Articles

Effect of Replacing Conserved Proline Residues on the EPR and NMR Properties of *Clostridium pasteurianum* 2[4Fe–4S] Ferredoxin

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ABSTRACT: Most of [4Fe–4S] proteins bind their metallic center by four cysteine residues, three clustered in a single stretch of seven amino acids and a remote fourth generally followed by a proline residue. Two such prolines in *Clostridium pasteurianum* 2[4Fe–4S] ferredoxin have been substituted by different amino acids and the resulting molecular variants studied with EPR and NMR spectroscopies. The isolated EPR contributions of the $[4Fe–4S]^+$ clusters do not change much in all variants. The exact positions or the number of features composing the fully reduced EPR spectra built by the two interacting $[4Fe–4S]^+$ $S = {}^1/_2$ systems vary slightly but, in none of the proteins in which either proline 19 or 48 were substituted, do they indicate a major difference either in the folding of the ferredoxin or in the electronic structure of its clusters. A subset of paramagnetically shifted NMR signals is significantly affected by these replacements at both redox levels. The corresponding protons belong to two cysteines liganding the cluster close to the substitution. These data, combined with the presently available three-dimensional information, form the basis for partial assignments of the most shifted resonances in the NMR spectra of such proteins. The positions of intermediate lines in the NMR spectra of semireduced ferredoxins depend on the difference between the redox potentials of the two clusters; this difference is sensitive to the substitutions of either conserved proline residue by lysine.

Iron–sulfur proteins have now been recognized in a large variety of biological processes, including reactions which do not apparently make use of the electron transfer properties of their active sites (Cammack, 1992). Still, a large majority of them are involved in various electron transfer chains, and those organized around [4Fe–4S] clusters are ubiquitous from bacteria to mammals.

The detailed knowledge of the relationships between the structure of [4Fe–4S] proteins and their electron transfer properties would be a major step forward in the study of electron transfer in biology. Indeed, the complexity of the electronic structure of iron–sulfur clusters (Noodleman & Case, 1992) may have been one of the reasons why the mechanism of electron transfer in such proteins has remained less well known than for the other main classes of redox proteins, like cytochromes or copper proteins (Palmer, 1991). In such studies on relatively small proteins, NMR has proven to be uniquely instrumental, as the spectroscopic properties of the protons surrounding the cluster are sensitive both to the nearby electronic spin and to the spatial arrangement of the protein [e.g., Lecomte et al. (1989)]. With the availability of complete assignments of NMR spectra for a redox protein, it may be expected that the electron transfer pathway could be defined as the region most perturbed upon electron exchange.

The 2[4Fe–4S] ferredoxin (Fd)[1] from *Clostridium pasteurianum* is one of the most extensively studied [4Fe–4S] proteins, mainly because of its modest size (55 amino acids) and the availability of the high-resolution crystallographic structures of closely related proteins (Adman et al., 1973, 1976; Backes et al., 1991; TranQui et al., 1991). It has also long been used as a benchmark [4Fe–4S] protein in NMR studies (Phillips & Poe, 1973; Packer et al., 1977; Gaillard et al., 1987; Bertini et al., 1990; Busse et al., 1991). However, it has so far been extremely difficult to assign the observed NMR signals to sequence-specific residues. Recent attempts have led to some proposals and, more importantly, have established which signals originate from the same cysteine (Bertini et al., 1990, 1991, 1992a; Busse et al., 1991).

A clear picture of the arrangement of protons, as derived from NMR spectra, would probably constitute a powerful tool in further investigating the main spectroscopic and biological properties of Fd. As an alternative to methods relying on the observation of polarization transfer between correlated protons on 2D NMR spectra, site-directed mutants of *C. pasteurianum* Fd on strongly conserved proline residues close to the clusters have been used here. Their combined EPR and NMR signatures have sorted some of the liganding cysteines out. The potential candidates for the residues perturbed by the mutations have been analyzed with the presently available structural information and have allowed us to deduce partial sequence-specific assignments for the most paramagnetically shifted signals. Moreover, this work is a further illustration of the power of NMR spectroscopy in giving access to otherwise out of reach properties like the slight difference between the redox potentials of the two clusters of 2[4Fe–4S] Fd.

MATERIALS AND METHODS

A synthetic gene encoding *C. pasteurianum* Fd (Davasse & Moulis, 1992) was used to obtain the mutated forms of the

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[1] Abbreviations: Fd, ferredoxin; NOE, nuclear overhauser effect; COSY, correlated spectroscopy.

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protein. For genes modified on the codon for P-19, the *Nhe*I–*Hpa*I (*Nhe*I–*Sty*I in the case of the {PN-DD} double mutant) oligonucleotide fragment was removed by endonuclease digestion, and the resulting cleaved plasmid pUCFd2 (Davasse & Moulis, 1992) was ligated with a duplex bearing the mutation. In the case of the plasmid encoding P48K, the complete synthetic gene was reassembled from the oligonucleotides used to obtain pUCFd2 (Davasse & Moulis, 1992), except for the oligonucleotide containing the CCG codon for P-48 and the complementary one which were replaced by oligonucleotides containing the AAA codon for lysine and its complement instead. The presence of the correct substitutions was checked by restriction analysis, when applicable, and by DNA sequencing with the dideoxynucleotide chain termination method (Sanger et al., 1977). Two residues, P-19 and P-48, of the native protein have been substituted in this work, and the resulting molecular variants will be named P19X and P48Y in the following, X and Y being the amino acids replacing proline. The molecular form in which P-19 and N-21 are both replaced by aspartate residues is designated by {P19D-N21D}.

