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Coordination of the [2Fe-2S] Cluster in Wild Type and Molecular Variants of *Clostridium pasteurianum* Ferredoxin, Investigated by ESEEM Spectroscopy[†]

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ABSTRACT: The [2Fe-2S] ferredoxin from *Clostridium pasteurianum* contains five cysteine residues in positions 11, 14, 24, 56, and 60. This pattern is unique, and a combination of site-directed mutagenesis and spectroscopy is therefore being implemented to identify the ligands of the [2Fe-2S] cluster. The possible involvement of ligands other than cysteine in some molecular variants of this ferredoxin has been considered, histidines being likely candidates. Therefore, the three histidine residues in positions 6, 7, and 90 of the amino acid sequence have been individually and collectively replaced by alanine or valine. The mutated ferredoxins have been purified and were all found to contain [2Fe-2S] clusters of which the UV−visible absorption spectra were identical to that of the wild-type protein. The H6A/H7A/H90A triply mutated ferredoxin was further characterized by EPR and by ESEEM spectroscopy and was found to differ only marginally from the wild-type protein. The ESEEM spectra of wild-type ferredoxin displayed weak ^{14}N hyperfine interactions at the three principal *g*-factors of the [2Fe-2S] center. The estimated ^{14}N coupling constants ($A_{iso} = 0.6$ MHz; $e^2qQ \sim 3.3$ MHz) indicate that the ESEEM effect is most likely due to ^{14}N from the polypeptide backbone. ^2H_2O ESEEM spectra showed that the [2Fe-2S] cluster is accessible for exchange with solvent deuterons. ESEEM spectra of the previously characterized C24A and C14A/C24A variants have been recorded and were also found to be very similar to those of the wild-type protein. There was no evidence for coordination of the [2Fe-2S] cluster by [^{14}N]histidine or other ^{14}N nuclei, in either wild-type or mutant forms of the ferredoxin. By these criteria, the environment of the [2Fe-2S] center is not distinguishable from those in plant-type ferredoxins. Non-cysteinyl coordination most probably occurs only in the C14A/C24A variant, which contains no more than three cysteine residues. The data shown here indicate that the fourth ligand of the [2Fe-2S] cluster is neither a histidine residue nor another nitrogenous ligand. The possibility of oxygenic coordination for this molecular variant is discussed.

Proteins containing [2Fe-2S] clusters fulfill a variety of biological functions over a wide range of redox potentials (Cammack, 1992). This versatility rests upon several factors, which include the nature of the ligands binding the [2Fe-2S] cluster to the polypeptide chain. Indeed, the presence of two histidine ligands in the Rieske-type proteins (Gurbiel et al., 1989, 1991) is the major cause of a *ca*. 500−700 mV redox potential upshift, as compared to those [2Fe-2S] proteins that have complete cysteinyl coordination (Cammack, 1992). Thus, exploration of the ligation of [2Fe-2S] clusters is an important aspect of studies aimed at understanding the biological role of these structures (Moulis et al., 1996).

A significant example of such investigations is being developed on the [2Fe-2S] ferredoxin from the anaerobic eubacterium *Clostridium pasteurianum* (*Cp* 2Fe Fd).[1] The polypeptide chain of this protein consists of 102 amino acid residues, which comprise 5 cysteines at positions 11, 14, 24, 56, and 60 (Meyer et al., 1986; Meyer, 1993). As the three-dimensional structure of *Cp* 2Fe Fd is unknown, assignments of the [2Fe-2S] ligands on the basis of sequence similarities with other [2Fe-2S] proteins are not possible because of the uniqueness of this ferredoxin (Meyer, 1988). Instead, site-directed mutagenesis and various spectroscopic techniques have been used, and these have shown that cysteine 14 is not necessary for coordination of the [2Fe-2S] cluster and that cysteines 11, 56, and 60 are most probably ligands (Meyer et al., 1994).

The latter investigation also suggested that cysteine 24 was a ligand in the wild-type protein. However, as a [2Fe-2S] cluster was still assembled in the C24A variant, it was proposed that cysteine 24 could be replaced by cysteine 14, or possibly by a non-cysteinyl ligand. A recent study demonstrated that cysteine 14 is indeed promoted to the fourth ligand in the C24A variant (Golinelli et al., 1996), in a similar fashion as for the C20A variant of Fd I of *Azotobacter vinelandii* (Martín et al., 1990). Further studies demonstrated that one [2Fe-2S] cluster per subunit was still

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[1] Abbreviations: *Cp*, *Clostridium pasteurianum*; EPR, electron paramagnetic resonance; ENDOR, electron nuclear double resonance; ESEEM, electron spin-echo envelope modulation; Fd, ferredoxin; FT, Fourier transform; PCR, polymerase chain reaction; WT, wild-type.

