Cysteine Ligand Swapping on a Deletable Loop of the [2Fe-2S] Ferredoxin from *Clostridium pasteurianum*†

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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, and 60). In previous investigations, a combination of site-directed mutagenesis and of spectroscopic techniques showed that cysteines 11, 56, and 60 are ligands of the [2Fe-2S] cluster in the wild type protein and that cysteine 14 is not, but the status of cysteine 24 remained unclear. New mutated forms of this ferredoxin have been obtained and characterized. The data show that cysteine 24 is a ligand of the cluster in the wild type protein. When cysteine 24 is mutated into alanine, it is replaced as a cluster ligand by cysteine 14. The fourth ligand of the cluster can also be a cysteine residue newly introduced in position 16 when both cysteines 14 and 24 are replaced by alanine. These results suggest that the region encompassing cysteines 14 and 24 is a solvent-exposed flexible loop, in agreement with structure predictions. A number of nondeleterious deletions of variable length (3−14 residues) have been performed in the region of residues 17−32. The deletions were found to modify only marginally the spectroscopic properties of the [2Fe-2S] cluster but resulted in variations of its redox potential over a range of nearly 100 mV. This is the first instance of ligand swapping in a [2Fe-2S] protein, and the first time in any ferredoxin that a large loop has been excised from the structure without preventing the assembly of the iron−sulfur chromophore. Some of the molecular variants described here also highlight the similarities between the *C. pasteurianum* [2Fe-2S] ferredoxin and the 25 kDa subunit of the proton-translocating NADH:ubiquinone oxidoreductase of *Paracoccus denitrificans*.

Iron−sulfur clusters of the [2Fe-2S] type occur in a wide range of proteins involved in electron transfer, catalysis of redox reactions (Cammack, 1992; Matsubara & Saeki, 1992), assembly of metallic active sites (Fu et al., 1994), or regulation of gene expression (Hidalgo & Demple, 1994). They are present either as sole prosthetic groups in small redox proteins (Matsubara & Saeki, 1992) or as components of proteins containing multiple redox sites (Cammack, 1992; Johnson, 1994). [2Fe-2S] proteins are remarkable because of the diverse distributions of the cluster ligands in their sequences. From this viewpoint, the [2Fe-2S] ferredoxin from the nitrogen-fixing saccharolytic anaerobe *Clostridium pasteurianum* (*Cp*[1] 2Fe Fd) has long been unique and has therefore attracted much interest (Meyer et al., 1986a,b; Meyer, 1988, 1993; Fu et al., 1992). The gene encoding this protein has been expressed in *Escherichia coli* (Fujinaga & Meyer, 1993), and all of its five cysteine residues in positions 11, 14, 24, 56, and 60 of the 102-amino acid sequence have been mutated into serine or alanine (Fujinaga et al., 1993; Meyer et al., 1994). These data have provided the unequivocal assignment of cysteines 11, 56, and 60 as ligands of the cluster, the characterization of serine-ligated [2Fe-2S] clusters (Fujinaga et al., 1993; Meyer et al., 1994), and the uncovering in the latter of unprecedented magnetic properties (Crouse et al., 1995).

The preparation and characterization of a number of new molecular variants of the *Cp* 2Fe Fd are reported here. For each mutant, the excited state electronic structure and the ground state vibrational properties of the $S = 0$ $[2Fe\text{-}2S]^{2+}$ clusters in the oxidized proteins have been investigated by UV−visible absorption and resonance Raman (RR) spectroscopies, respectively. The excited and ground state electronic properties of the $S = {}^1/_2$ $[2Fe\text{-}2S]^{+}$ cluster in the reduced proteins have been investigated by variable-temperature magnetic circular dichroism (VTMCD) and EPR spectroscopies, respectively. These data have allowed the identification of all ligands of the cluster in the WT protein and in various mutants thereof. A new case of ligand swapping has been disclosed, an occurrence that was unprecedented in [2Fe-2S] ferredoxins. Furthermore, in the region of the two exchangeable ligands, cysteines 14 and 24, deletions of up to 14 amino acids can be performed without considerably destabilizing the protein. The effects of these mutations on the structural, electronic, and redox properties of *Cp* 2Fe Fd are discussed. The numerous molecular variants described here also enlighten relationships in sequence and active site structure between *Cp* 2Fe Fd and other [2Fe-2S] proteins.

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[1] Abbreviations: Fd, ferredoxin; *Cp, Clostridium pasteurianum;* CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; *Pd, Paracoccus denitrificans;* PCR, polymerase chain reaction; RR, resonance Raman; VTMCD, variable-temperature magnetic circular dichroism; WT, wild type.

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Table 1: Generation of Mutated Genes[a]

mutation[b]	shorthand notation[c]	starting plasmid	mutagenic oligonucleotide[d]
C14A/L16C/C24A	C16	C14A/C24A	5′TTGCTGCTTTCCATTACATCTAGCACTAG3′
C14A/L16C/C24A/Δ19−21	C16/Δ19−21	C16	5′GGAGTAAGCAAAACC/TCCATTACATCTAGC3′
C14A/L16C/C24A/Δ19−23	C16/Δ19−23	C16	5′GAATTTTTGGAGTAAGC/TCCATTACATCTAGC3′
C14A/L16C/Δ19−28	C16/Δ19−28	C16	5′CTACAATTTCAACGGA/TCCATTACATCTAGC3′
C14A/L16C/Δ19−30	C16/Δ19−30	C16	5′GAATGTTTCTACAATTTC/TCCATTACATCTAGC3′
C14A/L16C/Δ19−32	C16/Δ19−32	C16	5′CCATGAATGTTTCTAC/TCCATTACATCTAGC3′
C14A/L16C/Δ19−34	C16/Δ19−34	C16	5′CTTCCATGAATGT/TCCATTACATCTAGC3′
Δ19−30	Δ19−30	wild type	5′GTTTCTACAATTTC/TCCATTAAGTCTACA3′
Δ19−32	Δ19−32	wild type	5′CATGAATGTTTCTAC/TCCATTAAGTCTAC3′
Δ17−30	Δ17−30	wild type	5′GTTTCTACAATTTC/AAGTCTACAACTAG3′
Δ15−32	Δ15−32	wild type	5′CATGAATGTTTCTAC/ACAACTAGTACAAAC3′

