^{1}H NMR Spectra of Vertebrate [2Fe–2S] Ferredoxins. Hyperfine Resonances Suggest Different Electron Delocalization Patterns from Plant Ferredoxins†

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ABSTRACT: We report the observation of paramagnetically shifted (hyperfine) proton resonances from vertebrate mitochondrial [2Fe–2S] ferredoxins. The hyperfine signals of human, bovine, and chick [2Fe–2S] ferredoxins are described and compared with those of *Anabena* 7120 vegetative ferredoxin, a plant-type [2Fe–2S] ferredoxin studied previously [Skjeldal, L., Westler, W. M., & Markley, J. L. (1990) *Arch. Biochem. Biophys. 278*, 482–485]. The hyperfine resonances of the three vertebrate ferredoxins were very similar to one another both in the oxidized state and in the reduced state, and slow (on the NMR scale) electron self-exchange was observed in partially reduced samples. For the *oxidized* vertebrate ferredoxins, hyperfine signals were observed downfield of the diamagnetic envelope from +13 to +50 ppm, and the general pattern of peaks and their anti-Curie temperature dependence are similar to those observed for the oxidized plant-type ferredoxins. For the *reduced* vertebrate ferredoxins, hyperfine signals were observed both upfield (−2 to −18 ppm) and downfield (+15 to +45 ppm), and all were found to exhibit Curie-type temperature dependence. This pattern and temperature dependence are distinctly different from those found with reduced plant-type ferredoxins which have signals centered around +120 ppm with Curie-type temperature dependence, assigned to cysteines which interact with Fe(III), and signals centered around +20 ppm with anti-Curie temperature dependence, assigned to cysteines which interact with Fe(II) [Dugad, L. B., La Mar, G. N., Banci, L., & Bertini, I. (1990) *Biochemistry 29*, 2263–2271]. These results indicate that the contact-shifted resonances in the reduced vertebrate ferredoxins detect different spin magnetization from those in the reduced plant ferredoxins and suggest that plant and vertebrate ferredoxins have fundamentally different patterns of electron delocalization in the reduced [2Fe–2S] center.

The vertebrate ferredoxins are small (13–14 kDa), [2Fe–2S] proteins found in the mitochondria of steroid metabolizing tissues. They function to transfer reducing equivalents from NADPH oxidoreductases to cytochrome P450 enzymes involved in the biogenesis of steroid hormones, the formation of vitamin D metabolites, and the production of bile acids. Ferredoxins of the [2Fe–2S] type are also found in cyanobacteria and blue-green algae and in the chloroplasts of higher plants where they participate in photosynthetic electron transport. For the plant-type ferredoxins, X-ray crystallographic studies have established that the iron–sulfur center has a structure as shown in Figure 1 with the iron atoms ligated to four cysteine residues (Tsukihara et al., 1981, 1990; Rypniewski et al., 1991). A crystal structure has not been determined for any of the vertebrate ferredoxins, but the general similarity of their spectroscopic properties to the plant ferredoxins and to model [2Fe–2S] clusters is consistent with this type of structure [reviewed by Spiro (1982) and by Matsubara et al. (1986)]. In addition, chemical modification studies using bovine ferredoxin have implicated specific cysteine residues in iron coordination (Tuls et al., 1987; Cupp & Vickery, 1988). The amino acid sequence alignments given in Figure 2 show regions of primary structure similarity between the two classes of ferredoxins.

While the iron–sulfur clusters of vertebrate and plant ferredoxins are believed to have like structures, the two ferredoxin classes exhibit some significant differences in their physicochemical properties. The oxidation–reduction potential of the vertebrate ferredoxins (~−270 mV) is considerably more positive than that of the plant ferredoxins (~−400 mV) (Estabrook et al., 1973; Cammack et al., 1977). This and differences in magnetic susceptibility (Kimura et al., 1970; Palmer et al., 1971), EPR *g* tensor (Bertrand et al., 1987), magnetic circular dichroism (Thompson et al., 1977), and resonance Raman spectra (Yachandra et al., 1983; Mino et al., 1987; Han et al., 1989) must reflect detailed structural and electronic differences between the cluster types.

