several bands arising from overtones and combinations of fundamental modes were observed (Meyer et al., 1984). This is consistent with previously published evidence demonstrating that the recombinant protein is identical with the native one (Fujinaga & Meyer, 1993). Resonance Raman spectra of the C11S mutant could not be obtained, partly because of a strong background fluorescence and partly because of the relatively low chromophore content of the protein solution, which was, as discussed above, a consequence of the low stability of this mutated Fd. Resonance Raman spectra of the C14S (not shown) and C14A (Figure 5) Fds are only marginally different from that of the wild type Fd (Table 5), in keeping with the UV−vis absorption (Table 3) and EPR (Table 4) spectra which indicated that mutations on cysteine 14 had little bearing on the spectroscopic properties of the [2Fe-2S] chromophore.

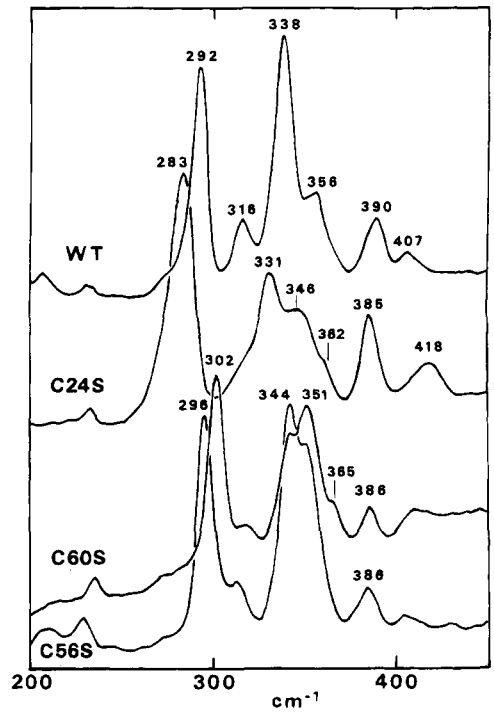

FIGURE 4: Resonance Raman spectra, excited at 457.9 nm, of wild type and cys ⟹ ser-mutated [2Fe-2S] *C. pasteurianum* ferredoxins. Temperature 20 K. Scanning speed 50 cm^{-1}/min. Time constant 1.8 s. The spectra were obtained by the summation of eight scans, except for the wild type (6 scans) Fd.

In the resonance Raman spectra of the C24S (Figure 4) and C24A (Figure 5) Fds, most bands occur at lower frequency than in the spectra of the wild type Fd, with the exception of the 407 cm^{-1} band (wild type Fd) which is shifted to higher frequency by ca. 10 cm^{-1} (Table 5). Another noteworthy feature of the C24S and C24A Fd spectra is an increased bandwidth and asymmetry of the ca. 285 cm^{-1} band, as compared to its counterpart in the spectrum of the wild type Fd (Figure 4). The broadening of this band results in part from the presence of an additional component on its low frequency side. The resonance Raman spectrum of the C14A/C24A double mutant displays features that are shared by all of the Fds mutated at cysteine 24 and which distinguish them from the wild type and from the other mutated Fds: namely, a downshift of the 292 cm^{-1} band to 283−288 cm^{-1}, of the 370 cm^{-1} to 362−363 cm^{-1}, and an upshift of the 407 cm^{-1} band to 416−419 cm^{-1} (Table 5). This indicates that the chromophores of all these Fds have common structural characteristics. On the other hand, in the 310−350 cm^{-1} region, the spectrum of the C14A/C24A double mutant displays unique features (Figure 5). Interestingly, the resonance Raman spectra of the Fds mutated at cysteine 24 are reminiscent of those of the *in vitro* reconstituted wild type Fd (Meyer et al., 1992).

Resonance Raman spectra of the C56S and C60S proteins (Figure 4) display band patterns analogous to that of the wild type protein in the frequency region (200−450 cm^{-1}) where Fe−S stretching modes are expected (Meyer et al, 1984; 1986b). The differences concern mainly the strongest bands, at 292 and 338 cm^{-1} for the wild type protein, which are shifted to higher frequency in the spectra of both the C56S and the C60S proteins. Such shifts to higher frequency, which are expected for a S⟹O ligand substitution, are consistent with the attribution of these two bands to stretching modes of the Fe−Scys bonds (Han et al., 1989; Fu et al., 1992; see Discussion). Most of the other bands are either unshifted or shifted to lower frequency (Figure 4, Table 5).