[a] All mutagenic oligonucleotides are complementary to the coding strand. For each mutation the primers used for the first round of PCR were the mutagenic oligonucleotide and an oligonucleotide complementary to the noncoding strand upstream of the gene. For the second round of PCR, the primers were the product of the first round and an oligonucleotide complementary to the coding strand downstream of the gene. The PCR products were processed and cloned, and the mutated plasmids were characterized as described (Fujinaga et al., 1993; Meyer et al., 1994). [b] 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. The numbers designating the deletions are the first and the last residue that have been removed; e.g., in Δ19−23, residues 19−23 have been removed. The sequences of the deleted protein fragments can be inferred from Figure 1. [c] To be used throughout the article. [d] Mutated bases (differing from the wild type sequence) are underlined. Slashes indicate where the deletions have been introduced.

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 on 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 mutated plasmids were prepared as described in Table 1. The C14A, C24A, and C14A/C24A mutations have been reported previously (Meyer et al., 1994). The mutated plasmids were used to transform *E. coli* K38 (HfrC λ) cells harboring the pGP1-2 plasmid (Tabor, 1990). Overproduction and purification of the mutated proteins were carried out as reported (Meyer et al., 1994).

Redox titrations were performed in a glovebox, maintaining an oxygen concentration below 1.5 ppm. The reaction mixture (2.3 mL) contained 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.06 mM ferredoxin, and the following mediators: indigo disulfonic acid, safranine T, and benzylviologen, each at a concentration of 2.5 μM. The potential was measured between the platinum electrode and the Ag/AgCl reference electrode of a combined electrode. Ferredoxin was titrated in both the reductive and the oxidative directions, with stepwise additions of dithionite or ferricyanide, respectively. UV−visible absorption spectra in the 300−800 nm range were recorded at each step, and the absorbance at 420 nm, where the contribution of the mediators was negligible, was used for the calculations (Quinkal et al., 1994). The redox potentials were obtained by averaging the values yielded by the reductive and oxidative titrations, which in all cases differed from each other by less than 10 mV.

UV−visible absorption spectra were recorded on a Hewlett-Packard 8452 diode array spectrophotometer. RR spectra were recorded by collecting 90° scattering from the surface of a 10 μL frozen droplet of sample (20 K) on the cold finger of an Air Products Displex DE-202 closed cycle helium refrigerator, using lines from a Coherent Innova 10-W Ar^+ laser. The scattered light was analyzed using a ISA U-1000 double monochromator fitted with a cooled RCA 31034 photomultiplier tube with photon counting electronics. VTMCD spectra were recorded using a Jasco J-500 spectropolarimeter interfaced to an Oxford Instruments SM3 or Spectromag 4000 superconducting magnet. The 50% (v/v) glycerol that was added to the VTMCD samples to ensure the formation of an optical glass on freezing had no effect on the EPR or UV−visible absorption spectra of the samples. X-Band EPR spectra were recorded on a Bruker Instruments ESP 300D spectrometer equipped with an Oxford Instruments ESR 900 flow cryostat (4.2−300 K). The samples used for EPR and VTMCD studies were reduced anaerobically by sodium dithionite (2 mM final concentration) in a glovebox (<1 ppm O_2). Unless otherwise indicated, the samples for spectroscopic measurements were in 50 mM Tris-HCl buffer (pH 7.8).

RESULTS AND DISCUSSION

Identification of the Fourth Cysteine Ligand. Previous investigations had clearly shown that cysteines 11, 56, and 60 are ligands of the [2Fe-2S] cluster in wild type (WT) *Cp* 2Fe Fd and that cysteine 14 is not (Fujinaga et al., 1993; Meyer et al., 1994). The nature of the fourth ligand remained elusive. Mutagenesis results pointed to cysteine 24, since C24S and C24A mutations resulted in significant and identical changes in the UV−visible absorption and RR spectra of the oxidized protein. However, the changes were not sufficiently dramatic to rule out the possibility of a noncysteinyl fourth ligand with cysteine 24 involved in some form of interaction with the cluster. By analogy with Rieske proteins, histidines are potential [2Fe-2S] cluster ligands. However, this possibility has since been excluded by the observation that individual or collective mutations of the three histidines (at positions 6, 7, and 90) to alanine or valine

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                          *         *
Cp 2Fe Fd      7 HIFVCTSCRLNGKQQGFCYSKNSVEIVET 35
                         |
Pd 25K        92 HIQICGTTTCMICGAEDLIRVCKEKIAPE 120