As *C. pasteurianum* 2[4Fe–4S] Fd contains two similar clusters, the one ligated by cysteines 8, 11, 14, and 47 is referred to as cluster I and the one ligated by cysteines 18, 37, 40, and 43 as cluster II.

EPR spectra were recorded with a Varian E-109 spectrometer as already described (Moulis et al., 1984). ^{1}H-NMR experiments were carried out on a Bruker AM-400 spectrometer, with samples dissolved in 50 mM potassium phosphate buffer at (uncorrected) pH 7.5 prepared in 99.8% D_2O (CEA, Gif-sur-Yvette, France). NOE difference spectra have been recorded by irradiating the resonance of interest for 100 ms before the application of the observation pulse. All chemical shifts are referred to the signal of residual water set at 4.7 ppm.

RESULTS

EPR Spectra. As the EPR spectra of reduced 2[4Fe–4S] Fd result from the contribution of two magnetically interacting clusters (Mathews et al., 1974), the individual contributions from each center are more conveniently observed in samples titrated with ca. 20% electron-equivalent (Orme-Johnson & Beinert, 1969). Such spectra have been recorded for proteins in which P-19 has been replaced by four different amino acids (N, T, M, and K) and P-48 by K (Figure 1) as well as the {P19D-N21D} double mutant. They closely resemble the spectra obtained with the native protein (Moulis et al., 1984) and indicate that both clusters individually contribute very similar signals. The only discrepancy concerns the low-field component of P19K which shows a clear splitting (Figure 1) indicating slightly different rhombic signals for the two clusters. At higher fields, the inequivalence in P19K is revealed by broader and less resolved lines than for P19M (Figure 1).

The spectra of fully reduced Fd are displayed in Figure 2. In all variants, the intercluster interaction results in a complex signal analogous to that of the native protein (Mathews et al., 1974; Moulis et al., 1984). In the case of P19K, the inequivalence of the two clusters may be sought on the low-field part of the spectrum where at least one additional line occurs. For P48K, the strong feature on the low-field side of the main component is shifted compared to the other proteins (Figure 2). These changes in the EPR signals may indicate very slight variations in the interaction between the two clusters (Guigliarelli et al., 1993) and cannot valuably be discussed with the presently available description of such systems.

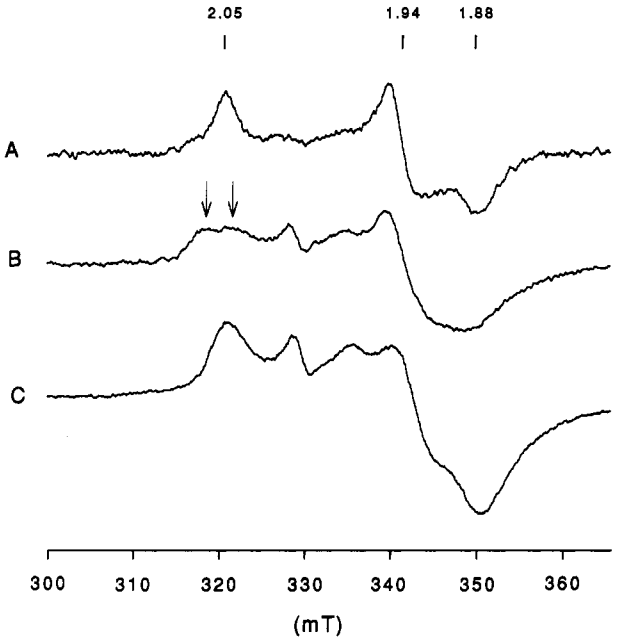

FIGURE 1: EPR spectra of semireduced proteins at 10 K. (A) P19M. (B) P19K. The arrows show the low-field components of the two rhombic signals. (C) P48K. Experimental conditions: radio-frequency = 9.226 GHz; modulation frequency = 100 kHz; modulation amplitude = 1 mT; and microwave power = 5 mW.

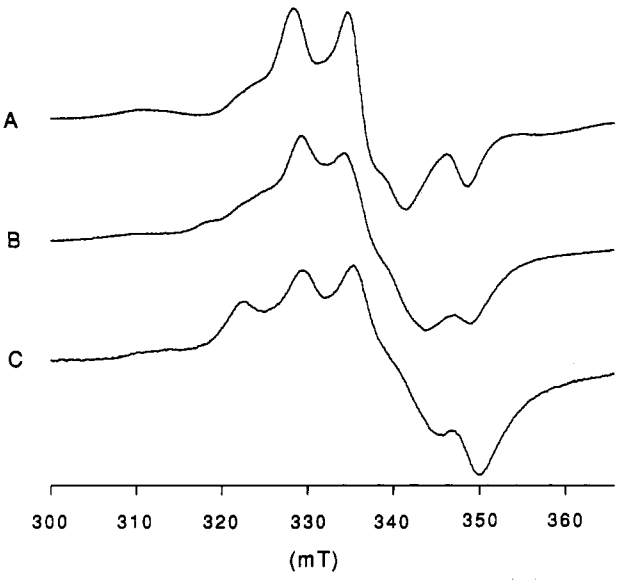

FIGURE 2: EPR spectra of reduced proteins at 10 K. (A) Native *C. pasteurianum* Fd. (B) P19K. (C) P48K. The experimental conditions are the same as those given in the legend to Figure 1 except for microwave power (2 mW).

