Human Ferredoxin: Overproduction in *Escherichia coli*, Reconstitution *in Vitro*, and Spectroscopic Studies of Iron−Sulfur Cluster Ligand Cysteine-to-Serine Mutants†

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Received February 26, 1996; Revised Manuscript Received May 16, 1996[⊗]

ABSTRACT: Human ferredoxin, the human equivalent of bovine adrenodoxin, is a small iron−sulfur protein with one [2Fe-2S] cluster. It functions, as do other vertebrate ferredoxins, to transfer electrons during the processes of steroid hormone synthesis. A DNA fragment encoding the mature form of human ferredoxin was cloned into an expression vector under control of the T7 RNA polymerase/promoter system. The protein was overproduced in *Escherichia coli*, and the [2Fe-2S] cluster was incorporated into the protein by *in vitro* reconstitution. The overall yield was ∼30 mg of purified, reconstituted ferredoxin per liter of culture. Four of the five cysteines in human ferredoxin are coordinated to the iron−sulfur cluster. First, the non-ligand cysteine (cysteine-95) was mutated to alanine, and then double mutants were created in which each of the other four cysteines (at positions 46, 52, 55, and 92) were mutated individually to serine. The wild-type ferredoxin and each of the five mutant proteins were studied by UV−visible spectroscopy and electron paramagnetic resonance spectroscopy. The EPR g_{av} values of all five mutants were very similar to that of wild-type human ferredoxin. In the reduced state, three of the cysteine-to-serine mutants exhibited axial EPR spectra similar to that of wild-type, but one of the double mutants (C52S/C95A) exhibited a rhombic EPR spectrum. The UV−visible spectroscopic properties of the wild-type and the C95A mutant ferredoxins were identical, but those of the other cysteine-to-serine mutant proteins of human ferredoxin were quite different from those of the wild-type protein and each other. These results, along with those from cysteine-to-serine mutations in other ferredoxins, provide the basis for a more comprehensive theoretical and practical understanding of the features important to the ligation of [2Fe-2S] clusters, although they do not yet permit determination of which two cysteines ligate Fe(II) and which ligate Fe(III) in the reduced protein.

Ferredoxins are a group proteins of that contain one or more iron−sulfur clusters and participate as electron carriers in biological electron transfer reactions. They display no classical enzymatic functions (Palmer & Reedijk, 1992). Ferredoxins typically are classified by the number and types of iron−sulfur clusters they contain. To date, three types of iron−sulfur clusters in ferredoxins have been identified by X-ray crystallography: [2Fe-2S] (Tsukihara, et al., 1978), [3Fe-4S] (Kissinger, et al., 1991), and [4Fe-4S] (Freer, et al., 1975). Within these categories, ferredoxins can be grouped into families on the basis of their amino acid sequence relationships, reduction potentials, biological source, and spectral properties (Cammack, 1992). For example, the [2Fe-2S] ferredoxins fall into three classes: plant-type, vertebrate-type, and bacterial-type ferredoxins. Plant-type [2Fe-2S] ferredoxins (prototypes include spinach, *Anabaena* 7120 vegetative and heterocyst, and *Spirulina platensis* ferredoxins) have reduction potentials around −300 to −460 mV and exhibit rhombic EPR[1] signals with $g_{av} = 1.96$ ($g_1 = 1.88$, $g_2 = 1.96$, $g_3 = 2.05$). Prototypes of vertebrate-type ferredoxins include bovine (adrenodoxin), chick, and human ferredoxins. Putidaredoxin, and the [2Fe-2S] ferredoxin from *Escherichia coli* share some of the properties of this group, but may eventually require separate classification. Vertebrate-type ferredoxins show typical axial EPR spectra with $g_{av} = 1.96$ ($g_1 = 1.94$, $g_2 = 1.94$, $g_3 = 2.02$); their reduction potentials are usually near −290 mV, around 200 mV higher than those of the plant-type [2Fe-2S] ferredoxins. The bacterial-type [2Fe-2S] ferredoxin has been isolated only from the nitrogen-fixing saccharolytic anaerobe *Clostridium pasteurianum*. It is a homodimeric protein with each subunit containing one [2Fe-2S] cluster. The reduced protein shows a rhombic EPR signal with *g* values of 1.92, 1.95, and 2.00; the reduction potential is about −300 mV.

In ferredoxins, the iron−sulfur clusters are bound to the protein by covalent bonds between iron atoms and the sulfur atoms of the thiolate side chains of four cysteine residues. Non-cysteinyl ligands have been reported in other classes of iron−sulfur proteins. For example, the [2Fe-2S] cluster in Rieske-type proteins is coordinated by the sulfur atoms

† Supported by National Science Foundation Grant MCB-9215142 and National Institutes of Health Grant GM35752.

