Optical, EPR, and ^{1}H NMR Spectroscopy of Serine-Ligated [2Fe–2S] Ferredoxins Produced by Site-Directed Mutagenesis of Cysteine Residues in Recombinant *Anabaena* 7120 Vegetative Ferredoxin†

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ABSTRACT: *Anabaena* 7120 vegetative ferredoxin is a plant-type [2Fe–2S] ferredoxin that contains only four cysteine residues. The four cysteines (Cys^{41}, Cys^{46}, Cys^{49}, and Cys^{79}), which ligate the iron–sulfur cluster, were mutated individually to serine. The wild-type and mutant apoprotein genes were overexpressed in *Escherichia coli*, and the iron–sulfur cluster was assembled *in vitro* by adding iron and sulfide. UV–vis, EPR, and ^{1}H NMR spectra were recorded on the wild-type ferredoxin and mutants. The optical spectra of all mutant proteins, in the oxidized state, differed from that of wild-type ferredoxin. Three of the mutant proteins ($Cys^{46}Ser$, $Cys^{49}Ser$, and $Cys^{79}Ser$) exhibited a rhombic EPR spectrum in the reduced state, but one ($Cys^{41}Ser$) showed a near-axial EPR spectrum. The ^{1}H NMR spectra of each of the four oxidized mutants contained a group of broad, hyperfine-shifted peaks between 20 and 30 ppm with anti-Curie temperature dependence. The pattern of these peaks was different for each mutant, and all were distinct from that of the wild-type ferredoxin. Because of problems with protein stability, it was possible to obtain NMR spectra of only two of the mutants when reduced. The downfield hyperfine ^{1}H NMR spectrum of the reduced $Cys^{46}Ser$ mutant resembled that of wild-type ferredoxin, but that of the $Cys^{49}Ser$ mutant did not. The hyperfine-shifted resonances of the ^{1}H NMR spectrum of the reduced $Cys^{46}Ser$ mutant were assigned on the basis of results from temperature dependence studies, measurements of nuclear Overhauser effect, and ^{1}H NMR spectra of the mutant labeled with [β-^{2}H]cysteine. Four hyperfine-shifted peaks of reduced $Cys^{49}Ser$ at 298 K were observed at 173, 120, 32, and 18 ppm. These peaks exhibited Curie-type temperature dependence and were tentatively assigned to protons from residues coordinated to Fe(III). The reduced $Cys^{49}Ser$ mutant showed an additional ^{1}H NMR peak at −15 ppm (at 298 K) with Curie-type temperature dependence whose origin is unknown at present. [2Fe–2S] clusters can be placed into three different classifications according to their EPR lines shapes, NMR spectra, and reduction potentials: plant type, vertebrate type, and Rieske type. The EPR and NMR results obtained here reveal that mutant $Cys^{46}Ser$ has a "plant-type" cluster but that mutant $Cys^{49}Ser$ has a "vertebrate-type" cluster. Cysteine to serine mutations have been employed in the past to probe whether particular cysteine residues participate as iron–sulfur ligands. The present results, which show that serine can substitute for cysteine as a ligand to an iron–sulfur cluster, indicate that results of such mutations should be interpreted with caution. Nature uses cysteine exclusively in preference to serine as cluster ligands in [2Fe–2S] ferredoxins apparently on account of the higher stability.

Ferredoxins belong to the class of non-heme iron proteins that also contain inorganic sulfur. To date, three types of iron–sulfur clusters in ferredoxins have been identified by X-ray crystallography; they are [2Fe–2S], [3Fe–4S], and [4Fe–4S]. In all types of ferredoxins, ligation of the iron–sulfur cluster to the polypeptide is provided solely by sulfur atoms from the thiolate side chains of cysteines. Mixed ligation by sulfur and nitrogen or oxygen has been reported in other classes of proteins that contain iron–sulfur clusters. For example, evidence from spectroscopic studies of Rieske-type [2Fe–2S] proteins has indicated that the iron–sulfur cluster is coordinated by two sulfurs from two cysteines and two imidazole nitrogen atoms from two histidines (Davidson et al., 1992; Gurbiel et al., 1989, 1991; Britt et al., 1991). In aconitase, the additional Fe atom in the [4Fe–4S] cluster, which accompanies conversion of its [3Fe–4S] cluster to a [4Fe–4S] cluster, is coordinated by one carboxyl oxygen, one hydroxyl oxygen, and one water molecule (Lauble et al., 1992). Recently, Werth et al. (1992) reported EPR[1] and redox studies of the $Cys^{65}Asp$ mutant of *Escherichia coli* fumarate reductase. Results from this work, coupled with the existence of an aspartic acid residue in the equivalent position of *E. coli*

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[1] Abbreviations used: COSY, homonuclear correlated spectroscopy; $Cys^{41}Ser$, cysteine at position 41 replaced with serine (other mutations are abbreviated in similar fashion); DTT, dithiothreitol; EPR, electron paramagnetic resonance; FNR, ferredoxin-NADP$^+$ reductase; g_e, the electronic *g* factor; IPTG, isopropyl thiogalactoside; LB medium, Luria–Bertani medium; NMR, nuclear magnetic resonance; NOESY, homonuclear nuclear Overhauser effect spectroscopy; pH*, pH value of a ^2H_2O-containing solution measured with a combination glass electrode calibrated with normal H_2O buffers and reported without correction; TMSP, sodium 3-(trimethylsilyl)propionate-*2,2,3,3-d*$_4$.

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succinate dehydrogenase, suggest that oxygen can be a ligand to a [2Fe–2S] cluster. By using site-directed mutagenesis, Werth et al. (1990) found that one of the coordinating cysteines of *E. coli* fumarate reductase can be replaced by serine without loss of activity. Many [2Fe–2S] cluster analogues with non-cysteine ligands have been synthesized and characterized (Mascharak et al., 1981; Beardwood et al., 1982).

Although site-directed mutagenesis has been used to investigate the roles of iron–sulfur ligands in other types of iron–sulfur cluster containing proteins [*E. coli* fumarate reductase (Werth et al., 1990), *E. coli* succinate dehydrogenase (Werth et al., 1992), *E. coli* dimethyl sulfoxide (DMSO) reductase (Rothery & Weiner, 1991), *E. coli* fumarate reductase (Manodori et al., 1992), *Rhodobactor capsulatus* Rieske protein (Davidson et al., 1992)], it is not yet known whether such replacements are possible in a ferredoxin. Moreover, the spectroscopic properties of a biological iron–sulfur cluster with mixed ligation have not yet been characterized. To study this problem, we have selectively mutated each of the four, active-site cysteines in *Anabaena* 7120 vegetative ferredoxin into serine, tyrosine, histidine, and alanine by using site-directed mutagenesis. These mutants have been studied systematically by several biophysical methods (Holden et al., 1993): optical, EPR, and NMR spectroscopy; X-ray crystallography; electron transfer rate analysis; and reduction potential measurements. In this paper, we present results of the Cys → Ser substitutions; the other mutants will be described elsewhere.

Anabaena 7120 vegetative ferredoxin is a plant-type ferredoxin with a molecular weight of 11 000. This protein contains one [2Fe–2S] cluster, which is ligated to the polypeptide through the S^{γ} atoms of its four cysteine residues (Cys^{41}, Cys^{46}, Cys^{49}, and Cys^{79}). As with other plant-type ferredoxins, it undergoes a one-electron redox reaction; its reduction potential is ca. –440 mV at pH 7 (Böhme & Schrautemeier, 1987; Hurley et al., 1993). The central physiological function of this ferredoxin is to serve as a terminal electron acceptor from photosystem I and subsequently to donate electrons in other redox reactions, such as $NADP^+$ photoreduction, cyclic photophosphorylation, nitrite and nitrate reduction, sulfite reduction, and (by the way of thioredoxin) in the light modulation of key enzymes involved in carbohydrate metabolism. Upon reduction, one of the two ferric ions is reduced to ferrous. NMR experiments with the reduced ferredoxin have shown that Cys^{41} and Cys^{46} are coordinated to Fe(II) and that Cys^{49} and Cys^{79} are coordinated to the Fe(III) in the reduced form (Skjeldal et al., 1991a).

In this study of *Anabaena* 7120 vegetative ferredoxin, we show that *in vitro* self-assembly of the iron–sulfur cluster occurs upon addition of iron and inorganic sulfur to the wild-type protein and also to mutants in which any one of the four active-site cysteines has been mutated to serine. (Since wild-type *Anabaena* 7120 vegetative ferredoxin contains only four cysteines, a cysteine cluster ligand cannot be recruited from elsewhere in the protein sequence.) We report the spectral properties of the mutant proteins as determined by optical, EPR, and NMR spectroscopy. The results from EPR and NMR studies have shown that reduced Cys^{46}Ser has a "plant-type" [2Fe–2S] cluster but that reduced Cys^{49}Ser has a "vertebrate-type" [2Fe–2S] cluster. The hyperfine-shifted resonances in the NMR spectra of reduced Cys^{46}Ser and Cys^{49}-Ser have been tentatively assigned. Details of the X-ray structure of oxidized Cys^{49}Ser and the results of electron transfer and reduction potential studies of these four Cys → Ser mutants will be reported elsewhere.