However, it should be emphasized that these effects are probably small and that the EPR spectra of all Fd variants examined here do not change significantly from that of the parent protein. Consequently, the ground spin state of the clusters ($S = {}^1/_2$) or the general trends of the magnetic interaction between the two centers are not very sensitive to these replacements.

NMR Spectra of Oxidized Fd. The NMR spectra of clostridial Fd are among the numerous examples in which the chemical shifts of the methylenic protons of the cysteines ligating the [4Fe–4S] clusters are a very sensitive probe of both the electronic properties of the nearby iron and of the arrangement of the cysteine side chains around the Fe–S clusters. As exemplified in Figure 3A, the most striking feature

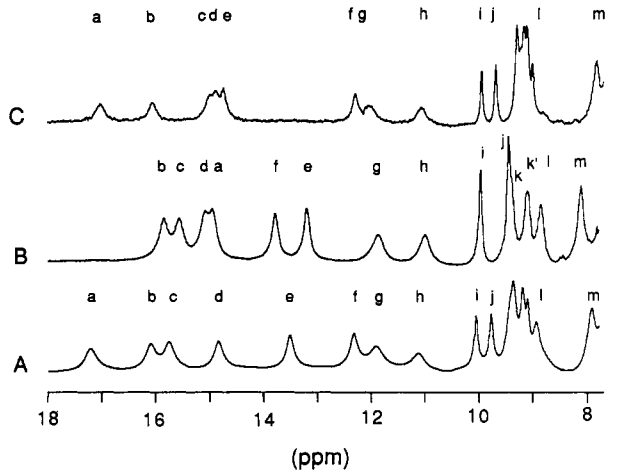

FIGURE 3: Low-field part of the ^{1}H NMR spectra of oxidized ferredoxins at 295 K. (A) Native *C. pasteurianum* Fd. (B) P19K. (C) P48K.

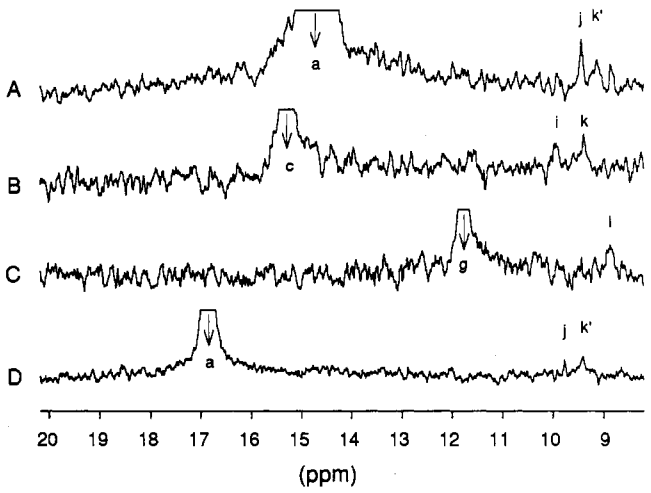

FIGURE 4: NOE difference spectra of oxidized proteins at 295 K. The irradiated lines are indicated by an arrow. (A–C) P19K. (D) P48K.

in the spectrum of *C. pasteurianum* 2[4Fe–4S] Fd is the presence between 10 and 20 ppm of eight well-resolved resonances [e.g., Phillips and Poe (1973), Packer et al. (1977), Gaillard et al. (1987), and Bertini et al. (1990)]. These signals have been assigned to one β-proton of each cysteine ligating the clusters (Packer et al., 1977; Bertini et al., 1991, 1992a; Busse et al., 1991).

The Fd variants in which either P-19 or P-48 have been substituted with K display NMR spectra similar to that of the parent protein (Figure 3), with eight lines between 10 and 20 ppm. However, some differences are clearly apparent between these spectra, as, for instance, the absence of the most shifted line at ca. 17 ppm in the spectrum of P19K (Figure 3B) or the change of the ca. 15.5–16 ppm doublet into a singlet for P48K (Figure 3C). Beside these differences, other features like the presence of two broad resonances between 11 and 12 ppm seem to be retained among the spectra of Figure 3. In addition, it has been found that the spectrum of the double mutant {P19D-N21D} is almost identical to that of P19K, except for some resonances which shift by *ca.* 0.2 ppm between the two mutated forms.

To analyze further these paramagnetically shifted protons, NOE difference spectra have been recorded (Figure 4). In the case of native Fd, two recent studies (Busse et al., 1991; Bertini et al., 1991) using a similar approach agree in

Table I: ^{1}H NMR Data of Oxidized Proteins

lines	pairing[a]	cluster[a]	native[b]	P19K[b]	Δ[c] (P19K)	P48K[b]	Δ[c] (P48K)
a	k', j	II	17.2	14.9	−2.3	17.0	−0.2
b		II	16.1	15.8	−0.3	16.0	−0.1
c	k, i	I	15.7	15.6	−0.1	15.0	−0.7
d		I	14.8	15.1	+0.3	14.9	+0.1
e		I	13.5	13.2	−0.3	14.7	+1.2
f		II	12.3	13.8	+1.5	12.3	0
g	l	I	11.9	11.9	0	12.0	+0.1
h	m	II	11.1	11.0	−0.1	11.0	−0.1
i	c, k	I	10.0	10.0	0	10.0	0
j	a, k'	II	9.7	9.4	−0.3	9.7	0
k	c	I	9.4	9.4	0	9.3	−0.1
k'	a	II	9.3	9.1	−0.2	9.3	0
l	g	I	8.9	8.9	0	9.0	+0.1
m	h	II	7.9	8.1	+0.2	7.8	−0.1