DISCUSSION

The [2Fe-2S] CpFd has previously been shown to be a dimer, each subunit containing one [2Fe-2S] cluster (Meyer et al., 1984, 1986a). The presence of binuclear iron−sulfur clusters in this Fd is supported by a considerable body of spectroscopic evidence reported previously (reviewed in Meyer et al. (1992)) as well as in this work. Furthermore, electrospray ionization mass spectra (ESI-MS) of this protein (E. Forest, Y. Pétillot, J.-M. Moulis, and J. Meyer, unpublished results) have recently been found to consist of only two components, with masses of 11 428 Da (monomeric apoprotein) and 11 600 Da (apoprotein plus two sulfur and two iron atoms, and minus four cysteinyl protons). The observation of the monomer only in the ESI-MS experiments, whereas the protein is dimeric in solution, is not unexpected since the quaternary structures of proteins are often not retained into the gas phase (Light-Wahl et al., 1994). These data confirm that the active site contains a Fe_2S_2 inorganic core and that there is one [2Fe-2S] cluster per subunit. They also show that the two clusters are not held between the two subunits: if they were, they would be lost as the two subunits dissociate, and only the apoprotein mass would be detected in the ESI-MS spectra. Additional information on the relative positioning of the two [2Fe-2S] clusters in the dimeric protein can be deduced from the EPR spectra at the reduced level (Cardenas et al., 1976; Fujinaga et al., 1993): since no magnetic interaction is observed, the two clusters are very likely to be at least 10 Å apart from each other. Therefore, their coordination spheres may be considered as independent units, and ligand reshuffling among them is very unlikely. Finally, gel filtration experiments on Sephadex G-75 (data not shown, see Methods) indicate that the proteins studied here, wild type and mutants, have apparent sizes differing by less than 10%. and, hence, are all dimers. For these reasons, it will be assumed that the quaternary structure has no bearing on the following discussion on the ligands of the [2Fe-2S] cluster.

Cysteine Ligands of the [2Fe-2S] Cluster. Cysteine 14 may be dismissed as a ligand of the [2Fe-2S] cluster, since the UV−vis (Table 3), EPR (Table 4), and resonance Raman (Table 5) spectra of both the C14S and C14A mutants are identical with those of the wild type Fd. This was rather unexpected, in view of the belonging of cysteine 14 to a CxxC (x being any amino acid) sequence segment which was to this day a ubiquitous bidentate ligand of [2Fe-2S] clusters in Fds (Matsubara & Saeki, 1992). This result underlines the present uniqueness of the [2Fe-2S] CpFd. The C14A-mutated Fd has been found, by gel filtration (data not shown, see Methods) to have the same apparent mass as the wild type Fd. Thus, the dimeric structure of this Fd is not dependent on cysteine 14.

As to cysteines 56 and 60, the spectroscopic data show that they are ligands of the [2Fe-2S] cluster: indeed, previously reported UV−vis (Table 3) and EPR (Table 4) spectra of the C56S and C60S mutants have been shown, by comparison with data obtained with synthetic analogues (Coucouvanis et al., 1984; Cleland & Averill, 1984; Beard-

Table 5: Resonance Raman Frequencies (cm^{-1}) of Wild Type and Mutated Forms of *C. pasteurianum* [2Fe-2S] Ferredoxin[a]

assignments[b]	wild type	C14S	C14A	C24S	C24A	C14A/C24A	C56S	C60S
$B_{3u}{}^T$	292	292 (0)[c]	293 (+1)	283 (−9)	285 (−7)	288 (−4)	296 (+4)	302 (+10)
$B_{1g}{}^B$	316	315 (−1)	316 (0)	316 (0)	312 (−4)	319 (+3)	313 (−3)	317 (+1)
$A_g{}^T$	338	338 (0)	339 (+1)	331 (−7)	330 (−8)	339 (+1)	344 (+6)	342 (+4)
$B_{1u}{}^T$, $B_{2g}{}^T$	356	354 (−2)	355 (−1)	346 (−10)[d]	350 (−6)[d]	345 (−11)[d]	351 (−5)	351 (−5)
$B_{3u}{}^B$	370[e]	370 (0)	370 (0)	362 (−8)	363 (−7)	362 (−8)	nd[f]	365 (−5)
$A_g{}^B$	390	390 (0)	390 (0)	385 (−5)	387 (−3)	388 (−2)	386 (−4)	386 (−4)
$B_{2u}{}^B$	407	406 (−1)	407 (0)	418 (+11)	416 (+9)	419 (+12)	405 (−2)	408 (+1)