MATERIALS AND METHODS

Enzymes, Chemicals, and E. coli Strains. Restriction enzymes were purchased from Promega (Madison, WI) and New England Biolabs (Beverly, MA). *Taq* DNA polymerase, T4 polynucleotide kinase, and T4 DNA ligase were from Promega (Madison, WI). All these enzymes were used according to the specifications of the manufacturers. *E. coli* strain JM103 was used for routine site-directed mutagenesis, *E. coli* strain HMS174 was used for plasmid storage, and *E. coli* strain BL21 (DE3)/pLysS was used as the host for protein expression. DTT was purchased from Sigma (San Diego, CA) or Boehringer-Mannheim Biochemicals (Indianapolis, IN). D,L-cystine-*3,3,3′,3′-d*$_4$ (98% D) was purchased from Cambridge Isotope Laboratories (Woburn, MA).

Plasmid Constructions and Site-Directed Mutagenesis. The DNA plasmid (pETa/F) encoding the entire gene region for *Anabaena* 7120 vegetative ferredoxin and used for protein expression was constructed as described elsewhere (Cheng et al., 1994). The pET9a vector used in its construction was purchased from Novagen (Madison, WI).

Mutagenesis was performed according to the published method of Kunkel et al. (1987). Oligonucleotides for mutagenesis and sequencing were synthesized on a 0.2-mmol scale in the DNA synthesis facility in the Department of Biochemistry, University of Wisconsin—Madison. The oligonucleotides used for mutagenesis differed from the wild-type gene sequence by single nucleotide substitutions: each oligonucleotide served to mutate one of the cysteines (Cys^{41}, Cys^{46}, Cys^{49}, or Cys^{79}) to serine. The synthetic oligonucleotides were purified by using QIAGEN-tip columns (QIAGEN, Chatsworth, CA). Mutagenesis was performed by using single-stranded M13 (mp18/pet9a/F) DNA (M13 mp18 with the *Eco*RI–*Sal*I fragment of the pET9a/F expression vector inserted) as a template. *E. coli* strain JM103 was used for the mutagenesis. The mutants were identified by DNA sequencing according to the Sequenase protocol (United States Biochemical Corp., Cleveland, OH) by using the standard dideoxynucleotide chain termination method (Sanger et al., 1977). After mutagenesis, the mutated *Eco*RI–*Sal*I DNA fragment containing the *petF* region was cloned back into pET9a to restore the complete expression vector. The plasmid DNA purified from *E. coli* strain HSM174 was transformed into the expression host cell, BL21(DE3)/pLysS.

Protein Production, Cluster Assembly, and Purification. LB medium (5 mL) supplemented with kanamycin (100 μg/mL) and chloramphenicol (34 μg/mL) was inoculated with a single colony picked from an LB plate. The culture was grown overnight at 37 °C. This culture was used to inoculate a 1-L LB–kanamycin–chloramphenicol culture. The culture was grown continually at 37 °C until the OD_{600} was 1.2; then 100 mg of IPTG/L of culture was added to induce ferredoxin production. After additional incubation at 37 °C for 4 h, the cells were harvested by centrifugation. The cell pellets were resuspended in 20 mL of 50 mM potassium phosphate buffer at pH 8 and then lysed by a freeze–thawing cycle. Triton X-100 was added to a final concentration of 0.1%. After sonication, ultrapure urea (Sigma) was added to the lysate to achieve 8 M concentration. The ferredoxin was then reconstituted by following the procedure described by Coghlan and Vickery (1991) for human placental ferredoxin. The reaction mixture was degassed under vacuum, and DTT was added to a final concentration of 100 mM. Under bubbling argon gas, $FeCl_3$, $Fe(NH_4)_2(SO_4)_2$, and Na_2S were then added gradually to a final concentration of 1 mM each. The mixture was incubated for an additional 15–20 min with stirring and

flushing with argon gas; next it was immediately diluted eightfold with degassed 50 mM potassium phosphate buffer, pH 7.4. The assembled ferredoxin was purified by ion-exchange chromatography followed by gel filtration according to the standard method (Ho et al., 1979). The [β-^{2}H]cysteine-labeled Cys^{46}Ser *Anabaena* 7120 vegetative ferredoxin was prepared as described by Cheng et al. (1994).

Concentrations of mutant proteins were calculated by using an extinction coefficient of 15 700 M^{-1} cm^{-1} at 278 (280) nm (Pueyo & Gomez-Moreno, 1991); it was assumed that the 278-nm extinction coefficients of the mutant ferredoxins were similar to that of wild-type ferredoxin since the mutations did not involve aromatic residues.

Spectral Instrumentation. Optical absorption spectra were obtained with a Hewlett-Packard 8452 A diode array spectrophotometer. Low-temperature EPR spectra were recorded at X-band with a Varian E Line spectrometer equipped with a Varian E102 microwave bridge. Precise measurements of the microwave frequency and the magnetic field strength were obtained by using a Hewlett-Packard 5245A frequency converter/5245L electronic counter and a Varian gaussmeter, respectively. The microwave power was calibrated by using a Hewlett-Packard 432A power meter. Sample temperatures were around 10 K and were regulated with an Oxford Instruments ESR-900 continuous-flow cryostat. ^{1}H NMR spectra were recorded on a Bruker AM-400 wide-bore NMR spectrometer. A 5-mm ^{1}H probe was used. A simple delay–90°–acquisition pulse sequence was employed for collecting 1D data. The conventional 180°–τ(delay)–90° pulse sequence (Vold et al., 1968), with the 180° pulse replaced by a composite 90°–180°–90° pulse (Freeman et al., 1980), was used for T_1 relaxation time measurements. The spin–lattice (T_1) relaxation times were calculated by fitting the intensity values to the equation $M_z = M_0[1 - 2\exp(-\tau/T_1)]$. ^{1}H chemical shifts are referenced to internal TMSP (sodium 3-(trimethylsilyl)propionate-*2,2,3,3-d*$_4$, 98% D, Cambridge Isotope Laboratories, Woburn, MA).

All EPR and NMR samples contained 0.4 mL of 3–5 mM ferredoxin in 100 mM sodium pyrophosphate buffer with 100 mM sodium chloride; the pH was 8.6. Proteins were reduced by introducing 2 mg of solid sodium dithionite into the sample tube under argon. For the NMR experiments, a deuterated buffer was used, which was prepared by exchanging the water with ^2H_2O by lyophilization. The final pH* value of the deuterated buffer was 8.5. The pH* values were measured with an Ingold (Wilmington, MA) combination glass electrode and are reported as uncorrected meter readings.

RESULTS

Self-Assembly of the Iron–Sulfur Cluster. The four Cys → Ser mutant proteins were produced in *E. coli* as apoproteins as described for wild-type ferredoxin (Cheng et al., 1994). The iron–sulfur cluster in each mutant protein was assembled by adding iron and inorganic sulfur exactly according to the procedure used for reconstitution of wild-type ferredoxin (Cheng et al., 1994). Mutant proteins were purified by the standard procedures used for wild-type ferredoxin. Mutant proteins were eluted from an anion exchange column (e.g., DE-53 cellulose (Whatman), DEAE Sephacel (Pharmacia), or Q-Sepharose (Phamacia)) with 0.4 M NaCl. The same time period was required to elute the mutant proteins from a gel filtration column (G-75, Phamacia) as for wild-type ferredoxin. The mutants behaved identically to wild-type ferredoxin on SDS/urea polyacrylamide gel (result not shown). The purified Cys^{49}Ser mutant showed the same red-brown color in the oxidized state as the wild-type ferredoxin. After reduction by solid sodium dithionite, the color changed to green-brown. The purified Cys^{41}Ser, Cys^{46}Ser, and Cys^{79}Ser mutants were green-brown in the oxidized state, and this color changed following reduction of these mutants by sodium dithionite. The color of Cys^{46}Ser in the reduced state was red-brown. Electron transfer studies showed that the oxidized Cys^{41}Ser, Cys^{46}Ser, and Cys^{49}Ser mutants could be reduced by deazariboflavin semiquinone and that *Anabaena* FNR could be reduced by the reduced Cys → Ser mutants just as with wild-type ferredoxin but with different kinetic parameters (J. K. Hurley, G. Tollin, H. Cheng, B. Xia, and J. L. Markley, unpublished results). Crystals of Cys^{49}Ser were obtained that were suitable for high-resolution X-ray crystallographic analysis, and X-ray analysis of this mutant showed that the iron–sulfur cluster ligand provided by the S$^{\gamma}$ of Cys^{49} in wild-type ferredoxin was replaced by the O$^{\gamma}$ of Ser^{49} in the mutant (B. L. Jacobson, H. M. Holden, H. Cheng, B. Xia, and J. L. Markley, unpublished results). The amide/α-proton "fingerprint" regions of the ^{1}H NMR COSY spectra of the oxidized Cys^{46}Ser and Cys^{49}Ser mutants in 90% ^1H_2O/10% ^2H_2O were found to be very similar to that of the wild-type ferredoxin (results not shown). The major cross-peaks in the ^{1}H NMR spectrum of the oxidized Cys^{46}Ser mutant in ^2H_2O superimposed on those of the wild-type ferredoxin (results not shown). On the basis of these results and those from the optical, EPR, and NMR studies presented below, we believe that the iron–sulfur cluster of each Cys → Ser mutation assembled spontaneously *in vitro* upon addition of iron and inorganic sulfur just as did that of the wild-type ferredoxin (Cheng et al., 1994).