[a] According to Bertini et al. (1992a) and Busse et al. (1991). [b] Shifts (ppm) at 295 K. [c] Differences in chemical shift between the native and the mutated proteins.

associating eight sets of two or three signals to each cysteine of the sequence. For the sake of consistency with these previous studies, the labeling of the lines used by the italian group, which later provided such associations at both redox levels (Bertini et al., 1992a), will be used throughout. Then the signals at the oxidized level of the parent protein are referred to by lower case letters, starting at low field (Figure 3, Table I). The two β-protons, and when possible the α-proton, of the eight cysteines of the native Fd were grouped (Bertini et al., 1991; Busse et al., 1991) as follows: (a, k', j), (b, y), (c, k, i), (d, z), (e, w), (f, x), (g, l), (h, m), where letters above m refer to peaks located at higher field than presented on Figure 3.

For P19K, the associations involving the nearly invariant resonances at 15.6 ppm (c) (Figure 4B), 11.9 ppm (g) (Figure 4C), and 11.0 ppm (h) have been confirmed; in addition, NOE with resonances at 9.4 and 9.1 ppm have been detected (Figure 4A) for the peak at 14.9 ppm which does not occur in this position for the native protein (Figure 3A).

For P48K, similar experiments have been carried out. Peak a has been associated with resonances at 9.7 and 9.3 ppm (Figure 4D), and NOEs involving resonances g and h have been detected as for P19K. In addition, the signal at 15 ppm, specific of P48K, can be associated with two other signals at 10 and 9.3 ppm.

Without even developing the analysis of the data at this point, it already appears that in the spectra of Figure 3, only a limited number of paramagnetically shifted resonances is perturbed by the substitution of the prolines and a larger number hardly senses the modification (Table I).

NMR Spectra of Reduced Fd. Due to the paramagnetic ground state of the $[4Fe–4S]^+$ cluster, the NMR spectra of reduced *C. pasteurianum* Fd span a larger range of chemical shifts than at the oxidized level. The low-field regions of the spectra recorded for the three molecular variants of Figure 3 are displayed in Figure 5. As for the oxidized spectra, some similarities, including the number of shifted resonances found beyond 25 ppm or the presence of a triplet as the most shifted feature in all spectra, can be noticed. However, there is no exact correspondence between the three spectra, which indicates that the mutations introduced near one ligand selectively perturb the behavior of only some resonances. Then the changes observed at the reduced level appear qualitatively similar to those occurring at the oxidized level, and these combined data have been used to assign the shifted lines detected in the spectra of Figures 3 and 5 (see Discussion).

NMR Spectra of Partially Reduced Fd. The coexistence in samples of clostridial Fd of both oxidized and reduced

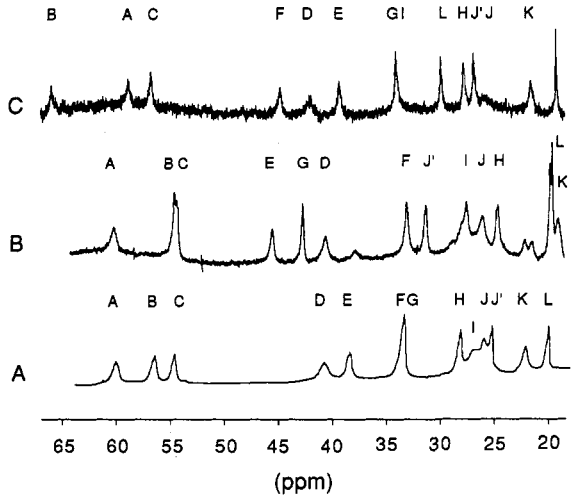

FIGURE 5: Low-field part of the ^{1}H NMR spectra of reduced proteins at 295 K. (A) Native *C. pasteurianum* Fd. (B) P19K. (C) P48K. The spectra were recorded on a spectrometer operating at 400 MHz except for panel A, 250 MHz.

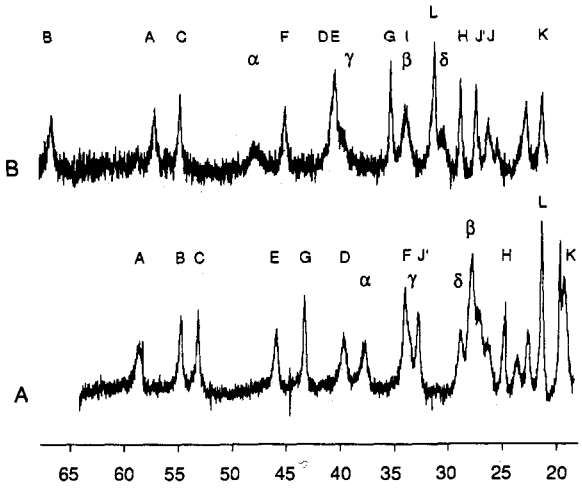

FIGURE 6: Low-field part of the ^{1}H NMR spectra of partially reduced proteins at 305 K. (A) P19K. (B) P48K. The intermediate peaks between the fully reduced and fully oxidized species are labeled with greek letters.

clusters translates into the occurrence of NMR lines which arise neither from the fully reduced nor from the fully oxidized protein (Phillips & Poe, 1977; Gaillard et al., 1987; Bertini et al., 1990). As the electron exchange between reduced and oxidized [4Fe–4S] clusters is fast on the NMR time scale, these additional resonances correspond to the coalesced lines of the oxidized and reduced species, and one of these peaks can be observed for each pair of signals associated with the same proton at both redox levels. The same type of resonances has been observed for the mutated forms of *C. pasteurianum* Fd (Figure 6), thus showing that electron self-exchange in partially reduced samples is not qualitatively changed by the substitution of the conserved proline residues studied here. Since these additional resonances are specific of clostridial Fd at an intermediate redox level and relate signals of the reduced and oxidized protein (Bertini et al., 1990, 1991, 1992a), they have been used below to check assignments proposed at each of these levels and to estimate the relative difference between the redox potentials of the two clusters.