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Table 1: Mutagenic Oligonucleotides and Reactions[a]

oligonucleotide	sequence	complementary to
O4	5′ TAATACGACTCACTATA 3′	noncoding strand
O9	5′ TTGGTAACTGTCAGACC 3′	coding strand
H6AV	5′ GTACAAACGAAGATGTGA(GA)CCTTTGGATTAACC 3′	coding strand
H7AV	5′ CAAACGAAGATG(GA)CATGCTTTGGATTAACC 3′	coding strand
H6A/H7A	5′ GTACAAACGAAGATGGCAGCCTTTGGATTAACC 3′	coding strand
H90AV	5′ GAAGAGATTGTAGAGTCTG(CT)TATCGAAAACGG 3′	noncoding strand

[a] Oligonucleotides O4 and O9 hybridize to regions upstream and downstream, respectively, of the *Cp* 2Fe Fd gene (Fuginaga et al., 1993; Meyer et al., 1994). Mutations are noted with the one-letter code for amino acids: the first letter indicates the original residue, the following number its position in the sequence, and the second letter the substituting residue. Mutated bases (differing from the wild-type sequence) are underlined. The bases in parentheses correspond to degenerate positions (H to A or V mutations). For the mutations of residues 6 and 7, the first round of PCR was run with O4 and the mutagenic oligonucleotide as primers. For the second round of PCR, the primers were O9 and the product of the first round. For the mutations of residue 90, the first round of PCR was run with O9 and the mutagenic oligonucleotide as primers. For the second round of PCR, the primers were O4 and the product of the first round. Experimental conditions as in Meyer et al. (1994).

assembled in a doubly-mutated ferredoxin having both cysteines 14 and 24 replaced by alanine (Meyer et al., 1994; Pétillot et al., 1995). Since four protein ligands are required for all known [2Fe-2S] clusters, the C14A/C24A variant must have a non-cysteine ligand (X). Histidine is a possible substitute for cysteinyl ligands, as in the Rieske [2Fe-2S] proteins (Gurbiel et al., 1989, 1991).

Electron spin-echo envelope modulation (ESEEM) spectroscopy allows for the measurement of nuclear hyperfine couplings, *e.g.*, from the [^{14}N]histidyl ligands of Rieske-type [2Fe-2S] clusters, which are not resolved in the EPR spectrum (Britt et al., 1991; Shergill & Cammack, 1994a; Shergill et al., 1995; Riedel et al., 1995). Therefore, in the present investigation, we have probed the ligand environment of the [2Fe-2S] clusters in wild-type and mutated forms (cysteine and histidine residues mutated to alanine) of *Cp* 2Fe Fd using the ESEEM technique.

MATERIALS AND METHODS

All common DNA manipulations were as described (Ausubel et al., 1988; Fujinaga & Meyer, 1993; Meyer et al., 1994). Enzymes were purchased from Boehringer Mannheim. 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 in which mutations were introduced was the pTCP2F plasmid (Fujinaga & Meyer, 1993), where a sequence encoding the *Cp* 2Fe Fd 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 mutagenic primers are listed in Table 1. The mutated plasmids were prepared as described (Meyer et al., 1994) and used to transform *E. coli* K38 (HfrC λ) cells harboring the pGP1-2 plasmid (Tabor, 1990). The production and purification of the mutated proteins were carried out as reported previously (Meyer et al., 1994).

For ESEEM studies of ^2H_2O-exchanged protein, 100−150 μL samples of protein were diluted in 50 mM HEPES, pD 7.4 (as measured by a pH electrode; the pH meter reading was not corrected for isotope effects), and reconcentrated using Centricon-10 microconcentrators (Amicon) at 4 °C. The dilution and ultrafiltration cycle was repeated 3 times to give a final ^2H_2O enrichment of 95−99%. The total time allowed for exchange with ^2H_2O was at least 24 h. The protein concentration per [2Fe-2S] cluster of each sample used for the EPR and ESEEM studies was 0.8 mM for WT and H6A/H7A/H90A, and 1 mM for C24A and C14A/C24A ferredoxins.