Among the assembled Cys → Ser mutants, Cys^{46}Cys was the most stable, as shown by lack of precipitation or color loss. The next most stable mutants were Cys^{49}Ser, Cys^{41}Ser, and then Cys^{79}Ser. Color bleaching, resulting from loss of the iron–sulfur cluster, was observed for Cys^{41}Ser and Cys^{79}Ser following their reduction by sodium dithionite. The half-time for color bleaching of Cys^{79}Ser was much shorter than that of Cys^{41}Ser.

Optical Spectroscopy. Figure 1 shows the absorption spectra of the four Cys → Ser mutants. The wavelengths of maximum absorbance and their ratios are summarized in Table 1. In addition to the absorption around 280 nm, the Cys → Ser mutants showed absorption bands between 300 and 600 nm similar to those of wild-type ferredoxin. These absorption bands arise from dipole-allowed sulfur (or oxygen) Fe charge transfer (Noodleman & Baerends, 1984). The most remarkable change was observed in the optical spectrum of Cys^{79}Ser, which did not exhibit a peak near 400 nm (Figure 1).

EPR Spectroscopy. EPR spectra of sodium dithionite reduced wild-type *Anabaena* 7120 vegetative [2Fe–2S] ferredoxin and the reduced forms of the four Cys → Ser mutants are shown in Figure 2. The calculated g values of the EPR signals are listed in Table 1. Three of the sodium dithionite reduced mutants (Cys^{46}Ser, Cys^{49}Ser, and Cys^{79}Ser) showed a rhombic EPR signal, but one reduced mutant (Cys^{41}Ser) exhibited a near-axial EPR signal (Figure 2). Pronounced differences were observed in the g value anisotropy of the mutants in comparison to those of the wild-type ferredoxin. The g_{av} values of 1.92 (for Cys^{41}Ser) and 1.95 (for others) were lower than that of wild-type ferredoxin (1.96). The Cys → Ser mutants also showed lower principal g values than the wild-type ferredoxin (1.96): 1.91 for Cys^{41}Ser, 1.95 for Cys^{46}Ser, 1.92 for Cys^{49}Ser, and 1.93 for Cys^{79}Ser. The EPR spectra of two of the mutants (Cys^{46}Ser and Cys^{79}Ser) exhibited more

Table 1: Optical and EPR Spectral Properties of Wild-Type *Anabaena* 7120 Vegetative Ferredoxin and Its Cysteine to Serine Mutants

	ferredoxin				
	wild type	C41S	C46S	C49S	C79S
optical absorbance maxima (nm)	466, 422, 332, 282, 278	456, 406, 328, 282, 276	468, 400, 326, 282, 278	458, 404, 348, 328, 282, 278	440, 322, 282, 278
relative ratio of optical absorbance maxima	A_{466}/A_{278}: 0.56 A_{422}/A_{278}: 0.60 A_{332}/A_{278}: 0.78	A_{456}/A_{276}: 0.40 A_{406}/A_{276}: 0.42 A_{328}/A_{276}: 0.74	A_{468}/A_{278}: 0.37 A_{400}/A_{278}: 0.47 A_{326}/A_{278}: 0.77	A_{458}/A_{278}: 0.39 A_{404}/A_{278}: 0.38 A_{328}/A_{278}: 0.64	A_{440}/A_{278}: 0.38 A_{322}/A_{278}: 0.59
EPR spectra (10 K) g values[a]	1.88 (g_1), 1.96 (g_2), 2.05 (g_3)	1.82 (g_1), 1.91 (g_2), 2.02 (g_3)	(1.76), 1.89 (g_1), (1.94), 1.95 (g_2), 2.02 (g_3)	1.90 (g_1), 1.92 (g_2), 2.02 (g_3)	1.89 (g_1), 1.93 (g_2), (1.98), 2.03 (g_3)

[a] For mutants Cys[46]Ser and Cys[79]Ser, which displayed heterogeneity, only the major components (g_1, g_2, and g_3) were used to calculate g_{av}.

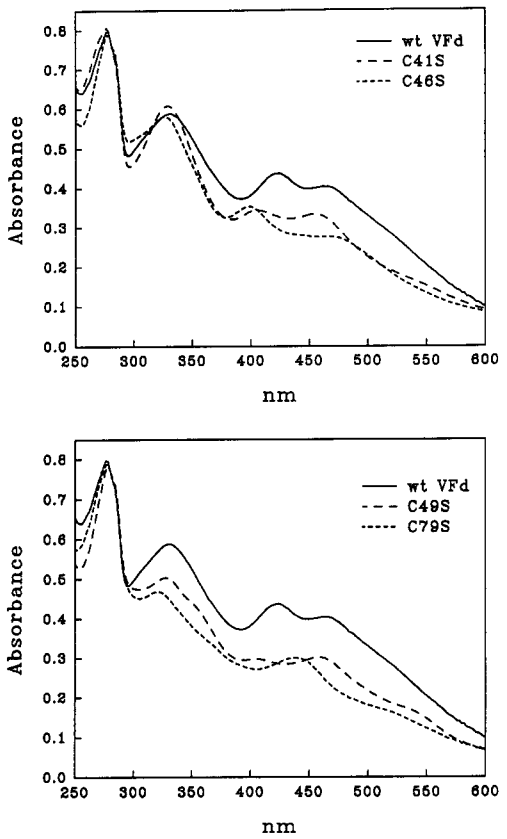

FIGURE 1: Optical spectra of wild-type ferredoxin and Cys → Ser mutant proteins in 50 mM NaCl and 50 mM phosphate buffer, pH 7.4. The concentrations of proteins were as follows: wild type, 4.91 × 10^{-2} mM; Cys[41]Ser, 3.24 × 10^{-2} mM; Cys[46]Ser, 7.20 × 10^{-2} mM; Cys[49]Ser, 3.90 × 10^{-2} mM; Cys[79]Ser, 2.33 × 10^{-2} mM. The spectra of mutants have been normalized to equal the absorption of wild-type ferredoxin at 278 nm to correct approximately for concentration differences.

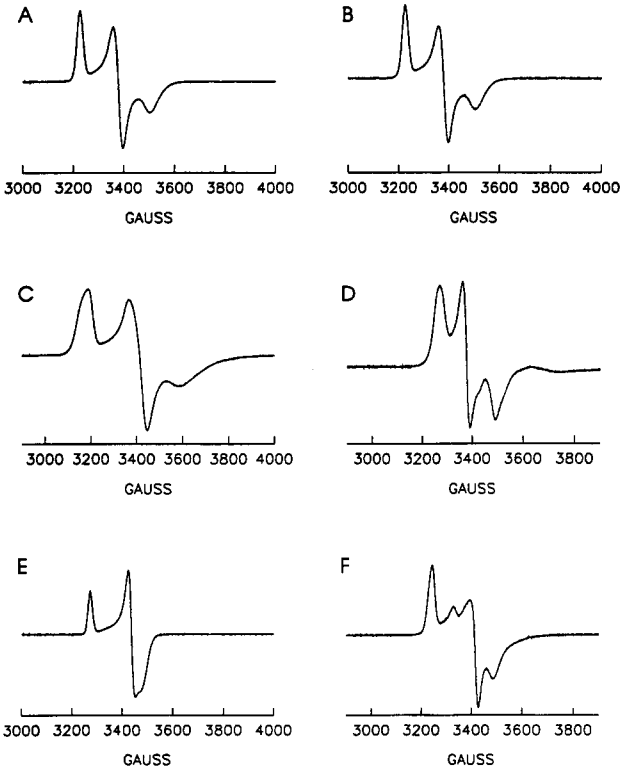

FIGURE 2: EPR spectra in the $g = 2$ region of sodium dithionite reduced, wild-type, and Cys → Ser mutant ferredoxins at 10 K. Samples consisted of 3–5 mM protein in 100 mM sodium pyrophosphate buffer and 100 mM sodium chloride with a pH of 8.6. Proteins were reduced by adding ~2 mg of solid sodium dithionite into the EPR cells under argon. Spectra were recorded at 9.23 GHz with varied receiver gains and modulation amplitudes. (A) Recombinant wild-type *Anabaena* 7120 vegetative ferredoxin. Receiver gain, 1.0 × 10^2; modulation amplitude, 4 G at 1-mW power. (B) Reconstituted, recombinant wild-type *Anabaena* 7120 vegetative ferredoxin. Receiver gain, 1.0 × 10^2; modulation amplitude, 4 G at 1-mW power. (C) Cys[41]Ser *Anabaena* 7120 vegetative ferredoxin. Receiver gain, 2.0 × 10^2; modulation amplitude, 4 G at 1-mW power. (D) Cys[46]Ser *anabaena* 7120 vegetative ferredoxin. Receiver gain, 2.5 × 10^2; modulation amplitude, 4 G at 1-mW. (E) Cys[49]Ser *Anabaena* 7120 vegetative ferredoxin. Receiver gain, 1.0 × 10^2; modulation amplitude, 4 G at 1-mW power. (F) Cys[79]Ser *Anabaena* 7120 vegetative ferredoxin. Receiver gain, 4.0 × 10^2; modulation amplitude, 2.5 G at 1-mW power.

than three turning points in their spectra. The extra features indicate heterogeneity in the samples. The origin of this heterogeneity is not known; however, the Cys[79]Ser mutant, which is the least stable, appears to have a higher fraction of a second spin system. The heterogeneity does not show up in the NMR spectra at room temperature, so it appears to be the result of a structural heterogeneity that develops or is frozen out at low temperature.