NMR spectroscopy is well suited for investigating the structure and electronic properties of these iron–sulfur proteins. The patterns of paramagnetically shifted ^{1}H resonances (hyperfine signals) and their temperature dependence, in par-

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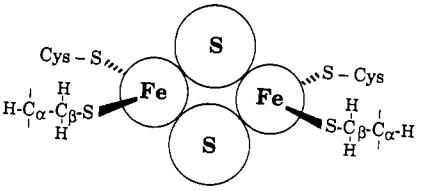

FIGURE 1: Structure of the [2Fe–2S] cluster. Two of the cysteine ligands are shown to illustrate positions of the C^{α} and C^{β} hydrogens.

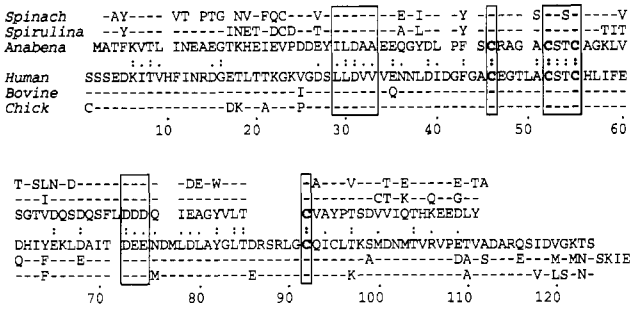

FIGURE 2: Sequence alignments of three vertebrate ferredoxins and three plant-type ferredoxins. For the vertebrate ferredoxins, residues identical with the human protein are indicated by dashes shown *below* that sequence; for the plant ferredoxins, residues identical with the *Anabena* protein are indicated by dashes shown *above* that sequence. Gaps have been introduced to improve alignment, and symbols indicate positions of amino acid identity (colon) or similarity (•) between the two ferredoxin classes. Cysteine residues that are proposed to ligate to the [2Fe–2S] cluster are indicated in boldface. Regions of sequence similarity are enclosed in boxes and include (using the numbering system of the vertebrate ferredoxins) the four cysteine ligands to the iron cluster at positions 46, 52, 55, and 92, a conserved pattern of residues from positions 29–33, and an acidic patch in the region of residues 72–74. Sources of the ferredoxin sequences are as follows: human (Mittal et al., 1988), bovine (Okamura et al., 1985), chick (Kagimoto et al., 1988), spinach (Matsubara & Sasaki, 1968), *Anabaena* 7120 vegetative (Alam et al., 1986), *Spirulina platensis* (Tanaka et al., 1976).

ticular, can provide detailed information on bonding and magnetic interactions within the [2Fe–2S] centers. The hyperfine ^1H NMR signals of the plant-type ferredoxins have been studied extensively. Poe et al. (1971) first observed hyperfine-shifted resonances in spectra of spinach and parsley ferredoxins and assigned them to $^1H^{\beta}$ nuclei of the cysteine ligands; differences in signal positions were attributed to differences in spin density at the β-carbons and to the distance and angular dependence of the shift interactions. For the reduced proteins, Poe et al. (1971) also observed differences in the temperature dependence of the resonances which suggested that the added electron is shared unequally between the two iron atoms; the effect of the localized valence of the reduced proteins on hyperfine shifts was treated theoretically by Dunham et al. (1971). Other plant and algal ferredoxins subsequently have been shown to exhibit similar hyperfine patterns.