DISCUSSION

The information content of the NMR spectra displayed by paramagnetic proteins needs to be sustained by sequence-specific assignments to be fully exploited. Indeed, the identification in the available primary and tertiary structures of the amino acids giving rise to the NMR lines is a major step forward in understanding the modulation of the [4Fe–4S] cluster properties by the polypeptide chain. Although 2D NMR is a very efficient tool for this purpose, as exemplified in the case of high-potential Fd (Bertini et al., 1992b; Nettesheim et al., 1992; Gaillard et al., 1992), no cross peak relating a proton of a cysteine ligand to a proton of another amino acid in low potential [4Fe–4S] Fd has yet been reported. The interpretation of the NMR spectra of *C. pasteurianum* 2[4Fe–4S] Fd has so far been relying mainly on the detection of COSY and NOESY cross peaks between protons belonging to the same cysteine (Bertini et al., 1991, 1992a; Busse et al., 1991), on the approximate 2-fold degeneracy of the NMR signals related to the pseudosymmetry of the structure (Busse et al., 1991), and on the possibility of detecting saturation transfer between the peaks corresponding to the same proton in the oxidized and reduced samples (Bertini et al., 1990, 1992a). Yet the variations of the spectra upon site directed mutations have been used here to eventually relate the paramagnetically shifted signals to the protons of sequence-specific cysteines.

Oxidized NMR Spectra. A close inspection of the spectra of Figure 3 easily reveals the major changes occurring upon mutating the proline residues neighboring C-18 and C-47. For P19K, an almost perfect one to one mapping of the eight most shifted signals can be established with those of the native protein, except for peaks labeled a and f, which experience marked up- and low-field shifts, respectively (Table I). Similarly, peaks c and e in P48K are significantly up- and down-shifted, respectively, as compared to the reference protein (Table I). These relationships between the spectra are borne out by NOE difference experiments which relate the most paramagnetically shifted peaks with those of the geminal or the α-proton of the same cysteine (Table I). Moreover, the availability of the double mutant {P19D-N21D} nicely confirms that signals a and f display the most noticeable shifts when P-19 is replaced.

On the basis of these data, it may be concluded that two protons previously associated with the cluster close to the mutation and belonging to different cysteines (Bertini et al., 1992a; Busse et al., 1991) are mainly perturbed by each proline substitution. For P19K, signals a and f arise from these protons while signals c and e are both perturbed in the case of P48K. One of these resonances may tentatively be assigned to one of the β-protons of the cysteine residue neighboring the mutations on these grounds only, but firm assignments require further analysis (see below).

Reduced NMR Spectra. Since the chemical shifts of the lines in the reduced spectra experience a larger contribution from paramagnetism, their variations as a function of temperature and their line widths may be used to complete the established associations for the native protein in the case of the different variants.

As a result of such analysis, the eight most downfield resonances, as well as some peaks occurring at higher fields, can unambiguously be traced between the spectra of Figure 5 (Table II). A few uncertainties remain for other peaks like the respective positions of I and J in the case of P19K, but these resonances are not very sensitive to the substitution of P-19 since they both have been attributed to cysteines of cluster I (Bertini et al., 1992a). Also, H and K have been attributed on the basis of the observation of their approximate invariance in position and in line widths; however, their Curie-type

Table II: ^{1}H NMR Data of Reduced Proteins

lines[a]	pairs	cluster[a]	native[b]	P19K[b]	Δ[c] (P19K)	F(T)[d]	P48K[b]	Δ[c] (P48K)	F(T)[d]
A(b)	?	II	60.3	60.1	−0.2	C	58.8	−1.5	C
B(e)	I	I	57.2	54.4	−2.8	AC	65.9	+8.7	AC
C(l)	D	I	54.8	54.5	−0.3	C	56.7	+1.9	C
D(g)	C	I	40.7	40.6	−0.1	C	42.1	+1.4	C
E(a)	G	II	38.5	45.5	+7.0	AC	39.3	+0.8	AC
F(c)	L	I	33.5	33.0	−0.5	AC	44.8	+11.3	AC
G(k′)	E	II	33.5	42.7	+9.2	AC	34.0	+0.5	AC
H(m)	K	II	28.0	24.6	−3.4	NC	27.7	−0.3	C
I	B	I	26.5	27.5	+1.0	NC	34.0	+7.5	C
J(d)	?	I	25.8	26.0	+0.2	NC	25.2	−0.6	NC
J′(f)	?	II	25.4	31.3	+5.9	AC	26.8	+1.4	AC
K(h)	H	II	22.0	19.0	−3.0	AC	21.6	−0.4	C
L(k)	F	I	20.0	19.7	−0.3	AC	29.9	+9.9	AC

[a] The lowercase letter indicates the corresponding signal in the oxidized spectra (Bertini et al., 1992a). [b] Shifts (ppm) at 295 K. [c] Differences in chemical shift between the native and the mutated proteins. [d] Dependence of the chemical shift with temperature: C for Curie, AC for anti-Curie, and NC for non-Curie behavior.

behavior in the native protein changes to an almost complete temperature independence for P19K in the 295–310 K temperature range.