Two theoretical models have been used to explain the EPR properties of [2Fe–2S] ferredoxins. The pioneering work of Gibson and co-workers (Gibson et al., 1966) predicted that antiferromagnetic coupling of the ferric iron to the ferrous iron causes two of the g values to be lower than g_e and the third to be higher than g_e. As with all reported EPR spectra of $[2Fe–2S]^+$ complexes, the EPR spectra of Cys → Ser mutants are consistent with this model.

In the Bertrand and Gayda analysis, the three g values of the EPR spectra are plotted as a function of χ, where $\chi = g_2 - g_3$, $g_1 > g_2 > g_3$ (Bertrand & Gayda, 1979, 1980; Bertrand et al., 1985). A near-linear correlation has been obtained for proteins and synthetic compounds with $g_{av} = 1.91$ (Bertrand et al., 1985) or $g_{av} = 1.96$ (Bertrand & Gayda, 1979), and the model was even found to hold for the selenium derivatives of these proteins (Bertrand & Gayda, 1980). The variation in g values for $[2Fe–2S]^+$ structures was explained by geometric variations at the ferrous site.

Figure 3 is a plot of the three effective g values of wild-type and Cys → Ser mutant *Anabaena* 7120 vegetative ferredoxins as a function of χ. Linear relationships were found between

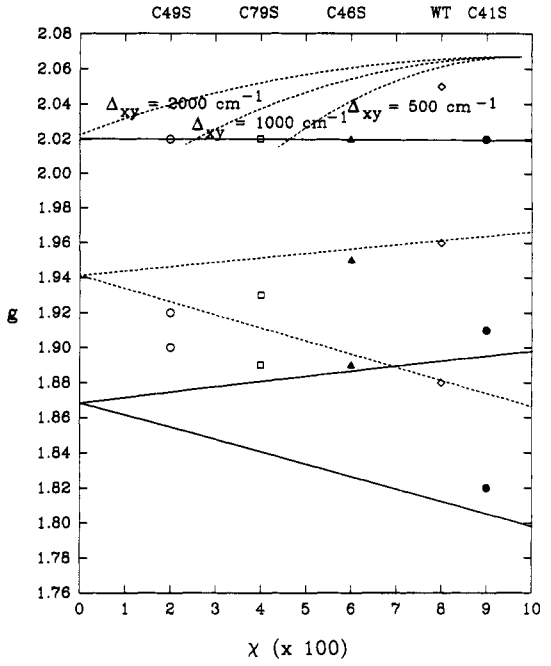

FIGURE 3: Bertrand–Gayda plot: experimental g values of wild-type ferredoxin and Cys → Ser mutant proteins as a function of $\chi = g_2 - g_1$ ($g_1 < g_2 < g_3$): (◇) wild-type *Anabaena* 7120 vegetative ferredoxin; (●) Cys^{41}Ser mutant; (▲) Cys^{46}Ser mutant; (○) Cys^{49}Ser mutant; (□) Cys^{79}Ser mutant. The dashed lines are theoretical curves calculated for the general trend of the variations of g_1, g_2, and g_3 within the $g_{av} \approx 1.96$ class, with $g_{1x} = 2.015$, $g_{16} = 2.034$, $g_{1z} = 2.030$, $\lambda_2 = 80$ cm^{-1}, $\Delta_{xy} = 6000$ cm^{-1}, and $\Delta_{yz} = 4000$ cm^{-1} (Bertrand et al., 1979). The solid lines are theoretical curves calculated for the $g_{av} \approx 1.91$ class, with $g_{1x} = 2.015$, $g_{1y} = 2.034$, $g_{1z} = 2.030$, $\lambda_2 = 80$ cm^{-1}, $\Delta_{xy} = 650$ cm^{-1}, $\Delta_{yz} = 1900$ cm^{-1}, and $\Delta_{xz} = 15000$ cm^{-1} (Bertrand et al., 1985).

g_2 or g_3 and χ for the three mutants with g_{av} of 1.95 (Cys^{46}Ser, Cys^{49}Ser, and Cys^{79}Ser). This linear correlation is different from that of [2Fe–2S] clusters characterized by $g_{av} = 1.96$ or 1.91. NMR results have shown that in the reduced wild-type *Anabaena* 7120 vegetative ferredoxin, Cys^{41} and Cys^{46} are coordinated to the ferrous iron and that Cys^{49} and Cys^{79} are coordinated to the ferric iron (Skjeldal et al., 1991a). The correlation between g values and χ described by the Bertrand and Gayda model only holds for changes that alter the geometry at the ferrous site of the [2Fe–2S]$^+$ cluster, not at the ferric site. Since the four Cys → Ser mutations studied here affect primarily either the Fe(II) site (Cys^{41}Ser and Cys^{46}-Ser) or the Fe(III) site (Cys^{49}Ser and Cys^{79}Ser), a more general theory is needed to describe the experimental results. Note that the values of the three g components for mutants Cys^{41}-Ser ($g_{av} \sim 1.92$) and Cys^{46}Ser ($g_{av} \sim 1.95$), as calculated from the EPR spectra, are very close to the theoretical values for clusters with a character of $g_{av} \sim 1.91$ and $g_{av} \sim 1.96$, respectively (Figure 3). These results suggest that a change in one of the atoms coordinated to the ferrous site from S to O can result in a change of the g_{av} value from ~1.96 to ~1.91. Thus, the Cys^{41}Ser and Cys^{46}Ser mutants represent two separate Bertrand–Gayda classes of [2Fe–2S]$^+$ cluster, one class with $g_{av} = 1.91$ and a second class with $g_{av} = 1.96$. The three g components of mutant Cys^{41}Ser (2.02, 1.91, and 1.82) are very similar to those reported for the Rieske iron–sulfur protein isolated from *Thermus thermophilus* (2.02, 1.90, and 1.80; Fee et al., 1984).

NMR Spectroscopy. Figure 4 shows ^{1}H NMR spectra of the wild-type ferredoxin and the four Cys → Ser mutants recorded in the oxidized state. The theoretical model for oxidized plant-type ferredoxin (Dunham et al., 1971) predicts

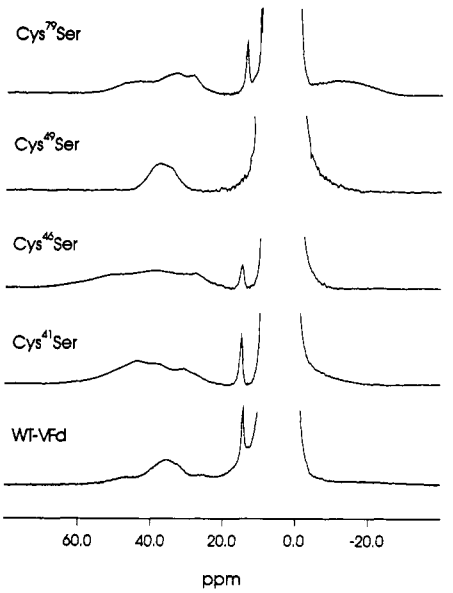

FIGURE 4: 400-MHz ^{1}H NMR spectra of oxidized wild-type ferredoxin and Cys → Ser mutant proteins at 298 K. Samples contained 3–5 mM protein in 100 mM sodium pyrophosphate buffer and 100 mM sodium chloride; the pH* was 8.5.

chemical shifts for the ^{1}H$^\beta$ resonances of cysteine ligands of ca. 15 ppm and chemical shifts for ^{1}H$^\alpha$ resonances of ca. 4 ppm, both with anti-Curie temperature dependence. Groups of broad, hyperfine-shifted peaks, similar to those of the wild-type ferredoxin, were observed between 20 and 60 ppm in the spectra of the four mutants (Figure 4). As expected, these peaks in each mutant exhibited anti-Curie temperature dependence (results not shown). They are assigned tentatively to protons from the amino acid residues ligated directly to the iron–sulfur cluster as in the wild-type ferredoxin (Cheng et al., 1993; Skjeldal et al., 1990, 1991a,b). The peak observed at ~14 ppm in the spectrum of the wild-type ferredoxin apparently is shifted into the diamagnetic envelope in the spectrum of the Cys^{49}Ser mutant (Figure 4). In addition to the hyperfine-shifted resonances in the downfield region, a broad, hyperfine-shifted peak at −15 ppm (upfield of the diamagnetic region) was observed in the spectrum of the Cys^{79}-Ser mutant. The origin of this unusual peak near −15 ppm, which displayed anti-Curie temperature dependence, is unknown at present; selective isotopic labeling will be needed for its assignment.