EPR spectra were recorded at X-band on a Bruker ESP 300 EPR spectrometer with a TE102 rectangular cavity. The temperature was controlled using an Oxford Instruments ESR 900 liquid helium flow cryostat fitted with a ITC 4 temperature controller. Pulsed EPR measurements were recorded at X-band on a Bruker ESP 380 spectrometer, with a dielectric variable-Q resonator ($Q \sim 100$, corresponding to a minimum dead time of ~100 ns) and an Oxford Instruments CF 935 liquid helium cryostat. ESEEM data were acquired with a three-pulse ($90°-\tau-90°-T-90°$) stimulated-echo sequence using a phase cycling routine, as described elsewhere (Shergill et al., 1991). For the optimal detection of ^{14}N nuclei, the τ-value (the time between the first and second 90° microwave pulse) was matched as closely as possible to a multiple of the proton Larmor frequency, in order to suppress modulations due to weakly-coupled protons (Mims & Peisach, 1981). The ESEEM data were generally recorded at three magnetic field settings, corresponding to the principal *g*-factors of the reduced [2Fe-2S] cluster, and Fourier-transformed using the Bruker pulsed spectra manipulation routines (ESP 380 and WinEPR software). Other recording conditions are given in the figure legends.

RESULTS

EPR of Wild-Type and Molecular Variants of Cp 2Fe Fd. Figure 1 shows EPR spectra of WT and mutant *Cp* 2Fe Fd, recorded at 12 K. The [2Fe-2S] cluster of WT Fd displayed a rhombic spectrum with features at $g_{zyx} = 2.004$, 1.948, and 1.922 ($g_{av} = 1.96$) (Figure 1a). As reported previously, slight shifts in the *g*-factor were observed with C24A and C14A/C24A ferredoxins, to $g_{zyx} = 2.004$, 1.954, and 1.923 and $g_{zyx} = 2.001$, 1.956, and 1.917, respectively (Figure 1b,c) (Meyer et al., 1994). Small changes in the EPR signal line widths were observed upon exchange of the ferredoxins into a ^2H_2O environment (Figure 1, dotted lines), indicative of an exchangeable hydrogen atom(s) in the immediate vicinity of the [2Fe-2S] cluster. The interaction of protons with the [2Fe-2S] cluster in these ferredoxins is currently being investigated in a separate study using ^{1}H and ^{2}H electron nuclear double resonance (ENDOR) spectroscopy.

^{14}N ESEEM of Wild-Type and Molecular Variants of Cp 2Fe Fd. Nitrogens are abundant in proteins, and those

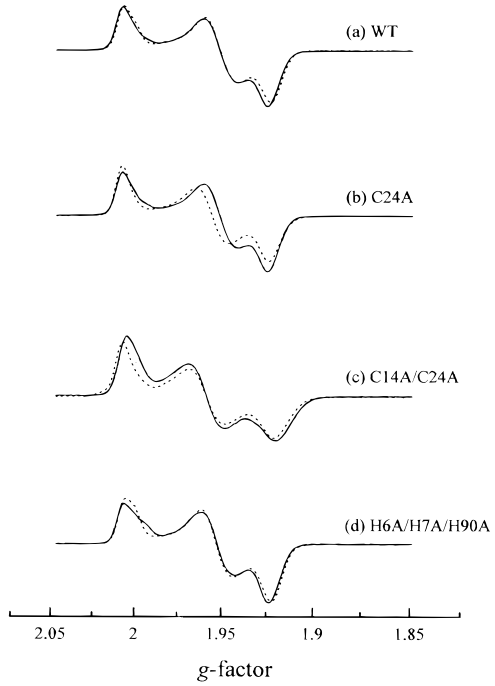

FIGURE 1: X-band EPR spectra of [2Fe-2S] *Cp* 2Fe Fd, in H_2O buffer (solid line) and ^2H_2O buffer (dotted line). (a) WT, (b) C24A, (c) C14A/C24A, and (d) H6A/H7A/H90A ferredoxin. Each protein sample was reduced under an argon atmosphere by incubation with 4 mM dithionite for 2 min. [2Fe-2S] concentration: (a, d) 0.8 and (b, c) 1.0 mM. The feature observed at $g = 2.00$ in Figure 1d is assigned to a free radical resulting from excess dithionite addition. Instrument settings: microwave power, 20 μW; microwave frequency, 9.35 GHz; modulation frequency, 100 kHz; modulation amplitude, 0.49 mT; time constant, 81.92 ms; sweep rate, 0.596 mT/s; temperature, 12 K. Number of scans averaged, 2.