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⊗ Abstract published in *Advance ACS Abstracts,* July 1, 1996.

[1] Abbreviations used: EPR, electron paramagnetic resonance; ESEEM, electron spin echo envelope modulation; HuFd, human ferredoxin; IPTG, isopropyl thiogalactoside; LB medium, Luria−Bertani medium; MCD, magnetic circular dichroism; NMR, nuclear magnetic resonance; RR, resonance Raman; VFd, *Anabaena* 7120 vegetative ferredoxin; wt, wild-type.

S0006-2960(96)00467-9 CCC: $12.00

from each of two cysteines and by one of the imidazole-ring nitrogen atoms from each of two histidines (Davidson et al., 1992; Gurbiel et al., 1989, 1991; Britt et al., 1991). In aconitase, by contrast, one of the iron atoms of the [4Fe-4S] cluster is coordinated by one carboxyl oxygen, one hydroxyl oxygen from the substrate, and one oxygen from a water molecule; this iron is the one that is lost when the cluster is converted to a [3Fe-4S] cluster (Lauble et al., 1992; Beinert & Kennedy, 1993). Non-cysteinyl ligands appear to be present in some additional proteins that contain [4Fe-4S] clusters (Holm et al., 1992).

Several analogues of [2Fe-2S] clusters that contain non-sulfur ligands to the iron atoms have been synthesized (Macharak et al., 1981; Beardwood et al., 1982). Mutagenesis methods have been used to probe whether individual cysteine residues in proteins are cluster ligands and whether they can be replaced by other residue types. Werth et al. (1990) mutated each of the four ligand cysteines of the [2Fe-2S] cluster in *E. coli* fumarate reductase individually to serine and found that the cluster was incorporated spontaneously into all mutant proteins *in vivo*; results on the C65S mutant of *E. coli* fumarate reductase showed that the mutation did not result in significant perturbations of its spectroscopic, catalytic, or redox properties. The position analogous to Cys^{65} in *E. coli* fumarate reductase is occupied by an Asp residue in *E. coli* succinate dehydrogenase (Werth et al., 1992); this again suggests that oxygen can be a ligand to a [2Fe-2S] cluster.

Early site-directed mutagenesis studies of cysteines of ferredoxins focused on the identification of non-ligating cysteines (Gerber et al., 1990; Uhlmann et al., 1992; Fujinaga et al., 1993). Formation of the cluster following the mutation of a cysteine (usually to serine) generally was taken to indicate that the mutated residue was not a cluster ligand. However, all such mutants in the *Anabaena* 7120 vegetative ferredoxin, whose four cysteines are all ligated to the cluster (Rypniewski et al., 1991), yielded proteins that formed a cluster by *in vitro* reconstitution (Cheng et al., 1994). The resulting mutant proteins have been studied systematically by several additional biophysical methods (Holden et al., 1993): optical, EPR and NMR spectroscopy, X-ray crystallography, electron transfer rate analysis, and reduction potential measurement. In a more recent study of individual Cys-to-Ser cluster-ligand mutants of the [2Fe-2S] ferredoxin from *C. pasteurianum* (CpFd) (Meyer et al., 1994), the mutant proteins were purified directly from the *E. coli* cells and then used for optical, EPR, and resonance Raman spectroscopic investigations. Taken together, these studies indicate that the clusters of [2Fe-2S] ferredoxins with one serinate and three cysteinate ligands can be synthesized *in vivo* and form spontaneously *in vitro*. Recently, a Cys-to-Ser mutation has been reported for one of the iron ligands of rubredoxin (Meyer et al., 1995); wild-type rubredoxin contains a single iron ligated by four cysteines and no inorganic sulfur.

This paper describes investigations of Cys-to-Ser mutations in a vertebrate-type [2Fe-2S] ferredoxin, human ferredoxin (HuFd) which is found in the mitochondria of placenta and other tissues. HuFd functions as an electron carrier in the initial step of steroid hormone biosynthesis. The mature form of HuFd consists of 124 amino acid residues. Its five cysteine residues are located at positions 46, 52, 55, 92, and 95; Cys^{95} has a free thiol group, and the other four cysteines function as ligands to the [2Fe-2S] cluster (Cupp & Vickery, 1988). The EPR spectrum of reduced wt HuFd exhibits an axial signal with g values of 1.94, 1.94, and 2.02. The reduction potential of the iron−sulfur cluster is −260 mV at pH 7.4 (A. Weber-Main, M. T. Stankovich, and L. E. Vickery, personal comunication), and its value is pH dependent with a pH_{mid} of 7.2 (Cooper et al., 1973). It had been proposed that a conserved histidine residue (His^{56}) adjacent to one of the cluster-ligated cysteines might be the responsible pH titratable group; however, this was ruled out by a recent NMR study of the histidine residues of HuFd (Xia et al., 1995). Figure 1 presents alignment of the amino acid sequences of human ferredoxin (HuFd), putidaredoxin (Pdx), and *Anabaena* 7120 vegetative ferrredoxin (VFd).