          Cp 2Fe Fd     53 NTGCFGICSQGP 65
                              |   |
          Pd 25K       134 EVECLGACTNAP 145
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FIGURE 1: Alignment of the sequences of *Cp* 2Fe Fd and *Pd* 25K in the regions surrounding the cysteine ligands of the [2Fe-2S] clusters. The cysteine residues that were known to be ligands of the clusters prior to the present report are shown in boldface and are underlined: cysteines 11, 56, and 60 for *Cp* 2Fe Fd (Meyer et al., 1994) and cysteines 96, 101, 137, and 141 for *Pd* 25K (Crouse et al., 1994; Yano et al., 1994b). Cysteines 14 and 24 of *Cp* 2Fe Fd are starred. The alignment of the first segment has been extended to residue 35 of *Cp* 2Fe Fd in order to allow the identification of the residues removed in the various deletions described in this work.

have no effect on the UV−visible, EPR, and pulsed EPR spectra of the [2Fe-2S] cluster (J. Shergill, M.-P. Golinelli, R. Cammack, and J. Meyer, unpublished observations). Alternatively, the modest changes in the spectroscopic properties associated with the C24S and C24A mutations could be reconciled with replacement of cysteine 24 as a cluster ligand by cysteine 14 in these variants. This hypothesis was supported by the observation that the C14A mutation was without effect when performed on the WT protein but caused significant changes when carried out on the C24A protein, thus yielding the C14A/C24A double mutant (Meyer et al., 1994).

Further support for the cysteine 14/cysteine 24 ligand swapping has been obtained from new series of mutations described hereafter. Some of these mutations were designed on the basis of similarities between *Cp* 2Fe Fd and the 25 kDa subunit of the proton-translocating NADH:quinone oxidoreductase (NDH-1) of *Paracoccus denitrificans* (hereafter designated *Pd* 25K). The chromophores of the two proteins have very similar spectroscopic properties (Crouse et al., 1994) and display similar patterns of cysteine ligand distribution in their sequences (Crouse et al., 1994; Yano et al., 1994b). Indeed, although the overall sequence similarity of the two proteins is fairly low, at least three of the cysteine ligands, 11, 56, and 60 for *Cp* 2Fe Fd and 96, 137, and 141 for *Pd* 25K, can be superimposed (Figure 1). Only one of the ligands, cysteine 101, of *Pd* 25K (Yano et al., 1994b) has no counterpart in *Cp* 2Fe Fd; both cysteines 14 and 24 of the latter protein are offset relative to cysteine 101 of *Pd* 25K (Figure 1). Therefore, in an attempt to mimic the cysteine spacing occurring in *Pd* 25K, leucine 16 has been replaced by a cysteine in the C14A/C24A mutant of *Cp* 2Fe Fd; this new mutant (designated C16, see Table 1) has four cysteines in positions 11, 16, 56, and 60, all matching the ligands of *Pd* 25K, according to the alignments of Figure 1.

The most notable consequence of the incorporation of a cysteine in position 16 is a reversion of most properties of the C14A/C24A double mutant toward those of the wild type protein. This is particularly apparent in the oxidized UV−visible absorption spectra in which the loss in visible S-to-Fe(III) charge transfer intensity (relative to the protein band at 280 nm) and the frequency shifts that accompany the C14A/C24A mutation are reversed by the additional L16C mutation (Figure 2). Since there are only three cysteines in the C14A/C24A mutant protein, replacement of the noncysteinyl ligand by the cysteine incorporated at position 16 provides a logical interpretation of this result. The RR

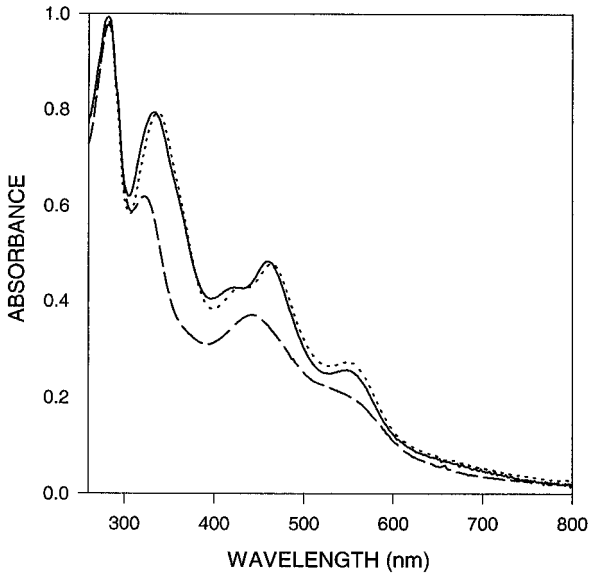

FIGURE 2: UV−visible absorption spectra at the oxidized level of wild type (dotted line), of C14/C24A (dashed line), and of C16 (solid line) mutated *Cp* 2Fe Fd. The protein concentrations were 5 mg/mL, and the medium was 0.5 M NaCl and 10 mM Tris-HCl buffer (pH 8.0). The optical path length was 1 mm.