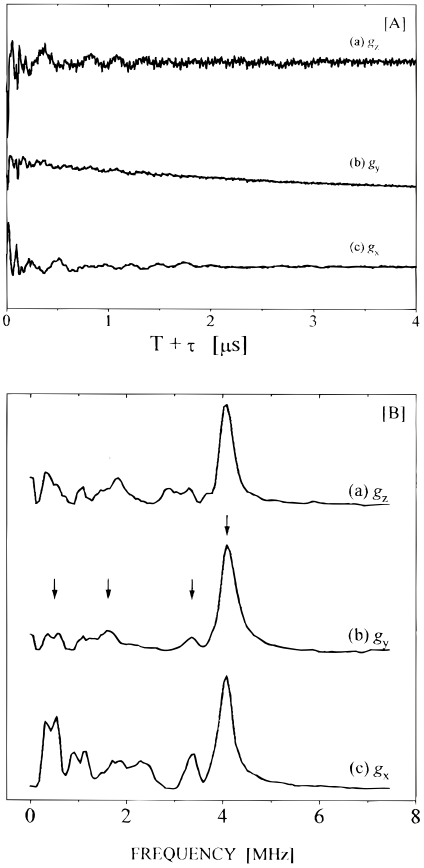

FIGURE 2: Three-pulse ESEEM spectra of WT *Cp* Fe Fd recorded at (a) g_z, (b) g_y, and (c) g_x. (A) Time domain and (B) Fourier transform data. [2Fe-2S] concentration was the same as in Figure 1. Instrument settings were: microwave frequency, 9.72 GHz; magnetic field (B_0), (a) 347.8, (b) 357.8, (c) 362.7 mT; microwave pulse power attenuation, 2.6 dB; pulse resolution in *x*-axis, 1024 points; pulse width (for a 90° pulse), 16 ns; bandwidth, 100 MHz; sample temperature, 3.9 K; shot repetition time, 20.48 ms; τ-value, (a, b) 136, (c) 128 ns. The arrows indicate four of the most prominent ^{14}N modulation frequencies detected at g_y.

interacting with the paramagnetic [2Fe-2S] cluster of *Cp* 2Fe Fd would be expected to show various degrees of hyperfine coupling (*A*). For example, ESEEM spectra showing relatively strong ^{14}N hyperfine couplings ($A = 3.4-5.5$ MHz) have been observed for the histidine ligands in Rieske-type [2Fe-2S] proteins (Britt et al., 1991; Shergill & Cammack, 1994a; Shergill et al., 1995; Riedel et al., 1995). Much weaker couplings ($A \sim 1.0$ MHz) have been detected with the peptide nitrogens in the plant-type [2Fe-2S] ferredoxins (Cammack et al., 1988; Britt et al., 1991; Shergill et al., 1991; Shergill & Cammack, 1994b).

Figure 2 shows the three-pulse ESEEM spectra obtained at the three principal *g* factors of WT *Cp* 2Fe Fd. The echo decay envelopes displayed rather shallow modulations (Figure 2A), which upon Fourier transformation revealed features in the 0−5 MHz region (Figure 2B). For example, the spectrum acquired at g_y exhibited major frequencies at *ca*. 0.46, 1.69, 3.39, and 4.07 MHz (Figure 2B, see arrows). Slight shifts in the position and relative amplitude of these features were observed on recording the ESEEM at either g_z or g_x, indicating some anisotropy of the hyperfine coupling. The shifts were small as the modulation frequencies detected are primarily a function of ^{14}N nuclear quadrupole interactions.

The interactions giving rise to the ESEEM effect include the hyperfine interaction (*A*), which provides information on the strength of the electron−nuclear coupling, and the quadrupolar interaction (e^2qQ), which is characteristic of the type of ^{14}N nucleus giving rise to the ESEEM. We have estimated the *A* and e^2qQ values for the ^{14}N nucleus interacting with the [2Fe-2S] cluster of WT *Cp* 2Fe Fd using the theoretical and graphical analyses of Dikanov and Tsvetkov (1992), by assuming that the two highest frequencies in the ESEEM spectra are ^{14}N double-quantum transitions ($\Delta M_I = 2$). These values, estimated using the data acquired at the three principal *g*-factors, are summarized in Table 2. The four-line ESEEM pattern of WT *Cp* 2Fe Fd is described by a nitrogen with $A_{iso} \sim 0.61$ MHz and $e^2qQ \sim 3.3$ MHz, indicating the absence of a strong superhyperfine interaction between the [2Fe-2S] cluster of WT Fd and ^{14}N. No significant change in e^2qQ was observed upon alteration of the *g*-factor (Table 2). The quadrupolar coupling falls within the range of values reported for ^{14}N-peptide in di- and tripeptide compounds ($e^2qQ = 3.0-3.4$ MHz for $\eta = 0.4-0.5$) (Edmonds & Speight, 1971; Hunt & Mackay, 1976).