Recently, signals from the full complement of $^1H^{\beta}$ and $^1H^{\alpha}$ nuclei of the four cysteine ligands in reduced *Anabena* 7120 vegetative ferredoxin have been resolved and assigned (Skjeldal et al., 1991). A consistent pattern has emerged for the hyperfine resonances of plant-type ferredoxins: (i) In the *oxidized* state, overlapping signals from all eight cysteine $^1H^{\beta}$ nuclei appear between +20 and +30 ppm, and all exhibit anti-Curie temperature dependence. (ii) In the *reduced* state, four Curie-type signals from the $^1H^{\beta}$ nuclei of the two cysteine residues ligated to Fe(III) appear between +140 and +90 ppm, and four anti-Curie-type signals from the $^1H^{\beta}$ nuclei of the two cysteine residues ligated to Fe(II) appear between +28 and +11 ppm and exhibit anti-Curie behavior (Dugad et al., 1990; Skjeldal et al., 1991). The cysteine $^1H^{\alpha}$ signals show smaller hyperfine shifts than the $^1H^{\beta}$ signals: those from the two cysteines ligated to Fe(III) are near +45 and +18 ppm (Curie temperature dependence), and those from the two cysteines ligated to Fe(II) are near +5 and +3 ppm (Skjeldal et al., 1991). Additional hyperfine signals have been assigned to amide protons that are hydrogen-bonded to the [2Fe–2S] center (Skjeldal et al., 1990a). A new development has been the assignment of a Curie-type hyperfine peak around +14 ppm to the $^1H^{\alpha}$ of arginine-43 on the basis of a network of two-dimensional ^1H–^1H NOE connectivities (Skjeldal et al., 1991). The backbone amide nitrogen of arginine-43 is within hydrogen-bonding distance to one of the sulfide atoms of the iron–sulfur cluster in the X-ray structrure of *Anabaena* ferredoxin (Rypniewski et al., 1991).

Paramagnetically shifted hyperfine signals of vertebrate ferredoxins have not been described previously,[1] although the results of a ^1H NMR study of the aromatic diamagnetic region of bovine adrenal ferredoxin (adrenodoxin) have been published (Greenfield et al., 1989). The folding topology of a related 2Fe–2S ferredoxin, putidaredoxin from *Pseudomonas putida*, has been determined from two-dimensional ^1H NMR studies of its diamagnetic region (Pochapsky & Ye, 1991). We report here the detection and characterization of hyperfine resonances from three vertebrate ferredoxins and compare the behavior of the signals to those of plant-type ferredoxins. We have found that the ^1H hyperfine signals from oxidized vertebrate ferredoxins are similar to those of plant-type ferredoxins but that significant differences exist between the hyperfine signals of reduced vertebrate and plant ferredoxins. The results indicate that while the general structures of the metal centers are likely to be similar, electron delocalization in the reduced states differs and must reflect subtle structural differences between the two classes of [2Fe–2S] proteins.

Experimental Procedures

Protein Samples. Bovine ferredoxin was isolated from adrenocortical tissue as described by Cupp and Vickery (1988). Human and chick ferredoxins were produced as recombinant proteins in *Escherichia coli* and purified as detailed in the original reports (Coghlan & Vickery, 1989; Brandt et al., 1990). Protein concentrations were determined by assuming E_{414} = 11 $(mM \cdot cm)^{-1}$ (Huang & Kimura, 1973) for each ferredoxin. Samples were stored in 50 mM Tris, pH 7.5. Prior to NMR measurements, samples were desalted on a Sephadex G-25 column (Pharmacia) equilibrated with 50 mM potassium [^2H]phosphate, pH 8.8, lyophilized, and dissolved in 99.9% ^2H_2O. The pH*[2] of the samples was adjusted to 8.6 by adding dilute ^2HCl or KO^2H. Quoted pH* values were determined at 20 °C and are meter readings uncorrected for the isotope effects at the glass electrode. Preparation and spectroscopy of the vegetative ferredoxin from *Anabena* sp. strain PCC 7120 have been described previously (Oh & Markley, 1990; Skjeldal et al., 1990a).

Reduced Ferredoxin. The protein samples were deaerated on a vacuum line and sealed under argon in screw-top, 5-mm NMR tubes (Wilmad). Reduction of the protein was carried out anaerobically by injecting a small molar excess of sodium dithionite (Skjeldal et al., 1989) from a syringe. The final pH*

[1] After this work was completed, a study appeared (Miura & Ichikawa, 1991) which reported a similar pattern of hyperfine signals from bovine ferredoxin (adrenodoxin).

[2] Abbreviations: pH*, pH meter reading (glass electrode calibrated with buffers in H_2O at natural abundance) of a solution in ^2H_2O uncorrected for isotope effects; TSP, 3-(trimethylsilyl)propionic acid.