spectra in the Fe−S stretching region of the WT, C24A, C14A/C24A, and the C16 variants all show distinctive features consistent with changes in the fourth cluster ligand in each case (Figure 3). Since the RR spectra of [2Fe-2S] centers are exquisitely sensitive to changes in the Fe−S_γ−C_β−C_α dihedral angles (Han et al., 1989; Fu et al., 1992), differences are expected and observed as a result of changes in the fourth coordinating cysteine residue (cysteine 24 in WT, cysteine 14 in C24A, and cysteine 16 in C16). In the C14A/C24A variant, which contains only three cysteine residues, noncysteinyl ligation must occur. It is conceivable that a histidine provides the fourth ligand in this mutant, and this would be consistent with the RR data, by comparison with the published spectra for Rieske type proteins (Kuila et al., 1992). However, pulsed EPR spectra show no evidence for histidine ligation in the C14A/C24A Fd (J. Shergill, M.-P. Golinelli, R. Cammack, and J. Meyer, unpublished observations). An alternative would be oxygenic coordination (e.g., serine or solvent). By analogy with the C56S and C60S mutants (Meyer et al., 1994), this might be expected to result in a significant upshift (6−10 cm^{-1}) in the lowest-energy mode which occurs between 284 and 290 cm^{-1}, since this mode has a major contribution from terminal Fe−S(Cys) stretching (Meyer et al., 1986b; Han et al., 1989). The absence of a pronounced upshift in this mode for the C14A/C24A mutant would seem to disfavor this possibility, but oxygenic coordination cannot be ruled out, in view of the paucity of RR data on structurally characterized [2Fe-2S] proteins with noncysteinyl coordination. The nature of the fourth cluster ligand in the C14A/C24A mutant is the subject of ongoing spectroscopic investigations.

The VTMCD spectra of the $S = {}^1/_2$ localized-valence $[2Fe\text{-}2S]^+$ clusters in the reduced WT, C24A, C14A/C24A, and C16 proteins (Figure 4) provide a means of assessing the ligation at the Fe(II) and Fe(III) sites (Fu et al., 1992, 1994; Crouse et al., 1994). The negative bands in the 300−350 nm region are attributed to Fe(II)-to-S charge transfer and display the same pattern in all proteins investigated, indicating that the Fe(II) site is coordinated by two cysteines in

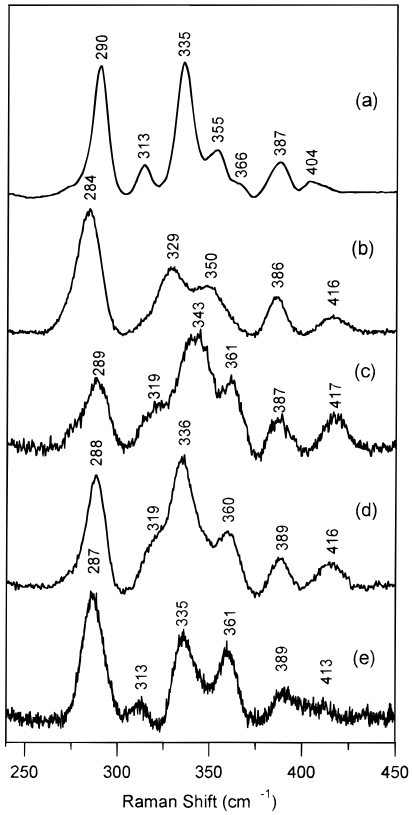

FIGURE 3: Low-temperature RR spectra of oxidized *Cp* 2Fe Fd and *Pd* 25K protein with 457.9 nm excitation: (a) *Cp* 2Fe Fd WT, (b) *Cp* 2Fe Fd C24A, (c) *Cp* 2Fe Fd C14A/C24A, (d) *Cp* 2Fe Fd C16, and (e) *Pd* 25K. For each sample, the cluster concentration was 1−2 mM and the medium was 50 mM Tris-HCl buffer (pH 7.8). The spectra were recorded at ∼20 K and are the sum of 7 scans (a), 30 scans (b), 30 scans (c), 27 scans (d), and 19 scans (e). Each scan involved advancing the spectrometer in 0.2 cm^{-1} increments and photon counting for 1 s/point with 6 cm^{-1} spectral resolution. Bands originating from the frozen buffer solution have been subtracted from each spectrum after normalizing the intensities of the "ice band" at 230 cm^{-1}. A linear ramp has been subtracted from the spectrum of the *Pd* 25 K protein to correct for the fluorescence background.

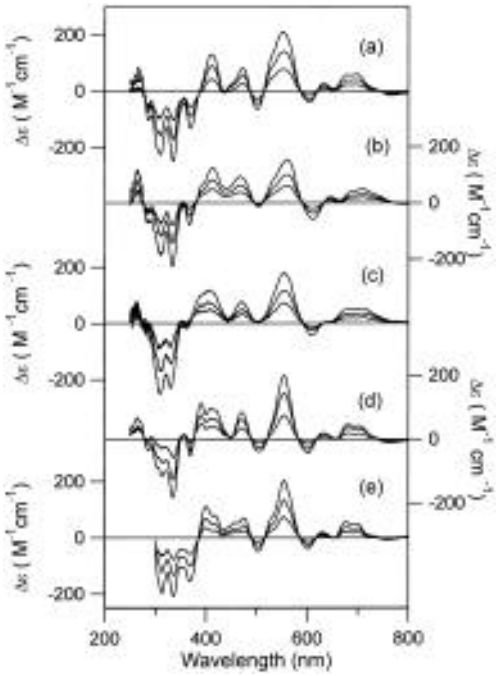

FIGURE 4: VTMCD spectra of dithionite-reduced *Cp* 2Fe Fd and *Pd* 25 K protein: (a) *Cp* 2Fe Fd WT, (b) *Cp* 2Fe Fd C24A, (c) *Cp* 2Fe Fd C14A/C24A, (d) *Cp* 2Fe Fd C16, and (e) *Pd* 25K. The spectra were recorded in 1 mm cuvettes with a magnetic field of 4.5 T (a−c and e) or 6.0 T (d) at temperatures of 1.6, 4.2, and 10 K. The intensity of all transitions increases with decreasing temperature. The samples were anaerobically reduced with sodium dithionite (2 mM final concentration). The cluster concentrations were in the range 0.3−0.5 mM, and the medium was 50 mM Tris-HCl buffer (pH 7.8), with 50% (v/v) glycerol (*Cp* 2Fe Fd samples) or 50 mM CHES buffer (pH 9.5), with 1 mM dithiothreitol and 50% (v/v) ethylene glycol (*Pd* 25K protein). $\Delta\epsilon$ values are based on the EPR spin concentrations of $S = {}^1/_2$ $[2Fe\text{-}2S]^+$ cluster.