In Figure 3, the ^{14}N ESEEM spectrum of WT *Cp* 2Fe Fd is compared with those from some of the molecular variants. In the spectra shown, the ESEEM was stimulated at the g_x feature of the EPR spectrum. The g_x spectra represent unique orientations of the [2Fe-2S] cluster with respect to the magnetic field, giving a 'single-crystal-like' pattern (Rist & Hyde, 1970). The ESEEM spectra of the C24A and C14A/C24A Fds displayed a shift of ≤0.37 MHz of the ∼3 MHz feature to lower frequency. A small shift of *ca*. 0.15 MHz

Table 2: Superhyperfine Coupling Constants Estimated for the ^{14}N-Nucleus Coupled to the [2Fe-2S] Cluster of *C. pasteurianum* Ferredoxin

		coupling constants (MHz)							
		A				e^2qQ^b			
protein	ligand	g_z	g_y	g_x	$A_{iso}{}^a$	g_z	g_y	g_x	e^2qQ
C. pasteurianum 2Fe Fd[c]									
WT		0.66	0.58	0.59	0.61	3.28	3.30	3.29	
C24A		0.86	1.00	0.61	0.82	3.04	3.08	3.09	
C14A/24A		0.82	0.90	0.61	0.78	3.04	3.07	3.13	
H6A/H7A/H90A		0.69	0.79	0.63	0.70	3.21	3.23	3.20	
Plant-type [2Fe-2S] clusters									
S. platensis ferredoxin[d]		0.85	0.96	1.22	1.01	3.12	3.58	3.37	
E. coli fumarate reductase[e]					1.10				3.30
Rieske-type [2Fe-2S] clusters									
Spinach cytochrome b_6f complex[f]	N(1)		3.75				2.5−2.9		
	N(2)		4.58				2.5−2.9		
Pseudomonas cepacia phthalate dioxygenase[g]	N(1)	3.28	3.85	5.77	4.28				2.60
	N(2)	4.56	4.99	6.99	5.49				2.30
P. putida benzene dioxygenase[h]	N(1)	3.36	3.50	3.82	3.56	2.17	2.46	2.78	
	N(2)	4.44	4.99	4.92	4.78	2.17	2.33	2.38	

[a] $A_{iso} = 1/3(A_{xx} + A_{yy} + A_{zz})$. [b] Quadrupolar couplings were determined assuming $\eta = 0.5$. [c] This work. [d] Shergill (1993). [e] Cammack et al. (1988). [f] Britt et al. (1991). [g] Gurbiel et al. (1989). [h] Shergill et al. (1995).

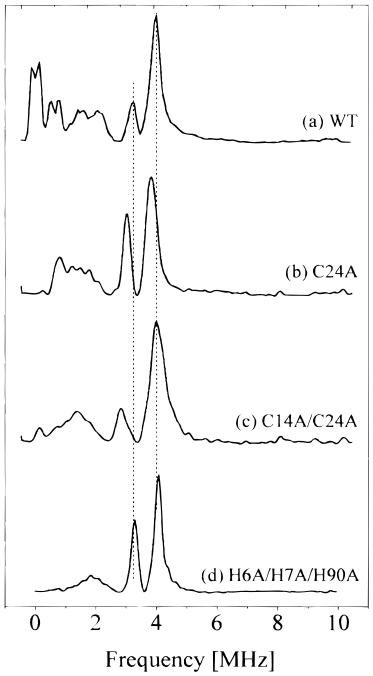

FIGURE 3: Comparison of the FT-ESEEM spectrum of (a) WT *Cp* 2Fe Fd with molecular variants (b) C24A, (c) C14A/C24A, and (d) H6A/H7A/H90A. [2Fe-2S] concentration was the same as in Figure 1. The three-pulse data were acquired at g_x. Temperature and instrument settings were as for Figure 2 except: B_0, (a) 361.5, (b) 361.0, (c) 362.0, and (d) 361.1 mT; microwave pulse power attenuation, 2.1 dB; pulse resolution in *x*-axis, 512 points; shot repetition time, 10.24 ms; τ-value, 128 ns.

of the double-quantum feature at *ca.* 4 MHz was also apparent with C24A Fd. Stimulating ESEEM at the g_z or g_y features of the EPR spectrum resulted in changes analogous to those illustrated for the WT protein (see Figure 2). The modulation frequencies detected (<4.2 MHz) with the C24A and C14A/C24A Fds are inconsistent with direct nitrogen coordination to the [2Fe-2S] cluster.