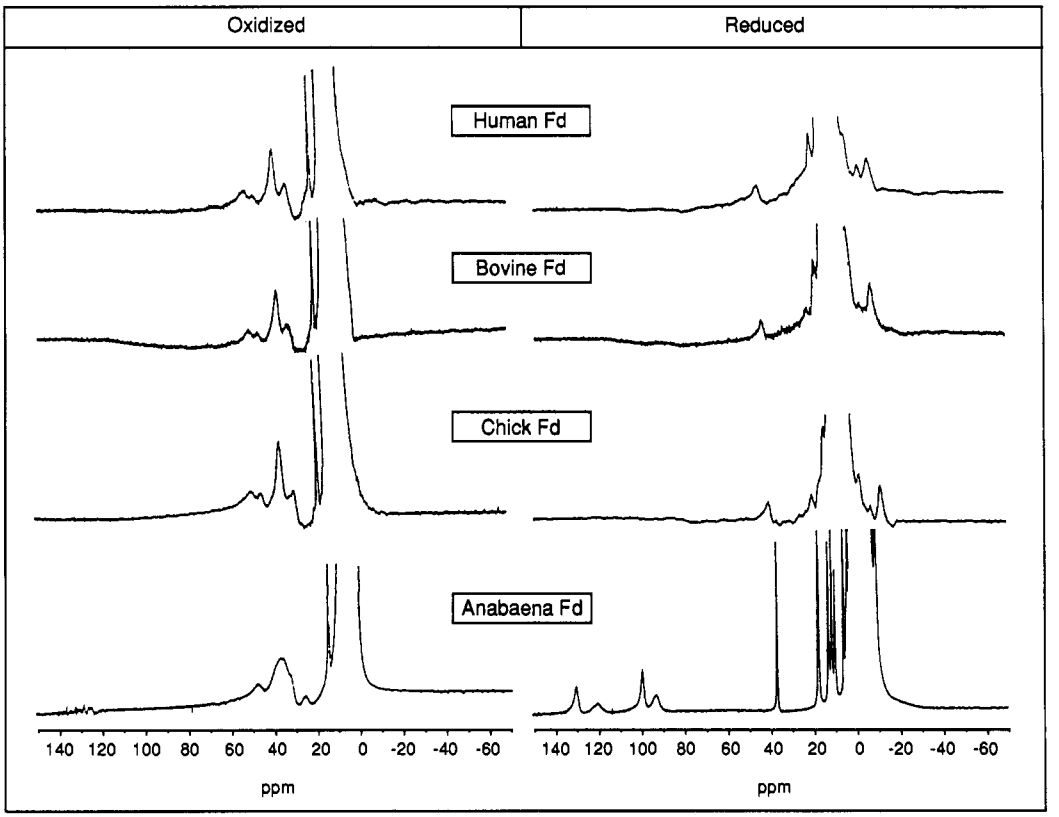

FIGURE 3: Comparison of 600-MHz ^{1}H NMR spectra of [2Fe–2S] ferredoxins in their oxidized and reduced states. At the scale shown, only the hyperfine-shifted resonances are resolved. All spectra were obtained at 300 K and at pH* 8.6. Data on *Anabaena* ferredoxin are from Skjeldal et al. (1990a).

of the reduced sample was measured after the NMR data were collected.

NMR Spectroscopy. ^{1}H NMR spectra were recorded with Bruker AM 500 and Bruker AM 600 spectrometers. Chemical shifts were measured relative to 3-(trimethylsilyl)propionic acid (TSP), and temperature was controlled to ±1 °C. The instrument spectral width was set to 300 ppm, and the data were digitized over 2–3 ms; no delay was used between the end of the observation pulse and the beginning of data acquisition. For measurements of spin–lattice relaxation times (T_1), the inversion–recovery pulse sequence (delay–180–t–90–acquisition) was used.