each case. The WT, C24A, and C16 proteins show a similar complex pattern of bands in the S-to-Fe(III) charge transfer region, 350−550 nm, that is indicative of bis-cysteine coordination at the Fe(III) site (Fu et al., 1992). The much simpler pattern of bands in this region that is observed for the C14A/C24A protein is similar to that found in the structurally characterized C49S mutant of *Anabaena* [2Fe-2S] Fd (B. R. Crouse, M. K. Johnson, B. Xia, and J. L. Markley, unpublished results) which has serinate coordinated in place of cysteinate at the Fe(III) site (Holden et al., 1994). Hence, the VTMCD supports the view that only the C14A/C24A mutant has a noncysteinyl cluster ligand and localizes this ligand on the nonreducible Fe site. Inspection of Table 2 shows that the EPR properties of the reduced WT and mutant proteins investigated in this work are remarkably invariant. This is in accord with the VTMCD results, since these mutations only perturb the ligation at the Fe(III) site, whereas the anisotropy of the EPR signal primarily reflects changes in ligation at the Fe(II) site (Bertrand et al., 1985; Werth et al., 1990; Fujinaga et al., 1993).

These combined spectroscopic data afford compelling evidence for complete cysteinyl ligation in the WT, C24A, and C16 proteins. This confirms the cysteine swapping explanation for the properties of the C24S and C24A mutants as previously suggested (Meyer et al., 1994). In conclusion, the coordination of the [2Fe-2S] cluster of *Cp* 2Fe Fd involves cysteines 11, 56, and 60 and a fourth cysteine in variable positions on the 14−24 segment of the sequence: 14 in the C24A mutant, 16 in the C16 mutant, or 24 in the WT.

The *Cp* 2Fe Fd has long been known to display an unusual and puzzling behavior in active site reconstitutions; unlike most other Fds, the reaction of the apoprotein with iron ions and sulfide yields a protein of which the UV−visible and RR spectra differ from those of the WT protein (Meyer et al., 1992). These discrepancies can be ascribed at least in part to the fact that the protein is a homodimer (Meyer et al., 1984; Pétillot et al., 1995). The data recently collected with mutated *Cp* 2Fe Fd (Meyer et al., 1994; this work) suggest an additional reason for the difficulties met in reconstituting this protein; since both cysteine 14 and cysteine 24 can come within coordinating distance of the [2Fe-2S] cluster, the chemical reconstitution may yield a mixture of molecular forms having either cysteine 14 or cysteine 24 as the fourth ligand of the cluster. This, added to possible heterogeneities in the quaternary structure of the reconstituted Fd (M.-P. Golinelli and J. Meyer, unpublished data), may explain its unexpected UV−visible spectra and the broadening of most of its RR bands (Meyer et al., 1992).

Structural Implications of Ligand Swapping. Deletion Mutations. A single case of ligand swapping in a ferredoxin has previously been observed and characterized by X-ray crystallography (Martin et al., 1990) around the [4Fe-4S]

Table 2: Properties of Mutated Ferredoxins[a]

ferredoxin	stability[b] 80 °C	70 °C	50 °C	redox potential[c]	UV−visible absorption features (nm)				A_{450}/A_{280} ratio	EPR g values g_1	g_2	g_3
wild type	350	1100		−280 (−262)	336	420	464	550	0.50	2.01	1.95	1.92
C14A/C24A		75		−360 (−332)	322	442		560	0.37	2.00	1.96	1.92
C16	<1	70		−300	332	422	460	544	0.49	2.01	1.96	1.92
C16/Δ19−21	<1	140		−309	332	422	460	548	0.43	2.01	1.96	1.92
C16/Δ19−23	<2	490		−294	332	420	460	546	0.48	2.01	1.96	1.92
C16/Δ19−28	70			−364	334	422	460	546	0.45	2.01	1.96	1.92
C16/Δ19−30	45			−381	334	422	458	548	0.48	2.01	1.96	1.92
C16/Δ19−32[d]	<1		350	−326	332	−	458	560	0.14		nd	
C16/Δ19−34[d]		nd		nd	330	−	458	560	0.10		nd	
C24A	<1	50		−358 (−338)	332	420	456	544	0.47	2.01	1.95	1.92
Δ19−30	130			−357	332	420	458	542	0.49		nd	
Δ17−30		350		−341	332	418	452	544	0.48	2.01	1.95	1.92
Δ19−32		35		−336	334	420	450	542	0.42		nd	
Δ15−32[e]												

[a] nd represents not determined. [b] The thermal stability is the half-life (in minutes) of the chromophore, calculated from the kinetics of the A_{450} decrease at the indicated temperature, in anaerobic conditions. The solvent was 0.05 M NaCl and 10 mM Tris-Cl (pH 8.0), except for C16/Δ19−32 (pH 7.4). [c] Redox potentials in millivolts versus the standard hydrogen electrode (see Materials and Methods). The values in parentheses have been measured at pH 7.0 by S. E. J. Fawcett and F. A. Armstrong as described in Shen et al. (1993). [d] The low stability of these mutants impeded a satisfactory spectroscopic characterization. [e] This mutated form of the Fd could not be isolated.