Likewise, the assignments in the case of P48K are relatively straightforward with the exception of line J, which appears much broader than in the spectra of the native protein and of P19K (Figure 5, Table II). However, changing the proposed assignment for J (Table II) would displace it toward the diamagnetic region by an unduly large amount in view of the otherwise limited shifts of the resonances observed in the present studies (<12 ppm, Table II) and of the invariance of the corresponding signal at the oxidized level (line d, Table I). As for the increased line width of J in P48K, it is worth noticing that the signal has one of the shortest relaxation times of all resonances in the native protein (Bertini et al., 1992a) and a further shortening may not be excluded for P48K. Even if the assignement of J may not be considered as totally established, it does not affect the ultimate conclusions reached in the present studies.

It then appears from Table II that the replacement of P-19 by K mainly shifts resonances E, G, and J′ while that of P-48 by K induces the larger changes in the positions of B, I, F, and L. From the correlations established between resonances at both redox levels, these lines can be associated in pairs corresponding to geminal β-protons of cysteines as (E, G), (J′, ?), (B, I) and (F, L) (Bertini et al., 1992a). Therefore, the main effect of the studied mutations is to perturb a couple of cysteines liganding the nearby cluster. It should be noted that these cysteines correspond to those found above to be also affected at the oxidized level; this may be seen as a strong indication that the interpretation of the spectra of the mutated forms provided in Tables I and II is a correct basis for further analysis.

Tentative Assignments to Sequence-Specific Cysteines. The changes induced by the mutations on the chemical shifts of only some resonances in the NMR spectra most probably reflect a very localized perturbation sensed by the active sites. The liganding sequence of each [4Fe–4S] cluster in *C. pasteurianum* Fd is made of a cysteine triplet, C(8/37)*xx*C(11/40)*xx*C(14/43), where *x* is any amino acid, and a fourth distant cysteine (47/18). As the mutations studied are adjacent to the isolated cysteine, it is expected that the latter is among the ligands most sensitive to the structural or electronic modifications occurring upon replacing proline 19 or 48. The NMR results obtained at both redox levels (Tables I and II) then indicate that signals (E, G) corresponding to (a, k′) and (F, L) correlated to (c, k) are the most likely candidates for the β-protons of cysteines-18 and -47, respectively. The choice of (F, L) rather than (B,I) is based on the magnitude of the shift induced by the mutation relative to the chemical shift in the native protein, i.e., 11.3/33.5 (ca. 33%) and 9.9/20.0 (ca. 50%) compared to 8.7/57.2 (ca. 15%) and 7.5/26.5 (ca. 28%). The same argument for P19K is less strong, as (E, G) and (J′) vary in roughly the same proportions; however, in this case, one line (a) correlated to E appears most sensitive to the mutation at the oxidized level.

These assignments leave the (B, I)(e, w) and (J′, ?)(f, x) couples available for one of the three remaining cysteines of clusters I and II, respectively. No obvious reason explains why a single cysteine, in addition to that neighboring the mutation, responds to the substitution of P-19 or P-48. It may be noted, however, that resonance J′ associated with a proton of a cysteine liganding cluster II is evidently less sensitive than resonances B or F to the replacement of the proline (P-48) close to cluster I, but is at least as sensitive as resonances C or D associated with protons undoubtly close to cluster I (Table II). Symetrically, line B (cluster I) is shifted in P19K to a larger extent than line A (cluster II). Since the mechanical links between the two clusters in the structure involve the short 14–18 and 43–47 fragments (Adman et al., 1973), one may propose that these cysteines are the privileged sensors of any perturbation occurring on the other cluster. Following such argument, signals (B, I) and J′ would be assigned to the β-protons of cysteines 14 and 43, respectively. This line of reasoning is borne out by a constant feature in structures of low-potential 2[4Fe–4S] Fd, either directly derived from X-ray data or resulting from simulations in solution. The distances separating the β-carbon atom of C-18 of cluster II (respectively C-47, cluster I) from the β-carbon of the other cysteines roughly fall into two groups: one including C-14 of cluster I (C-43 and II, respectively) and C-43 of cluster II (C-14 and I, respectively) exhibits values below 6.7 Å and the other including the remaining cysteines of cluster II, C-37 (C-8 and I, respectively), and C-40 (C-11 and I, respectively), values generally well above 7.5 Å. Therefore, the cysteine side chains neighboring the prolines substituted in this work are significantly closer to two cysteines (one liganding the same cluster, the other one the distant cluster) than to the other cysteines of the protein. Such distance relationships are fully consistent with the NMR data obtained here (Table I and II) and give support to the proposed assignments. No additional spectroscopic evidence provides any indication as to the detailed assignments of the other cysteinyl protons.