Although all four Cys → Ser mutants can be reduced by sodium dithionite, as seen from the EPR spectra (Figure 2), the low stabilities of Cys^{41}Ser and Cys^{79}Ser in the reduced state at room temperature prevented us from studying the reduced forms of these two mutants by NMR. The other two mutants were investigated in their reduced states.

The ^{1}H NMR spectrum of sodium dithionite reduced Cys^{46}-Ser (Figure 6, bottom) resembled that of wild-type ferredoxin, but the magnitudes of the hyperfine shifts exhibited by the mutant were larger than those of wild-type ferredoxin (Figure 5). Thje four peaks that showed the largest (positive) hyperfine shifts (A–D) had approximately the same intensity, were broader, and had much shorter T_1 relaxation times than the other peaks. These peaks are similar to peaks A–D in the corresponding spectrum of wild-type ferredoxin in that they erxhibited Curie-type temperature dependence (Figure 7). Of the remaining hyperfine-shifted peaks, peaks H and K had Curie temperature dependence, whereas peaks E–G, I, and J displayed anti-Curie temperature dependence (Figure 7). Selective deuterium labeling was used to identify signals from

Table 2: T_1 Relaxation Times, Chemical Shifts, and Assignments of Hyperfine-Shifted Resonances in ^{1}H NMR Spectra of Sodium Dithionite Reduced Wild-Type *Anabaena* 7120 Vegetative Ferredoxin and Cys^{46}Ser Mutant[a]

wild-type ferredoxin				Cys^{46}Ser mutant			
peak desig	T_1* (ms)	assignment[b]	chem shift (ppm)[c]	peak desig	T_1 (ms)	assignment	chem shift (ppm)[c]
A	nd	Cys^{79}H$^{\beta}$	134.6	A	nd	Cys^{79}(Cys^{49})H$^{\beta}$	179.6
B	nd	Cys^{79}(Cys^{49})H$^{\beta'}$	125.0	B	nd	Cys^{79}(Cys^{49})H$^{\beta'}$	149.5
C	nd	Cys^{49}H$^{\beta}$	104.2	C	nd	Cys^{49}(Cys^{79})H$^{\beta}$	135.5
D	nd	Cys^{49}(Cys^{79})H$^{\beta'}$	98.0	D	nd	Cys^{49}(Cys^{79})H$^{\beta'}$	125.1
E	4.0	Cys^{49}H$^{\alpha}$	43.5	E	6.7	Cys^{41}H$^{\beta}$	87.5
F	6.8	Cys^{41}H$^{\beta 2}$	24.4	F	3.0	Ser^{46}H$^{\beta}$	72.7
G	3.8	Cys^{41}H$^{\beta 3}$	24.4	G	1.6	Ser^{46}H$^{\beta'}$	53.6
H	8.8	Cys^{46}H$^{\beta 2}$	19.7	H	4.9	Cys^{49,79}H$^{\alpha}$	55.6
I	3.5	Cys^{46}H$^{\beta 3}$	16.7	I	2.4	Cys^{41}H$^{\beta'}$	44.8
J	4.0	Cys^{79}H$^{\alpha}$	18.1	J	nd		20.2
K	5.0	Arg^{42}H$^{\alpha}$	12.6	K	nd		19.1

[a] T_1 relaxation times were measured at 286 K; nd indicates that the T_1 relaxation time for a peak was not determined. [b] Assignments for Skjeldal et al. (1991a). [c] Chemical shifts are reported from spectra recorded at 298 K of samples which contained 3–5 mg of protein in 100 mM sodium pyrophosphate buffer and 100 mM sodium chloride; the pH* was 8.5.

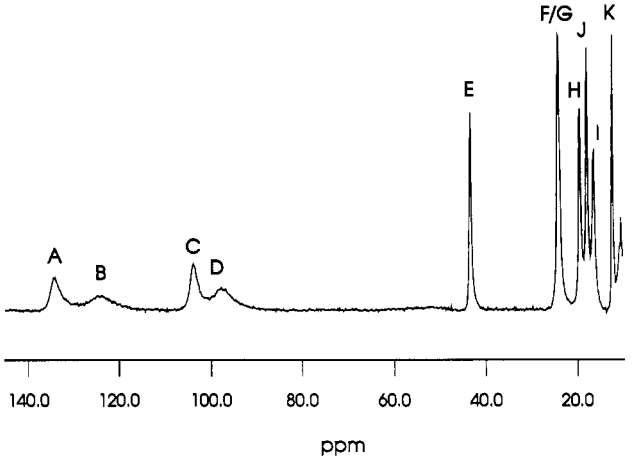

FIGURE 5: Hyperfine-shifted resonances in the downfield region of the 400-MHz ^{1}H NMR spectrum of sodium dithionite reduced wild-type *Anabaena* 7120 vegetative ferredoxin recorded at 298 K. The sample contained ~4 mM protein in 100 mM sodium pyrophosphate buffer and 100 mM sodium chloride; the pH* was 8.5. The sample was reduced by addition of ~2 mg solid sodium dithionite under argon.

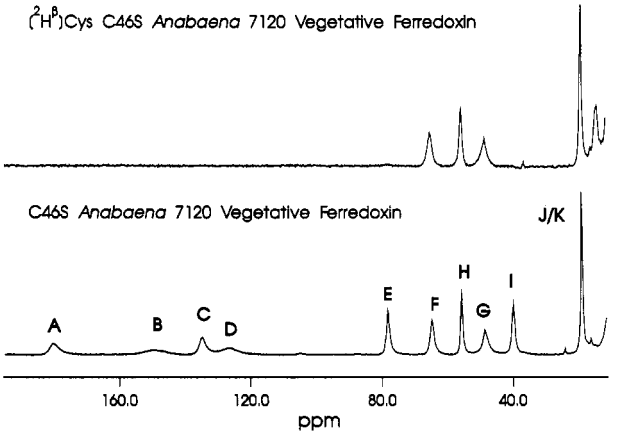

FIGURE 6: Hyperfine-shifted resonances in the downfield region of the 400-MHz ^{1}H NMR spectra of sodium dithionite reduced Cys^{46}-Ser *Anabaena* 7120 vegetative ferredoxin (bottom) and [β-^{2}H]cysteine-labeled Cys^{46}Ser *Anabaena* 7120 vegetative ferredoxin (top). The spectra were recorded at 286 K. The samples were prepared as described in the legend to Figure 5.

the cysteine β-protons. In the ^{1}H NMR spectrum of reduced [β-^{2}H]cysteine-labeled Cys^{46}Ser mutant, peaks A–D, E, F–G, and J/K were completely diminished, whereas peaks were observed without loss of intensity (Figure 6, top).

Peaks A–D, the four hyperfine proton peaks farthest downfield in the spectrum of reduced Cys^{46}Ser (Figure 6), which exhibit Curie temperature dependence and are identified as cysteine β-proton peaks from the selective deuteration experiment, are assigned to the two cysteine residues ligated to the same iron (Cys^{49} and Cys^{79}). The hyperfine proton peaks with anti-Curie temperature dependence are assigned to residues close to Fe(II). Those farthest downfield fall into two classes: (1) Peaks E and I, which are identified by deuterium labeling as cysteine β-protons, are sharper and exhibit a mutual cross-peak in the NOESY spectrum. These two peaks are assigned by difference to the β-protons of Cys^{41}. (2) Peaks F and G, which are shown by the deuterium labeling experiment (Figure 6) not to correspond to cysteine β-protons, are both broad and have short T_1 values (3 and 1.6 ms, respectively; Table 2). Peaks F and G are assigned to the β-protons of Ser^{46}; their shorter T_1 values, compared to those of the β-protons of Cys^{46} in the wild-type ferredoxin (8.8 and 3.5 ms), are consistent with the X-ray results for Cys^{49}Ser (B. L. Jacobson, H. M. Holden, H. Cheng, B. Xia, and J. L. Markley, unpublished results), which show that the Fe–O distance in the mutant (iron to the O$^{\gamma}$ of Ser^{49}) is shorter (1.8 Å) than the Fe–S distance (iron to the S$^{\gamma}$ of Cys^{49}) in the wild-type ferredoxin (2.3 Å). These results show that the same iron that is reduced in the wild-type protein is reduced in the mutant even though one of its sulfur ligands has been replaced by oxygen. Peak H in the spectrum of Cys^{46}Ser (Figure 6) showed a Curie-type temperature dependence similar to those of peaks A–D (Figure 7) and a T_1 value (4.9 ms) comparable to those of peaks E and J (both 4.0 ms) of wild-type ferredoxin assigned, respectively, to Cys^{49} ^{1}H$^{\alpha}$ and Cys^{79} ^{1}H$^{\alpha}$ (Skjeldal et al., 1991a). On the basis of this comparison, peak H in the spectrum of Cys^{46}Ser is attributed to the α-proton of either Cys^{49} or Cys^{79}.