cluster of *Azotobacter vinelandii* FdI. The replacement of cysteine 20 by cysteine 24 was shown to take place with relatively small (less than 2 Å) and localized displacements of the polypeptide backbone, due to the proximity of the two S_γ atoms of the relevant cysteine residues to one of the iron atoms of the cluster. Recently, cysteine 24 has been shown to become a ligand of the cluster even when cysteine 20 was replaced by serine, which indicated that ligand swapping was preferred over serine ligation (Shen et al., 1995). We had previously observed that the C24S and C24A variants of *Cp* 2Fe Fd had identical properties (Meyer et al., 1994). This shows that cysteine 14 takes over as the fourth ligand of the cluster in both mutants. Thus, in this case as well as in *A. vinelandii* FdI, ligand swapping is preferred over serine ligation.

In *Cp* 2Fe Fd, the fourth cysteine ligand can occur in various positions encompassed by residues 14−24. This region should therefore be a rather flexible loop allowing the cysteinyl sulfur atom of residues 14, 16, or 24, depending on the considered protein, to come within coordinating distance of the [2Fe-2S] cluster. This is consistent with sequence-based structural predictions (Meyer et al., 1986a) indicating that the region encompassing residues 15−30 is hydrophilic and poorly structured. Accordingly, this fragment may not be mandatory to stabilize the protein, and therefore, deletions of variable length were performed in this part of the sequence.

Two series of deletions were prepared (Tables 1 and 2). One (series C16/Δ) used the C16 mutant as starting material (therefore, cysteine 16 is the fourth ligand in all of them), and segments of 3−16 residues were deleted, starting at residue 19 (Table 2). For the other series (Δ), the WT gene was implemented. Thus, in this series, cysteine 14 is the fourth ligand of the cluster, since all of these deletions removed cysteine 24.

In the C16/Δ series, 3−12 residues could be deleted without impeding assembly of a [2Fe-2S] cluster in the protein. Since the spectroscopic properties of the C16/Δ mutants were practically identical with those of C16, only the RR (Figure 5) and the VTMCD (Figure 6) spectra of the C16/Δ19−30 mutant are shown for comparison with those of the C16 mutant. The EPR parameters for the

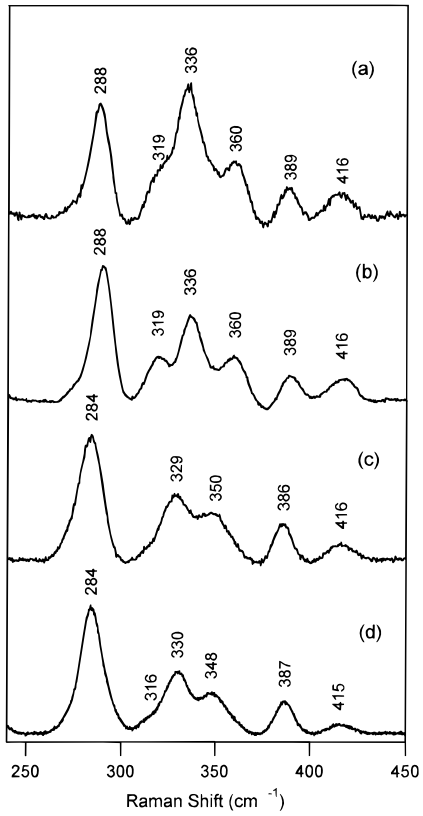

FIGURE 5: Low-temperature RR spectra of mutant forms of *Cp* 2Fe Fd with 457.9 nm excitation: (a) C16, (b) C16/Δ19−30, (c) C24A, and (d) Δ17−30. For each sample, the cluster concentration was 1−2 mM, and all were in 50 mM Tris-HCl buffer (pH 7.8). The spectra were recorded at ∼20 K and are the sum of 27 scans (a), 59 scans (b), 30 scans (c), and 18 scans (d). The data acquisition and handling were the same as described in Figure 3.

complete set of C16 deletion mutants are listed in Table 2. Collectively, these data demonstrate that the chromophores of the C16/Δ variants have nearly identical structures. Thus, the surprisingly wide range (ca. 90 mV, Table 2) of redox potentials spanned by these mutants must arise from factors independent of the chromophore structure. The length of the loop can be expected to have bearings on the redox potential of the [2Fe-2S] cluster by various means: solvent access to the vicinity of the cluster, polarity of its environ-