^{2}H ESEEM of Wild-Type and Molecular Variants of Cp 2Fe Fd. Features at the proton Larmor frequency, *i.e.*, ~14.8, 15.3, and 15.5 MHz, were also observed at g_z, g_y, and g_x, respectively (not shown), in the ESEEM spectra of WT *Cp* 2Fe Fd due to the incomplete suppression of the

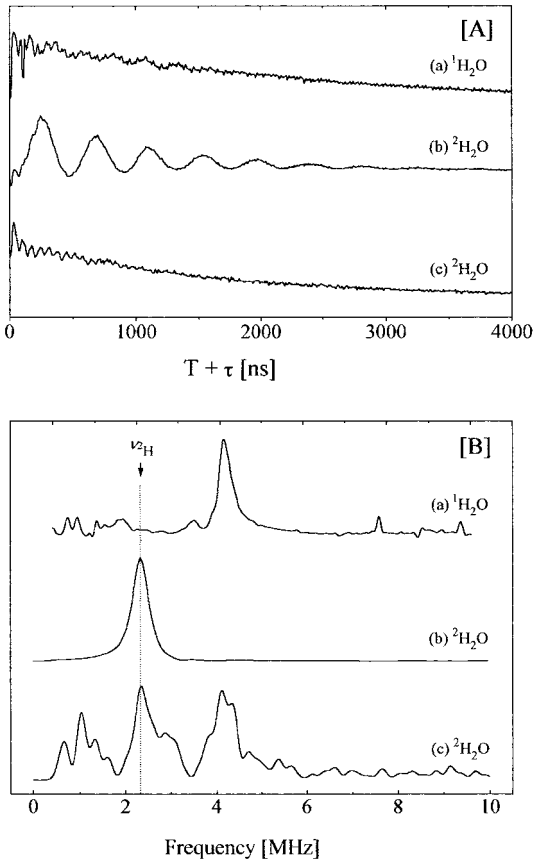

FIGURE 4: ESEEM spectra for WT *Cp* 2Fe Fd in (a) ^1H_2O and (b, c) ^2H_2O, acquired at $g_y = 1.948$. [2Fe-2S] concentration was the same as in Figure 1. Temperature and instrument settings were identical to those given in Figure 2, except: τ-value (a, b) 136 and (c) 440 ns.

frequency corresponding to the distant protons in the protein or water environment. Figure 4 compares the ESEEM spectra of WT Fd in ^1H_2O (spectrum a) and ^2H_2O (spectrum b), stimulated at the g_y position and recorded using a τ-value of 136 ns. Exchange of solvent to ^2H_2O resulted in the appearance of an additional deep modulation in the time domain envelope (Figure 4A), and the FT spectrum displayed a single broad feature centered at 2.335 MHz, corresponding to the ^{2}H Larmor frequency ($\nu_{^2H}$) (Figure 4B). The large

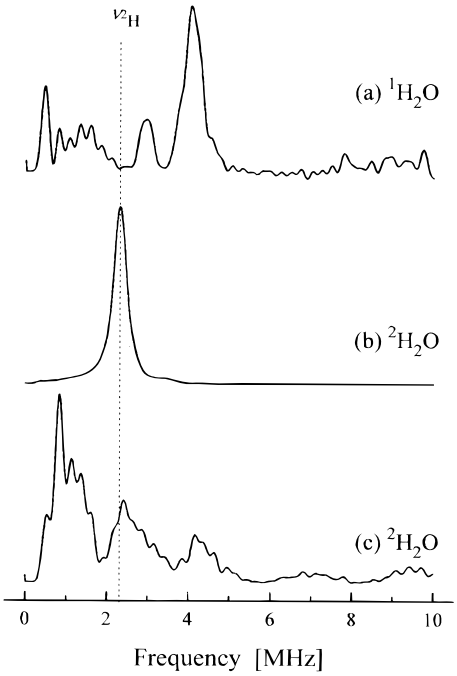

FIGURE 5: ESEEM spectra for C14A/C24A *Cp* 2Fe Fd in (a) ^1H_2O and (b, c) ^2H_2O, acquired at $g_y = 1.956$. [2Fe-2S] concentration was the same as in Figure 1. Temperature and instrument settings were identical to those given in Figure 3, except: τ-value (a, b) 136 and (c) 440 ns.

amplitude and line width of this feature indicates that the [2Fe-2S] cluster site of WT *Cp* 2Fe Fd is accessible for exchange with solvent deuterium, with a deuterium population in close proximity to the metallocenter. The ^{14}N frequencies observed in the 0−4.5 MHz region with WT Fd in ^1H_2O were clearly observed from the ^2H_2O sample at higher values of τ, when the $\nu_{^2H}$ transition no longer dominated the spectrum (Figure 4, spectra a and c). The ^2H ESEEM spectrum of the variant C14A/C24A also showed deep deuterium modulation (Figure 5), similar to that observed with WT.