[a] Spectra excited at 457.9 nm. [b] Assignments of the Fe−S stretching modes in the idealized D_{2h} point group of the $Fe_2S_2{}^BS_4{}^T$ chromophore (Han et al., 1989; Fu et al., 1992). B = bridging (inorganic sulfur), T = terminal (cysteinyl sulfur). [c] Frequency shift from the corresponding band of wild type protein in parentheses. [d] In all three of these spectra, this broad, and sometimes apparently composite (see Figure 5, spectrum of C24A Fd), band spreads over the 345−353 cm^{-1} region. [e] Previously detected in spectra of the native protein from *C. pasteurianum* (Meyer et al., 1986b). [f] nd = nondetected.

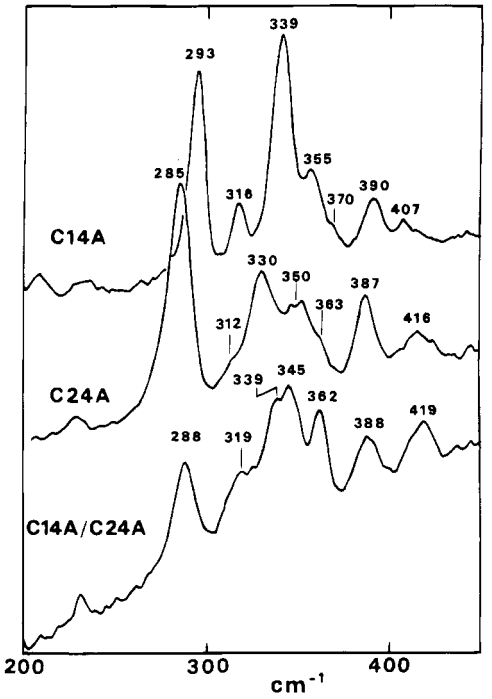

FIGURE 5: Resonance Raman spectra, excited at 457.9 nm, of cys ⟹ ala-mutated [2Fe-2S] *C. pasteurianum* ferredoxins. The conditions were as in Figure 4. Spectra are the summation of eight scans (C14A and C24A) or 16 scans (C14A/C24A).

wood & Gibson, 1985, 1992; Salifoglou et al., 1988), to be characteristic of oxygen-ligated [2Fe-2S] clusters (Fujinaga et al., 1993). Additional evidence is afforded here by the resonance Raman spectra of the C56S and C60S mutants: at least two bands assignable to stretching modes of the Fe−Scys bonds (Han et al., 1989; Table 5) were shifted to higher frequency upon cys ⟹ ser substitution, which is consistent with the replacement of a heavier (sulfur) ligand by a lighter one (oxygen) and with the shortening (or strengthening) of the metal−ligand bond upon replacement of sulfur by oxygen (Cleland & Averill, 1984). Among all of the Fd mutants discussed in this report, the C56S and C60S are the only ones of which the EPR spectra display a significantly greater g value anisotropy than that of the wild type Fd (Table 4). According to the model of Bertrand et al. (1985), variations in g values of $[2Fe\text{-}2S]^+$ clusters can be explained in terms of ligand field perturbations at the localized Fe(II) site. Therefore, as discussed for mutants of *E. coli* fumarate reductase (Werth et al., 1990), EPR spectra may be expected to undergo the greatest modifications upon mutagenesis of the ligands of the Fe(II) site. This would identify cysteines 56 and 60 as the ligands of the iron atom that is ferrous in reduced [2Fe-2S] CpFd.

The instability of the C11S mutant, as well as the hypsochromic shifts of some bands in its UV−vis spectrum (Figure 1), strongly suggest that cysteine 11 is a ligand of the [2Fe-2S] cluster. The observation that the EPR g values of the C11S Fd differ little from those of the wild type protein (Table 4) would suggest, according to the rationale used above for the assignments of cysteines 56 and 60 as ligands of the Fe(II) atom, that cysteine 11 is a ligand of the Fe(III) atom.

The plain assumption that the [2Fe-2S] cluster of CpFd is ligated by four cysteine residues would lead to the conclusion that the remaining cysteine, residue 24, is the second ligand of the Fe(III) atom. However, this inference is not supported by the observation that the [2Fe-2S] chromophore is assembled and stable in the C24A Fd. The case of cysteine 24 and the problem of the fourth ligand of the [2Fe-2S] cluster will be dealt with at the end of the Discussion.