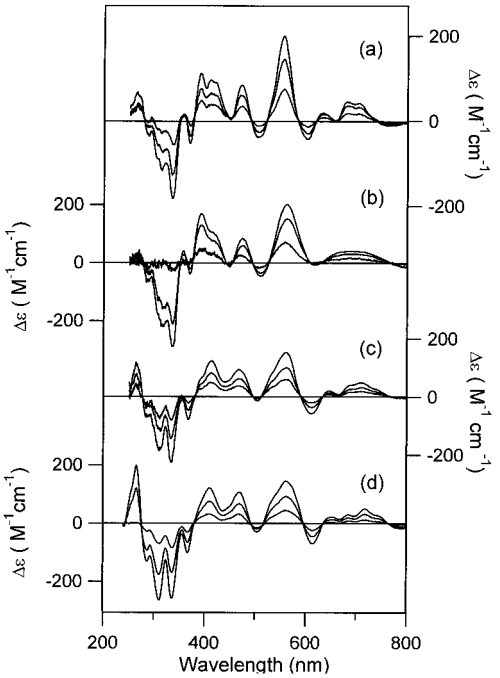

FIGURE 6: VTMCD spectra of dithionite-reduced mutant forms of *Cp* 2Fe Fd: (a) C16, (b) C16/Δ19−30, (c) C24A, and (d) Δ17−30. The spectra were recorded in 1 mm cuvettes with a magnetic field of 6.0 T (a and b) or 4.5 T (c and d) at temperatures of 1.6, 4.2, and 10 K. The intensity of all transitions increases with decreasing temperature. The samples were anaerobically reduced with sodium dithionite (2 mM final concentration). The cluster concentrations were in the range 0.3−0.5 mM, and the medium was 50 mM Tris-HCl buffer (pH 7.8), with 50% (v/v) glycerol. $\Delta\epsilon$ values are based on the EPR spin concentrations of $S = {}^1/_2$ $[2Fe\text{-}2S]^+$ cluster.

ment, and nearby charges. Indeed, at least two properties of the C16/Δ series of mutants appear to be somewhat correlated with redox potential variations. One is the thermal stability (Table 2), and the other one is the number of charged residues in the deleted segments. For instance, the removal of two (C16/Δ19−28 and C16/Δ19−30) positive charges results in a large decrease of the redox potential. Conversely, the subsequent removal of a negative charge (C16/Δ19−32) causes a relative increase of the redox potential (Figure 1 and Table 2).

In the Δ series, deletions no shorter than 12 residues were performed (Table 2). In view of the high conservation of the spectroscopic properties in the C16/Δ series (see above, Table 2), a systematic collection of all spectra was not undertaken in the Δ series. The positions of the maxima at 450−460 nm in the UV−visible absorption spectra (Table 2) show that the mutated Fd in this series can be grouped in two pairs, C24A and Δ19−30 on one hand and Δ17−30 and Δ19−32 on the other. Therefore, complete EPR (Table 2), RR (Figure 5), and VTMCD (Figure 6) data were collected for only one member of each pair, C24A and Δ17−30. The near identical VTMCD and RR spectra for the C24A and Δ17−30 mutants demonstrate that this 14-residue deletion has no effect on the structural and electronic properties for the cysteine 14-ligated $[2Fe\text{-}2S]^{2+}$ cluster. The variations of the redox potential in the Δ series are smaller than in the C16/Δ series, but it should be noted that the redox potential of the C24A variant, the parent of the Δ series, is already significantly more negative (by ca. 80 mV) than that of the WT. This suggests that the structural features determining the redox potential (see above) are different or vary in different ways in the C16/Δ and in the Δ series.

Stability of Mutated Ferredoxins. Most protein-engineering experiments involve amino acid substitutions. The implementation of insertions and deletions, which are expected to be more destabilizing than substitutions, is only marginal and most often limited to short (2 to 5 residues) fragments (Shortle & Sondek, 1995). The deletions reported here, of up to 14 residues in a sequence of 102 amino acids, are therefore quite unusual. They have probably been successful because they were targeted at a poorly structured and solvent-exposed region of the protein, as predicted from the sequence (Meyer et al., 1986a). Indeed, previous studies have shown that deletions and insertions are allowed in external loops rather than in internal helices and strands (Shortle & Sondek, 1995).

It should first be noted that WT *Cp* 2Fe Fd is more stable than all mutated forms characterized to this day. Then, among the proteins having full-length polypeptide chains, the stability decreases significantly when the fourth cysteine ligand is moved from position 24 to 16 (C16 mutant) or 14 (C24A mutant). In the C16/Δ series, where the size of the deletion has been increased stepwise from 3 to 16 residues, the stability is observed to increase up to 10 or 12 deleted residues and then to decrease sharply. In the Δ series, no short deletions have been tried, but a stabilizing effect of deletions involving 10−12 residues is also noted (Table 2).

The steep decrease of the stability when the deletions are longer than 14 residues might result from the inability of the protein framework to bear the removal of a sizeable (more than 15%) amount of its total length. However, more specific reasons are likely. In both series of deletions, the loss of stability appears to occur at the same point toward the C-terminus (between residues 32 and 34), suggesting that a stabilizing secondary structure may start in this region (a helix is predicted from the sequence). Toward the N-terminus, deletions up to the third neighbor of the second cysteine of the sequence are nondeleterious (residue 17 in the Δ series and 19 in the C16/Δ series). This is in keeping with the fact that the sequence segments closely neighboring the cysteine ligands are essential for the proper folding of the polypeptide chain around the iron−sulfur cluster (Matsubara & Saeki, 1992).

Similarities between Cp 2Fe Fd and the Pd 25K Subunit. By all spectroscopic criteria, *Cp* 2Fe Fd and *Pd* 25K have very similar active sites (Yano et al., 1994a; Crouse et al., 1994). Nevertheless, small differences are exhibited by their RR and VTMCD spectra (Crouse et al., 1994; Figures 3 and 4), and more importantly, the difference between their redox potentials exceeds 150 mV: −456 mV for *Pd* 25K (Yano et al., 1994a) and −280 mV for *Cp* 2Fe Fd (this work, Table 2). These differences must arise from differences in the folding of the polypeptide chains around the cluster. It was therefore anticipated that those of the molecular variants of *Cp* 2Fe Fd that were designed to mimic the cysteine ligand pattern of *Pd* 25K might show a closer resemblance to the latter. This prediction is borne out by both the spectroscopic and redox data. The displacements of the cysteine ligand 24 to positions 14 or 16, which result in a closer match with the cysteine ligand distribution in *Pd* 25K (Figure 1), shift the redox potential to more negative values by 80 and 20 mV, respectively (Table 2). Although these shifts do not nearly cover the redox potential gap between the two

proteins, they are in the right direction. Moreover, some of the deletion mutations result in even larger shifts; the redox potential of the C16/Δ19−30 variant (Table 2) is 100 mV more negative than that of the WT and only 75 mV more positive than that of *Pd* 25K.