Results

^{1}H NMR spectra of human, bovine, and chick ferredoxins in the oxidized and reduced states are presented in Figure 3 together with comparable spectra of *Anabena* ferredoxin. The hyperfine peaks were better resolved at 600 MHz than at lower fields as was found in earlier studies on *Anabena* ferredoxin (Skjeldal et al., 1990a). The spectra of each of the vertebrate ferredoxins are very similar to one another in both redox states, indicating conservation of structural features among these vertebrate species. Comparison of the spectra of the oxidized vertebrate proteins with oxidized *Anabena* ferredoxin shows that all exhibit a similar general pattern of downfield hyperfine peaks. The positions of the peaks of the reduced vertebrate ferredoxins, however, are very different from that of reduced *Anabena* ferredoxin, with hyperfine signals occurring in the upfield as well as downfield regions.

The hyperfine signals of oxidized human ferredoxin are shown in more detail and at two temperatures in Figure 4 (top panel). The signal at +13 ppm corresponds to a single proton intensity and has a T_1 of 2.9 ms and a line width of 500 Hz at 298 K and 500 MHz. The broad peak between +23 and +34 ppm has a line width of approximately 13 kHz and appears to arise from overlap of seven to eight proton resonances. Treatment of this broad signal as a single resonance yields a T_1 of approximately 430 μs at 298 K and 500 MHz. All signals exhibit anti-Curie behavior, shifting downfield, away from the diamagnetic region, as the temperature is increased from 280 to 310 K. The pattern of peaks, the T_1 values, and the temperature dependence are similar to those reported for the hyperfine peaks of oxidized plant-type ferredoxins [see Skjeldal et al. (1990a) and references cited therein].

The hyperfine signals of reduced human ferredoxin are shown in more detail and at two temperatures in the bottom panel of Figure 4. A total of five peaks were detected with line widths of 1–2 kHz; all but the peak at highest field, which appears to have an intensity of two protons, appear to correspond to single protons. Hyperfine signals from four protons occur upfield of the diamagnetic region, near −6, −12, and −18 (two-proton intensity) ppm at 280 K, and hyperfine signals from two protons occur downfield, near +13 and +40 ppm at 280 K. No signals could be detected in the +50 to +200 ppm region. This pattern is different from that of the plant-type ferredoxins, as exemplified by the *Anabaena* protein (Figure 3), which exhibit a characteristic pattern of downfield-shifted resonances between +10 and +200 ppm (Dugad et al., 1990; Skjeldal et al., 1990a). In addition, all of the hyperfine signals of human ferredoxin (as well as those of the bovine and chick proteins, data not shown) shift toward the diamagnetic region with increasing temperature. This Curie-type behavior for all of the hyperfine signals is different from that observed for the plant-type ferredoxins in which half of the hyperfine peaks assigned to cysteines exhibit Curie behavior and half exhibit anti-Curie behavior (Dugad et al., 1990; Skjeldal et al., 1990a, 1991).

The 600-MHz ^{1}H NMR spectrum of a sample of human

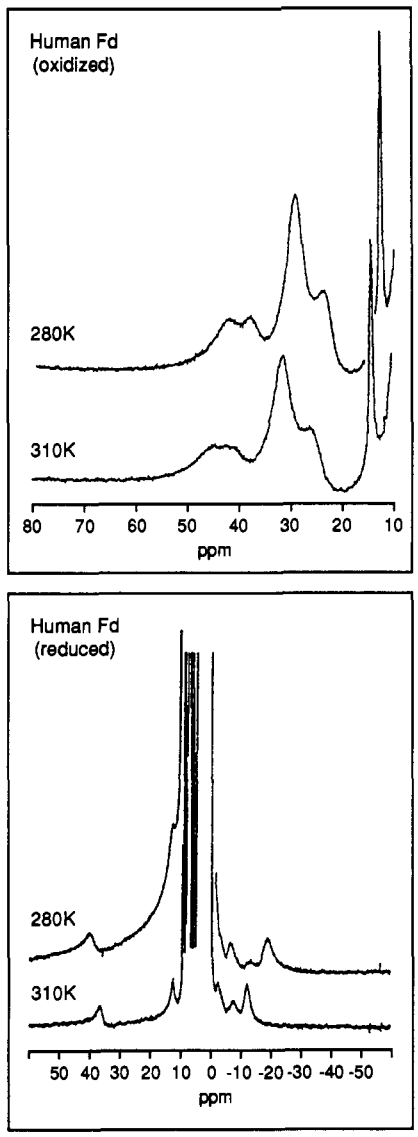

FIGURE 4: Temperature dependence of the hyperfine ^1H resonances (600 MHz) of human ferredoxin in its oxidized (upper panel) and reduced (lower panel) states.