Human ferredoxin has been overexpressed previously as a cleavable fusion protein in *E. coli* (Coghlan & Vickery, 1989) and in *Sacchromyces cerevisiae* (Seaton & Vickery, 1992) at protein yields of about 5 mg of protein per liter of culture. We increased this yield to around 30 mg per liter of culture by overexpressing the mature form of HuFd directly in *E. coli*. The product, a mixture of apo- and holoprotein, was converted fully to apoprotein, and then the iron−sulfur cluster was incorporated into the protein *in vitro* by the addition of iron and sulfide under anaerobic conditions. The fifth, non-ligand cysteine, Cys^{95} (Cupp & Vickery, 1988), was mutated first to alanine. Then each of the four ligand cysteines was mutated to serine individually in the C95A background. Contrary to a previous study of bovine ferredoxin (adrenodoxin) that indicated that variants with single Cys-to-Ser mutations at cysteines 46, 52, 55, and 92 do not support the formation of the iron−sulfur cluster (Uhlmann et al., 1992), each of the human ferredoxin mutants formed clusters under *in vitro* reconstitution conditions. The wild-type HuFd and the five mutant proteins were characterized by optical and EPR spectroscopy. Comparison of these results with those from analogous mutants of VFd revealed interesting differences between the clusters of vertebrate-type and plant-type ferredoxins.

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. DTT was purchased from Boehringer-Mannheim Biochemicals (Indianapolis, IN). *E. coli* strain JM103 was used for routine site-directed mutagenesis; *E. coli* strain HMS174 was used for plasmid storage; BL21-(DE3)/pLysS was used for protein expression. The protein expression vector pET9a was purchased from Novagen (Madison, WI).

Plasmid Constructions and Site-Directed Mutagenesis. The original plasmid pHFdx1 (Coghlan & Vickery, 1989) containing the gene for human ferredoxin was digested with restriction enzymes *Hin*dIII and *Bam*HI in order to excise the DNA sequence coding for HuFd. This fragment was then subcloned into the M13(mp19) vector. Two restriction enzyme sites, *Nde*I and *Bam*HI, were created by mutagenesis, immediately upstream and downstream of the mature HuFd structural gene, respectively. In addition, a starting codon was introduced just before the first codon of the mature HuFd gene. An *Nde*I restriction site inside the coding region of