Stability of Serine-Ligated [2Fe-2S] Clusters. The replacement of cysteine ligands by other residues could be expected to destabilize the [2Fe-2S] cluster. This was borne out by previous reports that the chromophore would not assemble in ferredoxins having cysteine ligands replaced by serine (Gerber et al., 1990; Uhlmann et al., 1992). Serine-ligated [2Fe-2S] clusters were nevertheless engineered into *E. coli* fumarate reductase (Werth et al., 1990, 1992) and possibly into a Rieske protein (Davidson et al., 1992). We thereafter characterized mutated forms of the *C. pasteurianum* Fd with, for the first time in an isolated iron−sulfur protein, serine-ligated [2Fe-2S] clusters (Fujinaga et al., 1993). Similar results have later been reported with *Anabaena* Fd (Cheng et al., 1994). We have now individually mutated all five cysteine residues of the [2Fe-2S] CpFd into serines and shown that [2Fe-2S] clusters are assembled in all of these mutants. These results indicate that binuclear iron−sulfur clusters can tolerate the replacement of at least one cysteine ligand by serine. At the same time it has been noted that the mutated Fds in which cysteines 11, 56, or 60 have been replaced by serine were less stable than the wild type protein (Fujinaga et al., 1993; this work). Similar effects of cysteine ⟹ serine mutations in Anabaena Fd have been reported (Cheng et al., 1994). This was not unexpected in view of the intrinsically greater lability of Fe−O bonds in these structures, as compared to the Fe−S ones (Cleland & Averill, 1984). As to the differences in stabilities between the various mutants, they are likely to result from specific

structural features in the vicinity of the [2Fe-2S] cluster: significant parameters in this respect may be solvent accessibility (which would favor solvolysis of the Fe−O bond), involvement of the S or O atom in hydrogen bonds, or the flexibility of the polypeptide chain segment surrounding the substituted residue. Indeed, the significantly shorter Fe−O bonds (1.8 Å , Cleland & Averill, 1984; Cheng et al., 1994), as compared to the Fe−S ones (2.3 Å , Tsukihara et al., 1990), would require at least a shift of the polypeptide chain in the neighborhood of the site of substitution. The observations that the C11S and the C79S mutated Fds are the least stable among the mutated forms of CpFd (this work) and *Anabaena* Fd (Cheng et al., 1994), respectively, will probably allow interesting comparisons to be made when a three-dimensional structure of *C. pasteurianum* Fd is available.

Resonance Raman Spectroscopy. Resonance Raman spectroscopy has afforded useful information on most mutant Fds described in this work. The spectra reported here also provide new insights into the currently available data and interpretations of the vibrational properties of [2Fe-2S] clusters (reviewed in Meyer et al. (1992) and Fu et al. (1992)). Resonance Raman band assignments for these chromophores were complicated by the low symmetry of the relevant point group and by the extensive coupling between the modes of the inorganic Fe_2S_2 core and those of the Fe−Scys bonds.

Considering the first point, we previously presented an analysis based on the numbers of observed bands, and on the numbers of those arising from totally symmetric modes, which led to the conclusion that the symmetry point group was probably as low as C_s (Meyer et al., 1986b). The numbers of bands, as well as their relative intensities, observed for the C56S and C60S Fds (Figure 4, Table 5) are very similar to those of the wild type protein. This indicates that the same, low symmetry point groups are experienced by all three chromophores. Since the only symmetry element that could possibly remain upon replacement of one sulfur ligand by an oxygen would be a plane containing the four terminal ligands, the point group involved here, including the wild type chromophore, must be at most C_s, thus confirming our previous analysis (Meyer et al., 1986b).

Strong coupling between the bridging (Fe_2S_2) and the terminal (Fe−Scys) modes has been inferred from the nearly uniform isotopic shifts observed for all the Raman bands upon $^{32}S \rightleftharpoons {}^{34}S$ substitution on the inorganic bridging S atoms (Meyer et al. 1986b; Han et al., 1989). This coupling is now further supported by the resonance Raman spectra of the C56S and C60S Fds, which show that the replacement of a single cysteinyl sulfur by oxygen results in shifts of all of the bands (Table 5). Furthermore, the observed shifts do not exceed 10 cm^{-1} (Table 5), whereas a shift of ca. 100 cm^{-1} is expected upon such an atomic replacement in a Fe−Y (Y = O, S) harmonic oscillator. These observations are consistent with the extensive coupling of the modes inferred from the $^{32}S \rightleftharpoons {}^{34}S$ isotopic shift measurements (Meyer et al., 1986b).