When the Cys^{49}Ser mutant was reduced by sodium dithionite, its ^{1}H NMR spectrum (Figure 8) became remarkably different from that of wild-type ferredoxin or that of the Cys^{46}Ser mutant (Figures 5 and 6). Instead, it resembled the spectra of reduced vertebrate ferredoxins reported by Skjeldal et al. (1991b). Four hyperfine-shifted peaks were resolved at 298 K at 173, 120, 32, and 18 ppm (Figure 8). These hyperfine peaks exhibited Curie-type temperature dependence (Figure 8). The peaks at 173 and 120 ppm were much broader than the other two peaks (at 32 and 18 ppm). We tentatively assign the peaks at 173 and 120 ppm to β-protons from two the residues directed ligated to Fe(III) and the peaks at 32 and 17 ppm to α-protons on the same residues. In the region upfield of the diamagnetic region, a broad peak was observed

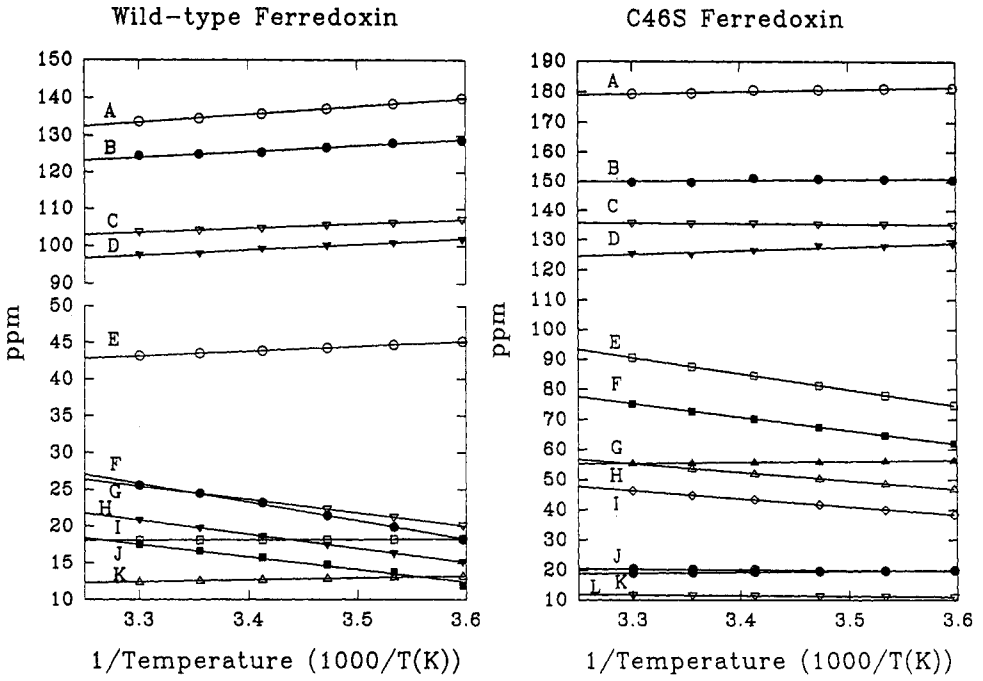

FIGURE 7: Temperature dependence of the hyperfine-shifted resonances in the downfield region of the 400-MHz ^{1}H NMR spectra of sodium dithionite reduced *Anabaena* 7120 vegetative ferredoxins: wild type (left) and Cys^{46}Ser (right).

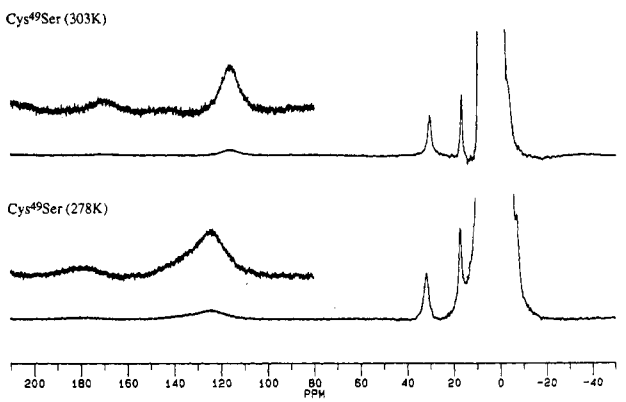

FIGURE 8: Hyperfine-shifted resonances in the 400-MHz ^{1}H NMR spectra of sodium dithionite reduced Cys^{49}Ser *Anabaena* 7120 vegetative ferredoxin obtained at different temperatures: 278 K (bottom) and 303 K (top). The sample was prepared as described in the legend to Figure 5.

at −1.7 ppm at 298 K which exhibited Curie-type temperature dependence.

Differences in the hyperfine shifts of wild-type, Cys^{46}Ser, and Cys^{49}Ser ferredoxins must arise from differences in electron distribution within the iron–sulfur clusters of these proteins. At this stage, we have no results that specify the oxidation state of the iron atom ligated to Ser^{49} in the reduced mutant Cys^{49}Ser.

DISCUSSION

Serine as an Iron–Sulfur Cluster Ligand in Ferredoxins. Although iron–sulfur proteins have been studied for many years, it still is unclear why ferredoxins that contain the same iron–sulfur cluster type (e.g., [2Fe–2S]) can have very different reduction potentials. Although the nature of the atoms ligated to iron must play a role in cluster stability and reduction potential, few experimental studies have investigated this issue. An obvious approach is to replace the cysteines with other amino acids. Although non-cysteine ligands have been found in other proteins containing [2Fe–2S] clusters (Gurbiel et al., 1991; Lauble et al., 1992; Werth et al., 1990, 1992) and although non-cysteine ligated analogues of [2Fe–2S] clusters have been synthesized and characterized by spectroscopy (Mascharak et al., 1981; Beardwood et al., 1982), a non-cysteine ligated iron–sulfur cluster in a natural ferredoxin has not been studied previously.

Of the 20 well-known natural amino acids, serine is the one most similar to cysteine. Upon replacing one of the cysteine ligands of *E. coli* fumarate reductase with serine by site-directed mutagenesis, Werth et al. (1990) found that the [2Fe–2S] cluster was assembled *in vivo*. To test the proposal that serine can replace cysteine as a ligand to the iron–sulfur cluster in a ferredoxin, we have chosen *Anabaena* 7120 vegetative ferredoxin as the wild-type ferredoxin and individually changed each of its four cysteine residues to a serine residue. *Anabaena* 7120 vegetative ferredoxin contains the simplest iron–sulfur cluster ([2Fe–2S]), and it has been studied well by many biophysical methods: e.g., X-ray crystallography (Rypniewski et al., 1991; Holden et al., 1993), EPR spectroscopy (Hutson et al., 1978), NMR spectroscopy (Cheng et al., 1993; Oh & Markley, 1990a,b, Oh et al., 1990; Skjeldal et al., 1990, 1991a,b), optical spectroscopy (Böhme & Schrautemeier, 1987), and reduction potential (Böhme & Schrautemeier, 1987). Its amino acid sequence contains no "free" cysteine which could become a replacement ligand to the cluster as occurred in the *Azotobacter vinelandii* 7Fe ferredoxin after mutation of one of the iron–sulfur ligated cysteines (Martin et al., 1990). Furthermore, wild-type ferredoxin has been produced in *Escherichia coli* in high level (~20 mg/L culture) by using a T7 promoter/T7 polymerase overproduction system (Cheng et al., 1994).

The results presented in this paper, coupled with those from X-ray crystallography (B. L. Jacobson, H. M. Holden, H. Cheng, B. Xia, and J. L. Markley, unpublished results) and electron transfer studies (J. K. Hurley, G. Tollin, H. Cheng, B. Xia, and J. L. Markley, unpublished results), clearly demonstrate that the iron–sulfur clusters of the four Cys → Ser mutants assemble spontaneously *in vitro* upon the addition of iron and inorganic sulfur. The optical, EPR, and NMR

spectra of the mutants have some characteristics in common with those of wild-type [2Fe–2S] ferredoxin. The mutants can be reduced by sodium dithionite. These results suggest that it is possible for serine to replace cysteine as an iron–sulfur cluster ligand in ferredoxins.