EPR does not provide a discriminating method for assessing the effects of the C16 mutation, since reduced *Cp* 2Fe Fd WT and C16 and the reduced *Pd* 25K protein exhibit very similar spectra with *g* values around 2.00, 1.95, and 1.92 [see Table 2 and Crouse et al. (1994)]. As discussed above, this is rationalized in terms of identical ligation at the Fe(II) site of the $[2Fe\text{-}2S]^+$ clusters. Comparisons of the RR (oxidized) and VTMCD (reduced) spectra of WT and mutant forms of *Cp* 2Fe Fd with those of the *Pd* 25K protein are shown in Figures 3 and 4, respectively. By both spectroscopic criteria, the C16 mutation (C16 and the C16/Δ series) has made the *Cp* 2Fe Fd [2Fe-2S] center even more like the *Pd* 25K [2Fe-2S] center. Compared to WT, the most significant change in the VTMCD spectra for the C16 and C16/Δ mutants is the emergence of a more pronounced positive band around 400 nm, and an equivalent feature is also observed in the *Pd* 25K protein. The RR spectra of the C16 and C16/Δ mutants of *Cp* 2Fe Fd differ from those of WT by an upshift of the 404 cm^{-1} band to 416 cm^{-1} and by the presence of a single band at 360 cm^{-1} instead of the band at 355 cm^{-1} and shoulder at 366 cm^{-1} (Figure 5). These features are identical to those observed in the same spectral region of *Pd* 25K.

Altogether, and rather unsurprisingly, the redox and spectroscopic differences between *Cp* 2Fe Fd and *Pd* 25K are decreased in those mutants of the former protein in which the sequence has been made to closely resemble *Pd* 25K. Thus, the discrepancies between the properties of the two Fe−S chromophores must arise mainly from differences in the polypeptide fold in the region of residues 15−30 of *Cp* 2Fe Fd. The latter portion of the protein can modulate its redox potential and is presumably a flexible and exposed loop. It is thus likely to play a role in the still unknown function of *Cp* 2Fe Fd, which may involve electron transfer and interaction with redox partners.

CONCLUSIONS

This investigation has allowed the completion of the cysteine ligand assignment of the [2Fe-2S] *C. pasteurianum* ferredoxin. Because of the uniqueness of this Fd, the identity of the ligands could not be derived from data collected with other proteins and required a comprehensive combination of site-directed mutagenesis and spectroscopy. Unexpectedly, although this protein contains a Cys-X-X-Cys run which is implemented as a bidentate ligand in a vast majority of [2Fe-2S] proteins (Matsubara & Saeki, 1992), only the first cysteine (number 11 in the sequence) of this pair is a ligand in the wild type *Cp* 2Fe Fd. The now-identified set of cysteine ligands for this Fd, namely residues 11, 24, 56, and 60, remains a unique pattern among iron−sulfur proteins.

One of the main difficulties encountered in this work has been the assignment of cysteine 24 as a ligand of the cluster, since no considerable modifications resulted from the replacement of this residue by serine or alanine. The reason for this has been shown to be the replacement of cysteine 24 by cysteine 14. Ligand swapping has not previously been observed in any [2Fe-2S] protein (Moulis et al., 1996), and the best-characterized precedent is that of the [4Fe-4S] cluster of *A. vinelandii* ferredoxin I, where ligand swapping has been shown by X-ray crystallography to involve cysteine residues 20 and 24 (Martin et al., 1990). In the molecular forms of *Cp* 2Fe Fd produced here, the possible ligands are not only cysteines 14 and 24 but also a cysteine introduced in position 16. This suggests that the relevant region of the protein, predicted to be a solvent-exposed loop, has a relatively large freedom of movement and a high coordinating power. Interestingly, all coordination patterns investigated here have afforded [2Fe-2S] clusters. Also, various nondeleterious deletions could be carried out in this loop. They show that a stretch of up to 14 residues (e.g., 17−30) can be deleted with neither severe impairment of protein stability nor significant modification of the spectroscopic properties of the chromophore. The most significant effect of these deletions is a variation of the redox potential over a range of nearly 100 mV, depending on the length of the deleted segment. The deletable loop can therefore be anticipated to be functionally important.

Some of the molecular variants of the *Cp* 2Fe Fd, in particular those having the fourth cysteine ligand in position 16, were aimed at mimicking the cysteine ligand pattern of the 25 kDa subunit of the proton-translocating NADH: quinone oxidoreductase of *P. denitrificans*. Interestingly, these mutations made the spectroscopic properties of the two proteins, of which the wild types are already very similar, converge further. The mutagenesis experiments described here and in previous studies show that the coordination of the [2Fe-2S] cluster in *Cp* 2Fe Fd can be manipulated to a surprisingly large extent. This property will be implemented to explore more thoroughly the effects of the polypeptide chains on these clusters and possibly to better understand the wide diversity of cysteine ligand patterns observed in [2Fe-2S] proteins.

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