Despite these difficulties, assignments of normal modes have been made, using the idealized D_{2h} point group as a gross approximation, for three [2Fe-2S] Fds, including the one from *C. pasteurianum* (Han et al., 1989; Fu et al., 1992). Thus, the bands at 292, 338, and 356 cm^{-1} were attributed to Fe−Scys stretching modes, whereas those at 316, 370, 390, and 407 cm^{-1} were assigned to stretching modes of the inorganic core (Table 5). The data for the C56S and C60S mutants are consistent with some of these assignments, since shifts to higher frequency expected for a $S \rightleftharpoons O$ substitution were observed only for two bands (292 and 338 cm^{-1}) assigned to Fe−Scys stretching modes. Also, two of the bands attributed to stretching modes of the inorganic core (316 and 407 cm^{-1}) underwent, as expected, the smallest shifts. An apparent discrepancy is noted for the 356 cm^{-1} band which is shifted to lower frequency, whereas it has been attributed to a Fe−Scys mode (Fu et al., 1992). However, the replacement of a cysteine ligand by a serine does not necessarily bear on all of the Fe−Scys stretching modes in the same way. This may be true in particular for the non totally symmetric modes to which the 356 cm^{-1} band has been assigned (Table 5). The shifts to lower frequency observed for several bands attributed to bridging modes (Figure 4, Table 5) may result from some uncoupling between the bridging and terminal modes upon replacement of a cysteine ligand by serine. A similar effect had previously been observed upon substitution of the inorganic sulfur atoms by the heavier element selenium in spinach [2Fe-2S] Fd (Meyer et al., 1986b).

Possible Noncysteinyl Ligation of the [2Fe-2S] Cluster in CpFd. The observation that cysteine 24 is not necessary for the assembly of the [2Fe-2S] chromophore suggests that either it is not a ligand or it is replaced by some other residue upon mutation of cysteine 24 into serine or alanine. In the latter case, a possible substitute of cysteine 24 could be cysteine 14. Alternatively, cysteine 24 could be replaced in the C24A mutant by a non cysteinyl residue. Possible schemes consistent with the experimental data are outlined in Figure 6.

According to path A, cysteine 24 would be replaced by cysteine 14 in the C24A Fd and by X in the C14A/C24A double mutant, X being a noncysteinyl ligand. In path B, cysteine 24 would be replaced by X in both the C24A mutant and the C14A/C24A double mutant. Path C would propose a noncysteinyl ligand X as the second ligand of the ferric ion, even in the wild type Fd. Cysteine 24 should then be near to the chromophore, possibly but not necessarily as a ligand of an iron atom, in order to account for the spectral modifications occurring in the C24S and C24A Fds. At first sight, path A would appear to afford the best explanation for the observation that the wild type, C24A, and C14A/C24A Fds all differ from each other. However, the differences between the C24A and the C14A/C24A mutants, in particular concerning the EPR (Figure 3, Table 4) and resonance Raman (Figure 5, Table 5) data, are moderate enough to be rationalized within the frameworks of paths B or C. Whatever the relevance of the paths shown in Figure 6, a noncysteinyl ligand X has to be involved at some stage, possibly even in the wild type protein. Some indications pertaining to the identity of X may be drawn from the resonance Raman data. The spectra of all Fds mutated on cysteine 24 display downshifts of most of their resonance Raman bands. In particular, the 292 cm^{-1} band of the wild type protein, attributed to the totally symmetric (A_g) Fe−Scys stretching mode, shifts by −4 to −9 cm^{-1} (Table 5). Whereas these shifts may result from a variety of structural changes, one of the latter might be the replacement of cysteine 24 by a weaker (or heavier) ligand. A reasonable