Reduction Potentials. The present results provide a test of the importance of the nature of atoms coordinated to the iron–sulfur cluster in determining the reduction potential. Details of the reduction potential studies will be published elsewhere (A. Weber-Main, M. T. Stankovich, H. Cheng, B. Xia, and J. L. Markley, manuscript in preparation), but the preliminary results show the following: The reduction potentials of wild-type and recombinant-reconstituted *Anabaena* 7120 vegetative ferredoxin are identical within experimental error (–360 mV). The reduction potential of the Cys^{46}Ser mutant is slightly lower (–378 mV) than that of the wild-type ferredoxin, and that of Cys^{49}Ser is considerably higher (ca. –262 mV). The lower stability of Cys^{41}Ser in its reduced state has made it difficult to determine its reduction potential, and no attempts have been made to measure the reduction potential of Cys^{79}-Ser, the least stable mutant. It is interesting that an S-to-O atom substitution has a larger effect at position 49 (Cys^{49}Ser mutant), where the cysteine is ligated to Fe(III) in both redox states, than at position 46 (Cys^{46}Ser mutant), where the cysteine is ligated to the iron which is reduced to Fe(II) in the reduced state.

EPR Spectra and the Nature of the Ligands. By reference to their EPR spectra and reduction potentials, [2Fe–2S] clusters in proteins can be placed into three different classifications: (1) The plant-type [2Fe–2S] cluster is found in ferredoxins from plants and cyanobacteria (e.g., spinach [2Fe–2S] ferredoxin, *Anabaena* 7120 vegetative ferredoxin, *Anabaena* 7120 heterocyst ferredoxin, *Spirulina platensis* ferredoxin, etc.). This class of cluster shows a rhombic EPR signal in the reduced state with g_{av} = 1.96. The reduction potential of the cluster is between –300 and –460 mV. (2) The vertebrate-type [2Fe–2S] cluster is found in vertebrate ferredoxins (e.g., adrenodoxin and human placental ferredoxin) and in some bacterial ferredoxins (i.e., *Escherichia coli* [2Fe–2S] ferredoxin (Ta & Vickery, 1992) and putidaredoxin (Cushman et al., 1967)). An axial signal with g_{av} = 1.96 is seen in the EPR spectrum of this class cluster type in the reduced state. Clusters in this class have reduction potentials of ca. –270 mV. (3) Rieske-type [2Fe–2S] clusters are ligated by sulfurs from two cysteines and imidazole nitrogens from two histidines. This cluster type, which is found in Rieske proteins and in some dioxygenases, exhibits a rhombic EPR signal with g_{av} = 1.91. Of the three, this has the highest reduction potential: +150 to +300 mV for Rieske proteins or between 0 and –150 mV for the Rieske-like clusters found in some dioxygenases.

The EPR spectrum of reduced Cys^{46}Ser (Figure 2D) exhibited a rhombic signal similar to that of wild-type ferredoxin; as noted above, the reduction potential of this mutant is only slightly lower than that of the wild-type ferredoxin. By contrast, replacement of Cys^{49} with Ser yielded a reduced cluster with a near-axial EPR signal similar to those of vertebrate-type ferredoxins. The g values of this mutant (1.90, 1.92, 2.02) were comparable to those reported by Coghlan and Vickery (1989) for human ferredoxin (1.92, 1.92, 2.03), and the g_{av} value of 1.95 of Cys^{49}Ser was nearly identical to that for human ferredoxin (1.96). Interestingly, the reduction potential of mutant Cys^{49}Ser (ca. –262 mV; A. Weber-Main, M. T. Stankovich, H. Cheng, B. Xia, and J. L. Markley, manuscript in preparation) is also more like that of vertebrate ferredoxins (typically –270 mV). Sodium dithionite reduced Cys^{41}Ser exhibited an anisotropic EPR signal with g values of 1.82, 1.91, and 2.02. Features of this EPR signal are similar to those of "Rieske-type" proteins ($g_{1,2,3}$ = 1.80, 1.90, and 2.02, respectively; Fee et al., 1984). Its g_{av} value of 1.92 also is comparable to those of "Rieske-type" proteins (g_{av} = 1.91).

The results discussed above suggest that there is a relationship between reduction potentials and EPR spectra of [2Fe–2S] proteins. A cluster with an axial EPR signal should have a lower reduction potential than one with a rhombic EPR signal. This qualitative relationship holds true for Cys^{46}-Ser, Cys^{49}Ser, and the wild-type *Anabaena* 7120 vegetative ferredoxins as well as for the serine-replaced *E. coli* fumarate reductase mutants (Werth et al., 1990).

NMR Spectroscopy of Cys^{46}Ser and Cys^{49}Ser. The distribution of hyperfine-shifted resonances of Cys^{46}Ser (Figure 6, bottom) is similar to that of wild-type ferredoxin (Figure 5), although the hyperfine shifts of the mutant are larger that those of wild-type ferredoxin. However, their temperature dependence and T_1 relaxation times are similar to those of the corresponding equivalent resonances of wild-type ferredoxin (Table 2 and Figure 7). The resonances assigned to corresponding protons of the wild-type and mutant ferredoxins exhibited similar temperature dependence of the kind predicted by theoretical models (Dunham et al., 1971; Banci et al., 1990).

The contact shift is usually expressed by the McConnell equation (McConnell & Chesnut, 1958):

$$\frac{\Delta\nu}{\nu_0} = -\frac{A_c\gamma_e}{\hbar\gamma_I}\frac{g_e\beta}{3kT}S(S+1)$$

where A_c is the contact shift constant, g_e is the electronic g factor, β is the Bohr magneton, γ_I and γ_e are the magnetogyric ratios for the nucleus and electron, respectively, S is the total electronic spin, k is the Boltzmann constant, T is the absolute temperature, and $\hbar$ is the modified Planck constant. Because the resonances arising from the protons on the iron-ligated residues in the ^{1}H NMR spectrum of Cys^{46}Ser mutant have larger hyperfine shifts than those of wild-type ferredoxin, the contact shift constants of the hyperfine-shifted resonances of Cys^{46}Ser must be larger than those of wild-type ferredoxin.

In ferredoxins, nuclei near the iron–sulfur cluster are relaxed by interactions between the nucleus and the unpaired electron on the iron atoms as described by the general equation (Banci et al., 1990):

$$T_{1M}^{-1} = KA_c f(\tau_{cj},\omega_j)\sum_i \langle S_j^2\rangle_i$$

where A_c, the hyperfine coupling constant, can be dipolar and contact in origin, and where $\langle S_j^2\rangle$ is calculated over all the S_j components of the spin levels. Thus, the nuclear relaxation depends on the hyperfine coupling and the distance between the nucleus and the iron atoms. The X-ray structure of Cys^{49}-Ser shows that the Fe–O(Ser) bond (1.8 Å) is shorter than the Fe–S(Cys) bond (2.3 Å) (B. L. Jacobson, H. M. Holden, H. Cheng, B. Xia, and J. L. Markley, unpublished data). Although one of the β-proton resonances (peak E) originating from Cys^{41} has a larger hyperfine shift than either of the two β-proton resonances of Ser^{46} (peaks F and G), the T_1 relaxation times of the Ser^{46} β-proton peaks are significantly shorter than those of the equivalent Cys^{46} peaks (H and I) of the wild-type ferredoxin, whereas the T_1 values of peaks E and I arising from the β-protons of Cys^{41} are similar to those of the equivalent peaks (F and G) in the wild-type ferredoxin

(Table 2). Therefore, the decrease in T_1 relaxation time must be due mainly to the fact that the two β-protons of Ser^{46} in Cys^{46}Ser are closer to the iron–sulfur cluster than the corresponding β-protons of Cys^{46} in the wild-type ferredoxin.

The ^{1}H NMR spectrum of the reduced Cys^{49}Ser mutant (Figure 8) is strikingly different from those of wild-type ferredoxin or Cys^{46}Ser. Four hyperfine-shifted peaks are resolved in the downfield region, and one is found upfield of the diamagnetic envelope. They all exhibit Curie-type temperature dependence. These peaks must arise from protons on residues near to the iron–sulfur cluster as seen in the ^{1}H NMR spectra of other [2Fe–2S] ferredoxins (Skjeldal et al., 1990, 1991a,b).

The two peaks with larger hyperfine shifts (at 173 and 120 ppm at 298 K) are considerably broader than the other two (at 32 and 18 ppm), and they are even broader than the peak near 40 ppm which is found in the ^{1}H NMR spectrum of human placental ferredoxin (Skjeldal et al., 1991b). The EPR spectrum and the results from the reduction potential measurement show that Cys^{49}Ser has a vertebrate-type [2Fe–2S] cluster. The theoretical treatment by Dunham et al. (1971) for reduced vertebrate ferredoxins 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). Therefore, the peaks at 173 and 120 ppm are assigned to the β-protons of the Fe(III)-ligated residues. Evidence from the ^{2}H NMR spectrum of [α-^{2}H]cysteine-labeled human placental ferredoxin (B. Xia, H. Cheng, V. M. Coghlan, L. E. Vickery, and J. L. Markley, unpublished results) suggests that the hyperfine-shifted resonance observed at 40 ppm in the ^{1}H NMR spectrum of reduced human placental ferredoxin (Skjeldal et al., 1991b) is from α-protons on the iron-ligated cysteines. Thus the two peaks at 32 and 18 ppm are tentatively assigned to α-protons on the Fe(III)-ligated residues. Both cysteine and serine have two $^1H^\beta$ and one $^1H^\alpha$. Only two of the four hyperfine-shifted peaks expected from the β-protons on the two residues ligated to Fe(III) were observed. This can be explained either by assuming that each of the two peaks represents more than one β-proton or by assuming that the other β-resonances are too broad to be detected, as was the case for the human placental ferredoxin (Skjeldal et al., 1991b). The assignment of the upfield-shifted peak cannot be specified at this stage. The temperature dependence of this peak suggests that it arises from one or more protons on residues close to the iron–sulfur cluster. The above question can be resolved by incorporating selectively ^{2}H-labeled cysteine into the protein (Cheng et al., 1990, 1994), and this work is underway.