A comment is needed about partial assignments at the oxidized level previously proposed by others (Busse et al., 1991). These authors developed an interpretation based

mainly on the presumed mechanism of spin delocalization between the Fe–S cluster and the cysteine protons and relying heavily on the X-ray crystallographic structure of the related Fd from *Peptostreptococcus asaccharolyticus*. However, this line of reasoning has provided conclusions at variance with those based on the present data. Indeed, the apparently most secure assignments obtained in these studies related lines (g, l) and (h, m) to the β-protons of C-14 and 43 partly because these resonances displayed the strongest cross peaks with their vicinal α-protons in magnitude COSY experiments (Busse et al., 1991), as expected from the protons farther from the nearby iron in the X-ray structure (Adman et al., 1973, 1976). However, it should be emphasized that the exact mechanism of spin delocalization in [4Fe–4S] proteins is still a matter of debate [e.g., Busse et al. (1991), Bertini et al. (1992b), and Mouesca et al. (1993)]. Also, it is not completely sure that the available crystal structures for this type of protein (Adman et al., 1973, 1976; Backes et al., 1991; TranQui et al., 1991) fully represent the structures in solution. Indeed, recent molecular dynamics simulations of the solution structure of a high-potential ferredoxin based on the model derived from X-ray data (Carter et al., 1974) have shown that the former was subjected to some displacements compared to the latter which were in better agreement with the observed NOEs (Banci et al., 1992). Similarly, our preliminary calculations on the present kind of low-potential ferredoxin have indicated that the orientation of some cysteine residues around the clusters experienced significant changes upon modeling of the structure in solution, in particular cysteines 11 and 40, which follow in the sequence relatively strained residues (Carter, 1977). Then, the arguments implicating the orientation of residues around [4Fe–4S] clusters should probably benefit from reexamination in light of the results of such calculations.

Considering the spin delocalization on the cysteine ligands, if the contact shifts primarily depend on the orientation of the side chains, variations of opposite signs would be expected for the signals of the geminal β-protons of the same cysteine in passing from the reference protein to the mutated forms (Busse et al., 1991; Bertini et al., 1992b). Although the shifts of the corresponding lines may be considered of opposite signs but of significantly different absolute values at the oxidized level (Table I), they generally are of the same sign at the reduced level (Table II). This most probably means that the perturbation induced on the chemical shifts of the protons sensitive to the mutations applies predominantly to the spin density on the liganded iron rather than on the orientation of the cysteines. In other words, although the changes in the detailed electronic properties of the clusters probably originate from a structural modification of the surroundings, the latter is certainly not large enough to strongly disorganize the cysteine side chains.

Effect of the Mutations on the Relative Redox Potentials of the Two Clusters. The intermediate peaks of the semireduced spectra (Figure 6) result from fast exchange between the reduced and oxidized forms of the same cluster. The chemical shifts of these lines depend on the lifetime at each redox level of the corresponding cluster. This provides a means of knowing which cluster is easier to reduce once assignments are available. Such analysis has been previously carried out for native *C. pasteurianum* Fd and has shown that cluster II, ligated by cysteines 18, 37, 40, and 43, had a less negative potential than cluster I by ca. 15 mV (Bertini et al., 1992a). Table III reports values of the parameter linking the position of the intermediate line with those of the fully oxidized and fully reduced corresponding resonances for a selected set of

Table III: ^{1}H NMR Data of Partially Reduced Proteins

lines	native[a]	d[b]	P19K[a]	d[b]	P48K[a]	d[b]
α(b,A)II	41.5	0.58	39.7	0.49	45.5	0.77
β(a,E)II	31.4	0.66	27.8	0.48	37.5	0.71
γ(e,B)I	27.7	0.33	33.0	0.45	33.8	0.48
δ(1,C)I	26.1	0.38	29.4	0.44	30.5	0.46

[a] Shifts (ppm) at 305 K. [b] $d = (\text{shift}_{SR} - \text{shift}_{Ox})/(\text{shift}_{Red} - \text{shift}_{Ox})$, where shift_{Ox}, shift_{Red}, and shift_{SR} stand for the chemical shift of corresponding lines at the oxidized, reduced, and partially reduced levels, respectively.

protons close to one cluster or the other. The fact that P19K exhibits fairly homogeneous values indicates that the redox potentials are very similar if not identical in this protein. On the contrary, the resonances associated with cluster II of P48K display far larger values than those of cluster I: this mutated form resembles the native protein with, perhaps, an even larger separation between the two redox potentials. These observations indicate that the microscopic redox potentials of the two clusters are sensitive to the breakage of the pseudo-2-fold symmetry at the level of proline 19/48 and that at least their relative values depend on the detailed structure of their surroundings. The kind of modulation on the redox potential induced by these changes would certainly benefit from the availability of other mutated forms in order to define its nature.

Conclusions. The substitution of the very conserved proline residues following one cysteine ligand in many [4Fe–4S] proteins has induced selective changes in the properties of paramagnetically shifted protons of *C. pasteurianum* Fd. In contrast, neither the ground spin state ($S = {}^1/_2$) of the clusters nor their magnetic interaction assessed by EPR spectroscopy have been strongly affected by these mutations. Indeed, only limited perturbations in the distribution of the spin density over the clusters upon replacing these conserved prolines may be deduced from the available data. Still, the latter effect most probably plays a major role in the shifts observed in the NMR spectra.

These observations have been used to propose part of the sequence specific assignments for the cysteine residues of a low-potential Fd, which have so far resisted analysis by conventional 2D NMR. Although site-directed modified proteins have already been used for interpreting NMR data of other paramagnetic systems [e.g., Rajarathnam et al. (1992)], the present work constitutes the first attempt at applying this method to a [4Fe–4S] protein. As this kind of Fd is among the proteins displaying the fastest relaxing protons, the present approach is certainly a very valuable complement to the observation of cross-correlations among paramagnetically shifted resonances. Precise NMR assignments are indeed a prerequisite to the detailed understanding of the solution structure of 2[4Fe–4S] Fd and of the changes occurring upon electron transfer, an almost completely obscure matter at the moment.