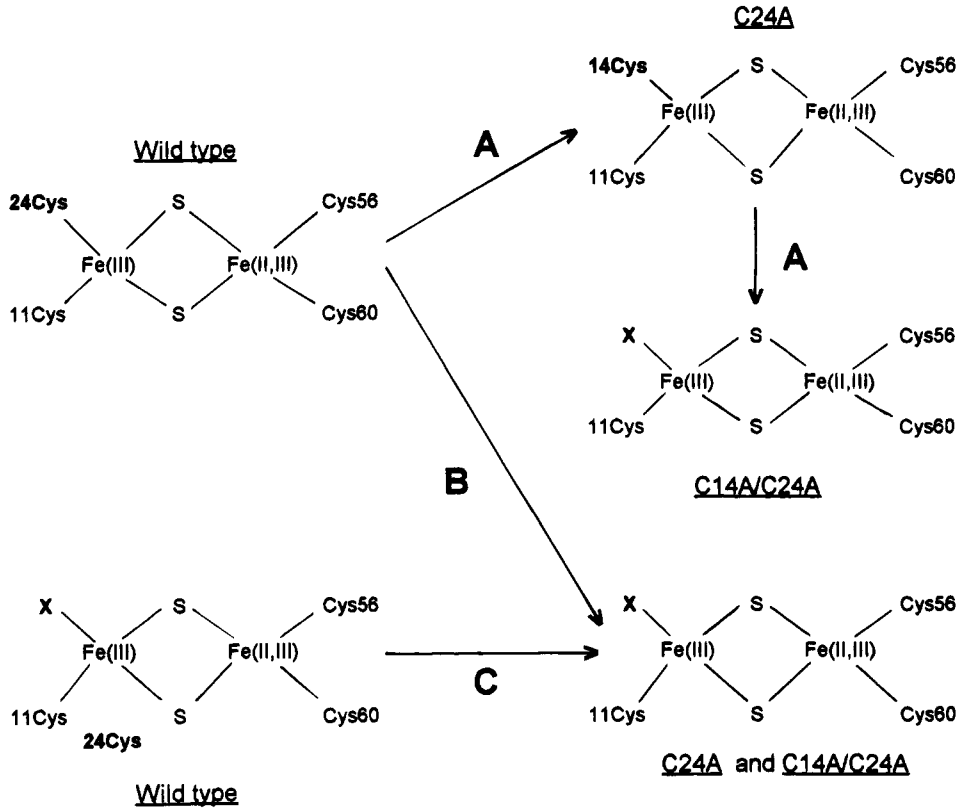

FIGURE 6: Putative ligations of the [2Fe-2S] chromophore to the polypeptide chain, as inferred from the site-directed mutagenesis experiments. The three proposed schemes describe ligand rearrangements that might take place upon the C24A mutation and upon the C14A/C24A double mutation (see text).

candidate as a substitute would then be histidine, of which three are present in the polypeptide chain in positions 6, 7, and 90 (Meyer et al., 1986a; Meyer, 1993). Indeed, in the resonance Raman spectra of Rieske-type proteins, in which two histidines have been reported to be the ligands of the same iron atom in the [2Fe-2S] cluster (Gurbiel et al., 1991), some bands, in particular those at ca. 270 cm^{-1}, which are assigned to modes of the Fe−Cys or Fe−His bonds, occur at frequencies lower by ca. 15 cm^{-1} than their counterparts of plant ferredoxins (Kuila et al., 1992). Whereas these data point to the possibility of histidine ligation in CpFd, ligands other than histidine should nevertheless remain within the scope of future investigations.

According to path C (Figure 6) the [2Fe-2S] chromophore would be bound to the polypeptide chain by cysteines 11, 56, and 60 and by an unidentified residue X, possibly a histidine (see above). Taking the latter hypothesis, an interesting comparison can be made with the 25 kDa subunit of the NADH−ubiquinone oxidoreductase from *Paracoccus denitrificans*, which has recently been expressed in *E. coli* (Yano et al., 1994). This polypeptide chain harbors a [2Fe-2S] cluster of which the UV−vis and EPR spectra bear some resemblance to those of the [2Fe-2S] CpFd (Yano et al., 1994). Furthermore, sequence alignments of the 25 kDa subunit with homologous proteins have shown that the following residues are conserved and therefore are potential ligands of the iron−sulfur cluster: H92, C96, C101, C137, and C141 (Yano et al., 1994). Although the sequences of the 25 kDa subunit and of the [2Fe-2S] CpFd are only marginally similar, cysteines 11, 56, and 60 (ligands of the iron−sulfur cluster) of the latter protein are spaced very similarly to cysteines 96, 137, and 141 of the former one. Superposition of the two sequences by using these three pairs of cysteine residues results in matching the conserved H92 of the 25kDa subunit with H7 of [2Fe-2S] CpFd. The hypothesis that histidine 7 might be a ligand of the [2Fe-2S] cluster in CpFd will be investigated by further mutagenesis and spectroscopic experiments. If it proves true, it would add up to the growing evidence (Beinert & Kennedy, 1993; Gurbiel et al., 1991; Holm, 1992; Kim & Rees, 1992; Thomann et al., 1991) for noncysteinyl ligation in an increasing number of iron−sulfur proteins.

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