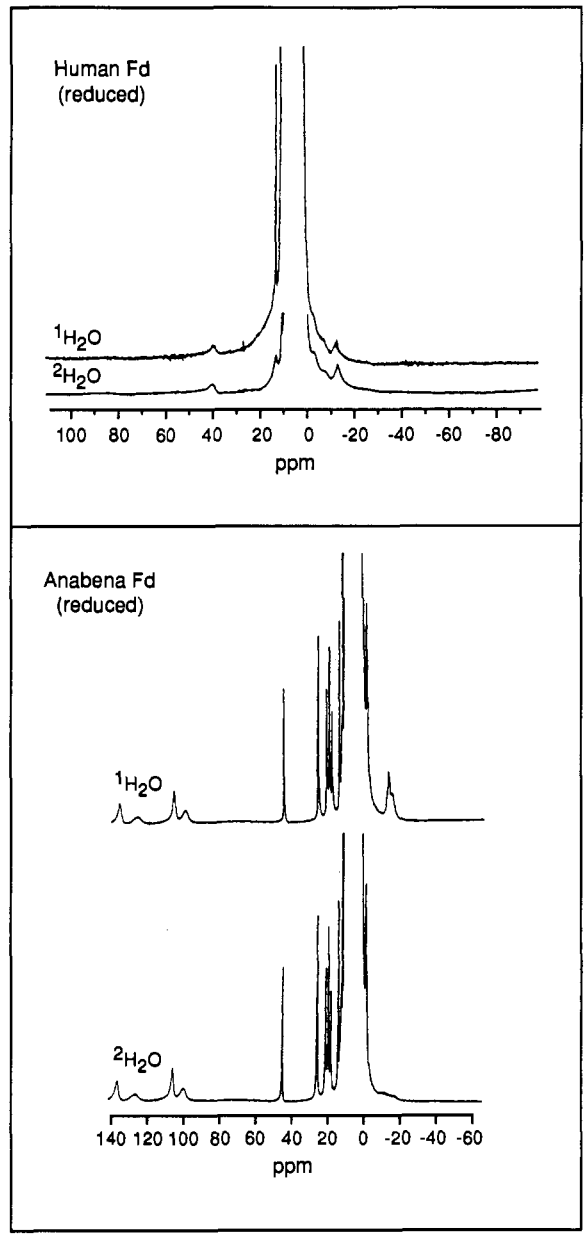

FIGURE 5: Exchange-labile hyperfine resonances in reduced ferredoxins. ^1H spectra at 600 MHz are compared in ^1H_2O and in ^2H_2O for reduced human ferredoxin (upper panel) and reduced *Anabena* ferredoxin (lower panel). Data on *Anabaena* ferredoxin are from Skjeldal et al. (1990a).

ferredoxin that was half-reduced with dithionite showed a superposition of the hyperfine lines from both the oxidized and reduced states (data not shown). This indicates that the electron self-exchange rate must be slow on the NMR time scale, i.e., that the lifetime of each redox state must be greater than 10 μs. The lifetimes must actually be considerably longer, >1 ms, because no saturation transfer was observed upon irradiation of any of the hyperfine peaks of the mixed redox state sample; if the self-exchange rate were faster than $1/T_1$ (T_1 values observed were in the range of 0.4–3 ms), saturation transfer would be expected. In this respect, the vertebrate ferredoxins resemble the plant-type ferredoxins, which also exhibit slow electron self-exchange in the absence of a catalyst such as methyl viologen [see Skjeldal et al. (1990a) and references cited therein].