Molecular Variants of Cp 2Fe Fd with Mutated Histidines. Several amino acids are considered as "safe" substitutions for histidine in site-directed mutagenesis (Bordo & Argos, 1991). As the purpose of the mutations in the present case is to verify the absence of [^{14}N]histidine ligands to the [2Fe-2S] cluster of *Cp* 2Fe Fd, as indicated by the ESEEM data, the list must be restricted to residues having chemically inert sidechains. With this limitation, the best substitutes are alanine or valine, in hydrophilic or hydrophobic environments, respectively (Bordo & Argos, 1991). In the absence of a crystal structure of *Cp* 2Fe Fd, the environment of the three histidine residues in positions 6, 7, and 90 can at best be predicted from the primary structure. Therefore, in a first round of mutagenesis, these three histidines were individually replaced by either alanine or valine.

Based on the purification yields of the mutated proteins, alanine was a better substitute than valine in positions 6 and 7, by a factor of 2. In contrast, in position 90, no clearcut difference was observed between the two substitutions. The H6A/H7A double mutant was then prepared, followed by the H6A/H7A/H90A and H6A/H7A/H90V triple mutants. All these single, double, and triple mutants had UV−visible absorption spectra identical with that of the WT ferredoxin (data not shown), indicating that the [2Fe-2S] chromophore was unaltered by the replacement of the histidine residues. Therefore, only the H6A/H7A/H90A triple mutant was further investigated by EPR and ESEEM spectroscopy. The EPR spectra of this variant were essentially identical with that of the WT ferredoxin (Figure 1), and the ESEEM spectra differed from WT only in the relative intensity of the lower frequency components (<3 MHz) (Figure 3).

Although the replacement of histidine by either alanine or valine did not alter the properties of the [2Fe-2S] cluster, the stability of the *Cp* 2Fe Fd was lowered. This was inferred from the observation that each added substitution decreased the purification yield of *Cp* 2Fe Fd by approximately a factor of 2. Thus, the triple mutants were obtained in yields 6−10 times lower than WT ferredoxin. Therefore, variants having mutations in addition to the triple histidine substitution could not be isolated. The latter included the H6A/H7A/C14A/C24A/H90A quintuple mutant, which was meant to afford direct evidence that histidine is not the fourth ligand of the C14A/C24A double mutant. Spectroscopic evidence supporting the latter point will be discussed below.

DISCUSSION

The ^{14}N ESEEM spectra of the [2Fe-2S] cluster in WT and molecular variants of *Cp* 2Fe Fd are similar to those observed for plant-type ferredoxins (Cammack et al., 1988; Britt et al., 1991; Shergill et al., 1991; Shergill & Cammack, 1994b). This confirms the similarities between the active sites of these proteins as suggested by their g_{av} values ($g_{av} \sim 1.96$ for *Cp* 2Fe Fd and plant-type Fd, as compared with $g_{av} \sim 1.91$ for Rieske-type [2Fe-2S] proteins) and their redox potentials (−280 mV, *Cp* 2Fe Fd; between −500 and −240 mV for plant-type Fd, compared with −140 to +350 mV for Rieske-type centers) (Golinelli et al., 1996; Mason & Cammack, 1992).

The ^{14}N superhyperfine couplings ($A_{iso} = 0.61$ MHz, $e^2qQ \sim 3.3$ MHz) estimated for WT *Cp* 2Fe Fd are relatively independent of magnetic field, and are consistent with ^{14}N nuclei weakly-coupled to the [2Fe-2S] cluster (Table 2). We observed no evidence for the directly-coupled nitrogens that have been attributed to the histidine ligands of the Rieske-type [2Fe-2S] clusters with features in the 5−8 MHz region of ESEEM spectra (Britt et al., 1991; Shergill & Cammack, 1994a; Shergill et al., 1995; Riedel et al., 1995). Furthermore, we note that the magnitude of the ^{14}N hyperfine coupling, $A_{iso} = 0.61$ MHz, detected with *Cp* 2Fe Fd is less than one-fifth that of the $A_{iso} = 3.5-5.5$ MHz couplings determined for the ^{14}N ligands of Rieske-type centers (Table 2). Moreover, the crystal structures of plant-type [2Fe-2S] ferredoxins indicate several nitrogens from the polypeptide backbone are close enough to form NH···S hydrogen bonds to the cluster (Rypniewski et al., 1991; Fukuyama et al., 1995). By these criteria, we conclusively assign the ESEEM spectra of WT *Cp* 2Fe Fd to a peptide (or possibly amino acid sidechain) nitrogen outside the first coordination sphere.