In contrast to the ^{1}H NMR spectra of the reduced ferredoxins, the spectra of oxidized wild-type and Cys → Ser mutants show patterns of hyperfine peaks (Figure 4) similar to that of the wild-type ferredoxin. As expected, all peaks exhibited anti-Curie temperature dependence as shown by the wild-type ferredoxin. These hyperfine resonances are assigned to the protons from iron–sulfur-ligated cysteine or serine residues. The hyperfine signals in the spectra of Cys^{41}-Ser, Cys^{46}Ser, and Cys^{79}Ser are less overlapped than those of the wild-type ferredoxin. Three peaks are resolved in the region between 20 and 50 ppm in the spectra of these mutants (Figure 4). Unlike other mutants, an additional anti-Curie peak was observed at –15 ppm in the spectrum of Cys^{79}Ser (Figure 4). The origin of this peak is unknown. It will be examined by incorporating selectively deuterated amino acids into the protein (Cheng et al., 1990, 1994).

Stability of the Mutants and Formation of the Iron–Sulfur Cluster. The stabilities of the Cys → Ser mutants were found to be lower than that of the wild-type ferredoxin as expected from the relative pK_a values of Cys and Ser. This probably explains the exclusive appearance of cysteine as the ligand in nature. Among the four Cys → Ser mutants, Cys^{46}Ser and Cys^{49}Ser were more stable than the other two. These cysteines are part of the conserved -Cys-X-X-Cys- sequence found in all known [2Fe–2S] ferredoxins. The stability differences among these mutants may provide some insight into protein folding and cluster formation.

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REFERENCES

Banci, L., Bertini, I., & Luchinat, C. (1990) *Struct. Bonding 72*, 113–136.

Beardwood, P., Gibson, J. F., Johnson, C. E., & Rush, J. D. (1982) *J. Chem. Soc., Dalton Trans.*, 2015–2020.

Bertrand, P., & Gayda, J. P. (1979) *Biochim. Biophys. Acta 579*, 107–121.

Bertrand, P., & Gayda, J. P. (1980) *Biochim. Biophys. Acta 625*, 337–342.

Bertrand, P., Guigliarelli, B., Gayda, J.-P., Beardwood, P., & Gibson, J. F. (1985) *Biochim. Biophys. Acta 831*, 261–266.

Böhme, H., & Schrautemeier, B. (1987) *Biochim. Biophys. Acta 891*, 1–7.

Britt, R. D., Sauer, K., Klein, M. P., Knaff, D. B., Kriauciunas, A., Yu, C.-A., Yu, L., & Malkin, R. (1991) *Biochemistry 30*, 1892–1901.

Cheng, H., Grohmann, K., & Sweeney, W. (1990) *J. Biol. Chem. 265*, 12388–12391.

Cheng, H., Westler, W. M., Bin, X., Oh, B.-H., & Markley, J. L. (1994) manuscript in preparation.

Coghlan, V. M., & Vickery, L. E. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 835–839.

Coghlan, V. M., & Vickery, L. E. (1991) *J. Biol. Chem. 266*, 18606–18612.

Cushman, D. W., Tsai, R. L., & Gunsalus, I. C. (1967) *Biochem. Biophys. Res. Commun. 26*, 577–583.

Davidson, E., Ohnishi, T., Tokito, M., Emmanuel, A.-A.-A., & Daldal, F. (1992) *Biochemistry 31*, 3342–3351.

Dunham, W. R., Palmer, G., Sands, R. H., & Bearden, A. J. (1971) *Biochim. Biophys. Acta 253*, 373–384.

Fee, J. A., Findling, K. L., Yoshida, T., Hille, R., Tarr, G. E., Hearshen, D. O., Dunham, W. R., Day, E. P., Kent, T. A., & Münck, E. (1984) *J. Biol. Chem. 259*, 124–133.

Freeman, R., Kempsell, S. P., & Levitt, M. H. (1980) *J. Magn. Reson. 38*, 453–479.

Gibson, J. F., Hall, D. O., Thornley, J. H. M., & Whatley, F. R. (1966) *Proc. Natl. Acad. Sci. U.S.A. 56*, 987–990.

Gurbiel, R. J., Batie, C. J., Sivaraja, M., True, A. E., Fee, J. A., Hoffman, B. M., & Ballou, D. P. (1989) *Biochemistry 28*, 4861–4871.

Gurbiel, R. J., Ohnishi, T., Robertson, D. E., Daldal, F., & Hoffman, B. M. (1991) *Biochemistry 30*, 11579–11584.

Ho, K. K., Ulrich, E. L., Krogmann, D. W., & Gomez-Lojero, C. (1979) *Biochim. Biophys. Acta 545*, 236–248.

Holden, H. M., Jacobson, B. L., Hurley, J. K., Tollin, G., Oh, B.-H., Skjeldal, L., Chae, Y. K., Cheng, H., Xia, B., & Markley, J. L. (1993) *J. Bioenerg. Biomemb.* (in press).

Hurley, J. K., Salamon, Z., Meyer, T. E., Fitch, J. C., Cusanovich, M. A., Markley, J. L., Cheng, H., Xia, B., Chae, Y. K., Medina, M., Gomez-Moreno, C., & Tollin, G. (1993) *Biochemistry 32*, 9346–9354.

Hutson, K. G., Rogers, L. J., Haslett, B. G., Boulter, D., & Cammack, R. (1978) *Biochem. J. 172*, 465–477.

Kunkel, T. A., Roberts, J. D., & Zakour, R. A. (1987) *Methods Enzymol. 154*, 267–382.

Lauble, H., Kennedy, M. C.; Beinert, H., & Stout, C. D. (1992) *Biochemistry 31*, 2735–2748.

Manodori, A., Cecchini, G., Schröder, I., Gunsalus, R. P., Werth, M. T., & Johnson, M. K. (1992) *Biochemistry 31*, 2703–2712.

Martin, A. E., Burgess, B. K., Stout, C. D., Cash, V. L., Dean, D. R., Jensen, G. M., & Stephens, P. J. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 598–602.

Mascharak, P. K., Papefthymiou, G. C., Frankel, R. B., & Holm, R. H. (1981) *J. Am. Chem. Soc. 103*, 6110–6116.

McConnell, H. M., & Chesnut, R. E. (1958) *J. Chem. Phys. 28*, 107–117.

Noodleman, L., & Baerends, E. J. (1984) *J. Am. Chem. Soc. 106*, 2316–2327.

Oh, B.-H., & Markley, J. L. (1990a) *Biochemistry 29*, 3993–4004.

Oh, B.-H., & Markley, J. L. (1990b) *Biochemistry 29*, 4012–4017.

Oh, B.-H., Mooberry, E. S., & Markley, J. L. (1990) *Biochemistry 29*, 4004–4011.

Pueyo, J. J., & Gomez-Moreno, C. (1991) *Prep. Biochem. 21*, 191–204.

Rothery, R. A., & Weiner, J. H. (1991) *Biochemistry 30*, 8296–8305.

Rypniewski, W. R., Breiter, D. R., Benning, M. W., Wesenberg, G., Oh, B.-H., Markley, J. L., Rayment, I., & Holden, H. M. (1991) *Biochemistry 30*, 4126–4131.

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

Skjeldal, L., Westler, W. M., & Markley, J. L. (1990) *Arch. Biochem. Biophys. 278*, 482–285.

Skjeldal, L., Westler, W. M., Oh, B.-H., Krezel, A. M., Holden, H. M., Jacobson, B. L., Rayment, I., & Markley, J. L. (1991a) *Biochemistry 30*, 7363–7368.

Skjeldal, L., Markley, J. L., Coghlan, V. M., & Vickery, L. E. (1991b) *Biochemistry 30*, 9078–9083.

Ta, D. T., & Vickery, L. E. (1992) *J. Biol. Chem. 267*, 11120–11125.

Vold, R. L., Waugh, J. S., Klein, M. P., & Phelps, D. E. (1968) *J. Chem. Phys. 48*, 3831–3832.

Werth, M. T., Cecchini, G., Manodori, A., Ackrell, B. A. C., Schröder, I., Gunsalus, R. P., & Johnson, M. K. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 8965–8969.

Werth, M. T., Sices, H., Cecchini, G., Schröder, I., Lasage, S., Gunsalus, R. P., & Johnson, M. K. (1992) *FEBS Lett. 299*, 1–4.