Although the present data cannot pretend to exactly define the structural modifications induced by the mutations, it appears that the conserved prolines are mandatory to create a specific environment for the nearby [4Fe–4S] cluster. This is demonstrated by the similarity of the NMR spectra recorded for P19K and the double mutant {P19D-N21D} and their marked differences with that of the native protein. Also, significant variations in the relative values of the redox potentials of the two clusters upon replacing these conserved prolines have been detected. The thorough functional significance of these features will be reported elsewhere, but it may already be suspected that the availability of various site-

directed mutants of *C. pasteurianum* Fd will certainly reveal important parameters in the properties of this type of electron transfer protein, as exemplified here with spectroscopic properties.

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REFERENCES

Adman, E. T., Sieker, L. C., & Jensen, L. H. (1973) *J. Biol. Chem. 248*, 3987–3996.

Adman, E. T., Sieker, L. C., & Jensen, L. H. (1976) *J. Biol. Chem. 251*, 3801–3806.

Backes, G., Mino, Y., Loehr, T. M., Meyer, T. E., Cusanovich, M. A., Sweeney, W. V., Adman, E. T., & Sanders-Loehr, J. (1991) *J. Am. Chem. Soc. 113*, 2055–2064.

Banci, L., Bertini, I., Carloni, P., Luchinat, C., & Orioli, P. L. (1992) *J. Am. Chem. Soc. 114*, 10683–10689.

Bertini, I., Briganti, F., Luchinat, C., & Scozzafava, A. (1990) *Inorg. Chem. 29*, 1874–1880.

Bertini, I., Briganti, F., Luchinat, C., Messori, L., Monnanni, R., Scozzafava, A., & Vallini, G. (1991) *FEBS Lett. 289*, 253–256.

Bertini, I., Briganti, F., Luchinat, C., Messori, L., Monnanni, R., Scozzafava, A., & Vallini, G. (1992a) *Eur. J. Biochem. 204*, 831–839.

Bertini, I., Capozzi, F., Ciurli, S., Luchinat, C., Messori, L., & Piccioli, M. (1992b) *J. Am. Chem. Soc. 114*, 3332–3340.

Busse, S. C., La Mar, G. N., & Howard, J. B. (1991) *J. Biol. Chem. 266*, 23714–23723.

Cammack, R. (1992) *Adv. Inorg. Chem. 38*, 281–322.

Carter, C. W., Jr., Kraut, J., Freer, S. T., Xuong, N.-H., Alden, R. A., & Bartsch, R. G. (1974) *J. Biol. Chem. 249*, 4212–4225.

Davasse, V., & Moulis, J.-M. (1992) *Biochem. Biophys. Res. Commun. 185*, 341–349.

Gaillard, J., Moulis, J.-M., & Meyer, J. (1987) *Inorg. Chem. 26*, 320–324.

Gaillard, J., Albrand, J.-P., Moulis, J.-M., & Wemmer, D. E. (1992) *Biochemistry 31*, 5632–5639.

Guigliarelli, B., Guillaussier, J., More, C., Sétif, P., Bottin, H., & Bertrand, P. (1993) *J. Biol. Chem. 268*, 900–908.

Lecomte, J. T. L., Smit, J. D. G., Winterhalter, K. H., & La Mar, G. N. (1989) *J. Mol. Biol. 209*, 235–247.

Mouesca, J.-M., Rius, G., & Lamotte, B. (1993) *J. Am. Chem. Soc. 115*, 4714–4731.

Mathews, R., Charlton, S., Sands, R. H., & Palmer, G. (1974) *J. Biol. Chem. 249*, 4326–4328.

Moulis, J.-M., Auric, P., Gaillard, J., & Meyer, J. (1984) *J. Biol. Chem. 259*, 11396–11402.

Nettesheim, D. G., Harder, S. R., Feinberg, B. A., & Otvos, J. D. (1992) *Biochemistry 31*, 1234–1244.

Noodleman, L., & Case, D. A. (1992) *Adv. Inorg. Chem. 38*, 424–470.

Orme-Johnson, W. H., & Beinert, H. (1969) *Biochem. Biophys. Res. Commun. 36*, 337–342.

Packer, E. L., Sweeney, W. V., Rabinowitz, J. C., Sternlicht, H., & Shaw E. N. (1977) *J. Biol. Chem. 252*, 2245–2253.

Palmer, G. A., Ed. (1991) *Structure and Bonding*, Vol. 75, Springer-Verlag, Berlin-Heidelberg.

Phillips, W. D., & Poe, M. (1973) in *Iron–Sulfur Proteins* (Lovenberg, W., Ed.) Vol. II, pp 255–285, Academic Press, New York.

Rajarathnam, K., La Mar, G. N., Chiu, M. L., & Sligar, S. G. (1992) *J. Am. Chem. Soc. 114*, 9048–9058.

Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463–5467.

TranQui, D., Fanchon, E., Vicat, J., Sieker, L. C., Meyer, J., Moulis, J.-M., Gagnon, J., & Duée, E. D. (1991) Abstract D049, Fifth International Conference on Bioinorganic Chemistry, Oxford, U.K.