As the ^{14}N ESEEM spectra and the coupling constants of the C24A, C14A/C24A, and H6A/H7A/H90A variants were similar to WT, their spectra can also be assigned to weakly-coupled peptide ^{14}N (see Table 2). These results conclusively demonstrate that a histidine residue cannot be the fourth ligand of the [2Fe-2S] cluster of C14A/C24A ferredoxin, and rule out direct nitrogen coordination by any other amino acid (residue X).

We note that the small increases observed in A_{iso} ($\leq +0.21$ MHz) with the molecular variants, with respect to WT, are consistent with a rearrangement of electron spin density with greater electron density now residing at the ^{14}N nucleus. These variations can tentatively be rationalized, at least in the case of the C24A and C14A/C24A variants, by changes in the distance(s) from the weakly-coupled peptide ^{14}N nucleus (nuclei) to the [2Fe-2S] cluster. Such changes are likely if the coupled nucleus belongs to the flexible and deletable region encompassing residues 17−30 (Golinelli et al., 1996). The absence of nitrogen ligands to the [2Fe-2S] cluster in WT *Cp* 2Fe Fd, as well as in all the molecular variants investigated here, is in agreement with inferences drawn from data obtained with deletion mutants, which have shown that in all of these proteins, with the notable exception of the C14A/C24A Fd, the cluster is coordinated by cysteines 11, 56, and 60, and by a fourth cysteine that may be located in several positions in the region encompassing residues 14−24 (Golinelli et al., 1996).

Non-Cysteinyl Ligation of the [2Fe-2S] Cluster in C14A/C24A Ferredoxin. To sum up, cysteinyl, histidyl, and ^{14}N-X coordination of the [2Fe-2S] cluster of C14A/C24A ferredoxin has been dismissed. Possible alternative ligands of the C14A/C24A Fd [2Fe-2S] cluster are methionine, carboxylic acids (aspartic or glutamic), and serine. Methionine ligation (there are three such residues in positions 1, 37 and 50) is the least likely, as there is no demonstrated case of methionine ligation in iron−sulfur proteins. It nevertheless has been hypothesized in Fe-hydrogenases (Meyer & Gagnon, 1991). Evidence of aspartate ligation has been obtained with a few mutated iron−sulfur proteins (Moulis et al., 1996) and in the [4Fe-4S] ferredoxin from *Pyrococcus furiosus* (Calzolai et al., 1995). Oxygenic coordination by serine has been demonstrated in site-directed mutants of [2Fe-2S] proteins, namely, *E. coli* fumarate reductase (Werth et al., 1990; Ackrell et al., 1992), *Cp* 2Fe Fd (Fujinaga et al., 1993; Meyer et al., 1994), and *Anabaena* Fd (Cheng et al., 1994). *Cp* 2Fe Fd contains a number of serine, aspartate, and glutamate residues, none of which can be excluded as possible candidates for the fourth ligand in C14A/C24A, since the structure of the protein is unknown.

Alternatively, exogenous oxygenic ligands from solvent, H_2O/OH^-, may be considered as candidate ligands of the C14A/C24A variant. Indeed, the [4Fe-4S] cluster of aconitase is coordinated by OH^- ($A_H \sim 3.3$ MHz) in the absence of substrate, and by H_2O in its presence ($A_H \sim 3.0$, 7.9 MHz) (Werst et al., 1990). ^2H-exchanged samples of both WT and C14A/C24A *Cp* 2Fe Fd displayed intense ESEEM with frequency ν^2_H due to exchangeable protons, similar to those observed in the plant-type [2Fe-2S] ferredoxins (Peisach et al., 1977; Orme-Johnson et al., 1983), lending support to a hydrogen-bonding explanation for the weakly-coupled ^{14}N. In addition, an appreciable sharpening of the EPR g_z and g_x features was noted with the ^2H-exchanged sample of C14A/C24A Fd (Figure 1c), with respect to WT and the other molecular variants investigated in this study. The [2Fe-2S] cluster in these proteins must, therefore, be accessible to solvent. This is in agreement with independent evidence showing that the region encompassing the cysteine 24 ligand is a flexible, solvent-exposed loop (Golinelli et al., 1996).

A large isotropic coupling ($A_H \sim 3.9$ MHz) with an exchangeable proton has been reported using Mims ENDOR for the [2Fe-2S] cluster of *Anabaena* 7120 Fd (Fan et al., 1992). Although the magnitude of this ^1H coupling indicates significant covalency for the hydrogen bond, there are no exogenous ligands to the plant-type [2Fe-2S] cluster (Rypniewski et al., 1991). Whether the ^2H feature detected for *Cp* 2Fe Fd is in part due to local ENDOR signals with $A(^2H) \neq 0$ cannot be determined from the ESEEM data presented in this study. Our current ENDOR studies are addressing the issue of H_2O/OH^- ligation in the double mutant.

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