The exchangeability of the protons giving rise to the hyperfine signals was also investigated to provide insight into their origin. For the *oxidized* form of the ferredoxins, the hyperfine resonances do not appear to arise from exchangeable protons: heating samples of human ferredoxin to 320 K for 20 min in ^2H_2O to remove slowly exchanging amide protons did not affect the signals (data not shown). This finding, which is similar to results with oxidized plant-type ferredoxins, is consistent with the signals observed in oxidized ferredoxin arising from carbon-bound hydrogens rather than amide hydrogens. The *reduced* ferredoxins, on the other hand, display exchange-labile as well as nonexchangeable hyperfine resonances. Figure 5 (top) shows spectra of reduced human ferredoxin obtained in ^1H_2O and ^2H_2O. The downfield signal near +40 ppm and the upfield signals from −2 to −18 ppm are unaffected, but the downfield peak near +13 ppm has lost significant intensity in the spectrum recorded in ^2H_2O. This readily exchangeable proton resonance may originate from amide hydrogen(s) in close proximity to the paramagnetic metal center. Comparison spectra of *Anabena* ferredoxin obtained in ^1H_2O and ^2H_2O (Figure 5, bottom) show that exchangeable protons are also observed for this protein; these, however, occur as upfield peaks at about −18 and −19 ppm; similar exchange-labile upfield peaks have been reported in spectra of two other plant-type ferredoxins and have tentatively been assigned to amide hydrogens of residues adjacent to the

cysteines involved in iron coordination (Dugad et al, 1990).

Discussion

All but one of the hyperfine ^1H resonances detected thus far in vertebrate ferredoxins appear to arise from nonexchangeable hydrogens. None of the signals from the oxidized vertebrate ferredoxins was affected by heating in ^2H_2O, and only one signal (at +13 ppm) was lost when reduced ferredoxins were treated similarly. These results suggest that all the nonexchangeable signals arise from carbon-bound hydrogens. The crystal structures of plant-type ferredoxins (Tsukihara et al., 1981, 1990; Rypniewski et al., 1991) indicate that the amide hydrogens of several residues close to the iron–sulfur center form H-bonds to the sulfide atoms or cysteine S atoms. The proton giving rise to the exchangeable, +13 ppm resonance in the vertebrate ferredoxins may be from an amide involved in this type of H-bond. Mino et al. (1987), in studying resonance Raman spectra of bovine ferredoxin, found that stretching vibrations of the [2Fe–2S] center are sensitive to ^2H substitution, suggesting that the vertebrate ferredoxins are likely to be extensively hydrogen-bonded similar to the plant-type ferredoxins. The most likely source of the nonexchangeable signals would seem to be the $^1H^\beta$ nuclei of the cysteine residues directly ligated to the iron–sulfur center (cysteines 46, 52, 55, and 92), although hydrogens on other residues near the [2Fe–2S] center cannot be ruled out. Cysteine hydrogens have been shown to be the source of the hyperfine signals in [3Fe–3S] and [4Fe–4S] centers in bacterial ferredoxins by incorporation of deuterium-labeled cysteine (Packer et al., 1977; Cheng et al., 1990). Recent assignments resulting from NOE studies of plant-type ferredoxins (Dugad et al., 1990; Skjeldal et al., 1991) have confirmed theoretical calculations that predicted large hyperfine shifts for the eight $^1H^\beta$ nuclei of the cysteines ligated to the [2Fe–2S] cluster (Dunham et al., 1971; Banci et al., 1989).

The hyperfine resonances of the oxidized vertebrate ferredoxins resemble those of the plant-type [2Fe–2S] ferredoxins. The chemical shifts are similar, +13 ppm and +20 to +40 ppm for both ferredoxins, and all show the same anti-Curie temperature dependence. It appears likely that the signals in the +20 to +40 ppm region correspond to the eight $^1H^\beta$ nuclei of the cysteines ligated to the iron–sulfur cluster, but it is not possible to determine the exact number because of overlap of the resonances; additional signals may also remain unobserved beneath the diamagnetic envelope. The aggregate T_1 value for the overlapping resonances, ~430 μs, is also similar to that found in an oxidized plant-type ferredoxin (Skjeldal et al., 1990a).

In contrast to the hyperfine signals from the oxidized ferredoxins, those from the two classes of reduced ferredoxins are strikingly different. Nonexchangeable hyperfine signals are observed both upfield and downfield of the diamagnetic region for the vertebrate ferredoxins; by contrast, plant-type ferredoxins exhibit a characteristic pattern of hyperfine signals downfield of the diamagnetic region. Moreover, the hyperfine signals of vertebrate ferredoxins all show Curie behavior, whereas half of the plant ferredoxin signals exhibit Curie behavior and half exhibit anti-Curie behavior.

Theoretical models for reduced plant-type ferredoxins (Dunham et al., 1971; Banci et al., 1989) predict that resonances whose contact-shifts arise from the Fe(III) center will exhibit Curie behavior whereas those whose contact-shifts arise from the Fe(II) center will display anti-Curie behavior. This model has been extremely successful in explaining the hyperfine signals of reduced plant-type ferredoxins, where the ligands to the two different iron atoms can be differentiated both by their chemical shifts and by their temperature dependence (Dugad et al., 1990). The theoretical treatment by Dunham et al. (1971) for reduced vertebrate ferredoxins (adrenodoxin) predicts that signals from the $^1H^\beta$ nuclei of cysteines ligated to Fe(II) will occur around −40 ppm (with Curie-type temperature dependence) and that those of cysteines ligated to Fe(III) will occur near +110 ppm (also with Curie-type temperature dependence).

The observed hyperfine peaks from the reduced vertebrate ferredoxins fit the theoretical predictions insofar as their presence both upfield and downfield of the diamagnetic region and in that all show Curie-type temperature dependence. Since the four hyperfine signals detected upfield have similar chemical shifts, they appear to arise from the nuclei of the cysteines ligated to Fe(II). They are not shifted upfield as far as prediced (Dunham et al., 1971), and thus the $^1H^\alpha$ resonances of the cysteines ligated to Fe(II) are probably buried under the diamagnetic envelope. The two signals shifted downfield correspond most likely to the $^1H^\alpha$ nuclei of the cysteines ligated to Fe(III). If this is correct, the four $^1H^\beta$ signals from these two cysteines should appear further downfield. Since we were unable to find additional resonances in the +50 to +200 ppm region sampled, the $^1H^\beta$ resonances either are broadened beyond detection or occur still further downfield of this region. The above resonance classifications can be tested by incorporating selectively ^2H-labeled cysteine (Cheng et al., 1990) into one of the vertebrate ferredoxins. With such assignments in hand, the magnetic susceptibility and hyperfine coupling parameters used in the chemical shift calculations (Dunham et al., 1971) can then be refined.

It should be possible to elucidate the molecular origin of the different magnetic and redox properties of the two classes of [2Fe–2S] ferredoxin. One striking difference between the two sequence families occurs at position 56 (in the vertebrate ferredoxin numbering system shown in Figure 1). This residue, which is adjacent to one of the cysteine ligands to the cluster, is histidine in all sequenced vertebrate ferredoxins. In plant-type ferredoxins, the residue at the homologous position is alanine, leucine, threonine, or asparagine (and never histidine or another positively charged residue) (Matsubara & Hase, 1983). However, mutation of residue 56 in human ferredoxin from histidine to glutamine (which is always neutral) or to arginine (which is always positively charged) had no effect on the pattern of hyperfine peaks (V. M. Coghlan, L. E. Vickery, L. Skjeldal, and J. L. Markley, unpublished results). These results appear to rule out His^{56} as contributing to the difference between vertebrate and plant-type ferredoxins.

A potentially interesting site is residue 47 (in the vertebrate ferredoxin numbering system shown in Figure 1). In the vertebrate ferredoxins, residue 47 is conserved as a glutamate. In the larger number of available plant-type ferredoxin sequences (Matsubara & Hase, 1983), the homologous residue (number 42 in the *Anabaena* ferredoxin numbering system) is arginine, with the sole exception of two ferredoxins from species of *Equiseum* (horsetail) in which residue 42 is glutamine. Mutagenesis studies of this residue will be of interest in view of recent evidence from *Anabaena* 7120 vegetative ferredoxin that Arg^{42} is hydrogen-bonded to a sulfide of the iron–sulfur cluster in the oxidized protein (Rypniewski et al., 1991) and that the Arg^{42} $^1H^\alpha$ experiences a hyperfine shift of about 8 ppm in the reduced protein (Skjeldal et al., 1991).

Registry No. Cys, 52-90-4.

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