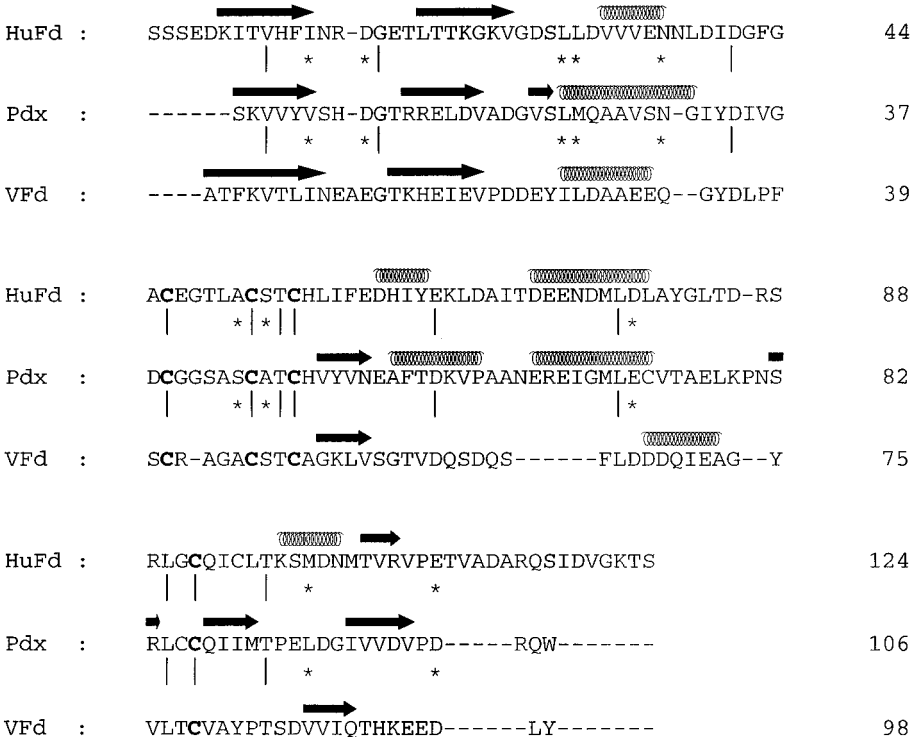

FIGURE 1: Amino acid sequence alignment and comparative secondary structure of representative [2Fe-2S] ferredoxins: human ferredoxin (HuFd), putidaredoxin (Pdx), and *Anabaena* 7120 vegetative ferredoxin (VFd). Conserved residues between human ferredoxin and vegetative ferredoxin and between human ferredoxin and putidaredoxin are indicated by vertical lines. The symbol "*" is used for indicating similar residues. Secondary structures shown are from HuFd (B. Xia and J. L. Markley, derived from unpublished NMR data), Pdx (Pochapsky et al., 1994), and VFd (Riepniewski et al., 1991). The CLUSTALW computer program was used to determine the protein sequence alignment (WWW address for CLUSTALW is http://glycine.ncsa.uiuc.edu:3104/wbt.dir/html/wbt.CLW02P.html).

HuFd was eliminated by changing the codon of Tyr^{82} from TAT to TAC. The DNA fragment coding for the entire mature HuFd gene was excised and cloned into the pET9a expression vector between the *Nde*I and *Bam*HI sites. This resulting plasmid, named HuFd/pET9a, was transformed into *E. coli* strain BL21(DE3)/pLysS for protein expression. The entire DNA sequence of the HuFd structural gene after incorporation into the expression vector was confirmed by standard, double-strand DNA sequencing.

For the production of each Cys-to-Ser mutation, the DNA fragment containing the coding sequence of HuFd, released from plasmid HuFd/pET9a with restriction enzymes *Eco*RI and *Sal*I, was cloned into the M13(mp18) vector. First, Cys^{95} was mutated into Ala. In this background, the codons of each of the other four cysteines were changed individually to serine codons by single-strand site-directed mutagenesis. Sequences of the oligonucleotides used in cloning and mutagenesis are provided as supporting information.

Protein Production, Cluster Assembly, and Purification. A single colony of *E. coli* BL21(DE3)/pLysS, containing plasmid HuFd/pET9a, was selected and grown overnight at 37 °C in 5 mL of LB medium with 100 mg of kanamycin/L and 34 mg of chloramphenicol/L. This overnight culture was used to inoculate 1 L of LB medium containing 100 mg of kanamycin/L and 34 mg of chloramphenicol/L. The culture was incubated at 37 °C until the A_{600} of the culture reached 0.6, and then IPTG was added to a concentration of 100 mg/L to induce protein expression. After induction, the culture was incubated at 37 °C for another 16 h, and then the bacteria were harvested by centrifugation. The cell pellet was resuspended in 50 mM phosphate buffer (pH 7.4) and stored at −20 °C until needed.

The frozen cells were melted and then lysed by a freeze−thaw cycle followed by sonication. Ultrapure urea was added to the cell lysate to a final concentration of 8 M. Reconstitution of the [2Fe-2S] cluster was achieved by the procedure described by Coghlan and Vickery (1991) and Cheng et al. (1995). The refolded holoprotein was then loaded onto a DE53 anion exchange column and eluted with 1 M NaCl in 50 mM phosphate buffer (pH 7.4). The protein was subjected to further purification by ion exchange chromatography (Q-Sepharose and DEAE-Sepharose) and gel filtration (Sephacryl S-100). Protein fractions with A_{414}/A_{276} ratios greater than 0.78 were considered to be more than 95% pure. The concentration of HuFd was estimated from the absorbance at 414 nm with $\epsilon_{414} = 11$ (mM cm)$^{-1}$. The overall yield of purified protein was ∼30 mg per liter of culture.

Spectroscopy. Optical absorption spectra were obtained with a Hewlett-Packard 8452A 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, respectively, by using a Hewlett-Packard 5245A frequency converter/5245L electronic counter and a Varian gaussmeter. 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.

All EPR samples contained 0.4 mL of 1−3 mM ferredoxin in 100 mM sodium phosphate buffer with 100 mM sodium chloride; the pH was 8.0. Proteins were reduced by introducing 2 mg of solid sodium dithionite into the sample tube under argon. Since the reduction caused a certain

degree of denaturation of all the Cys-to-Ser mutants, the exact concentrations were undetermined.

RESULTS

Protein Expression and [2Fe-2S] Cluster Assembly. The strategy adopted for overproducing HuFd was the same as that developed for preparing the *Anabaena* 7120 ferredoxins (Cheng et al., 1995). An expression vector HuFd/pET9a was constructed, which contained the entire gene coding for mature HuFd; this was transformed into *E. coli* strain BL21-(DE3) containing plasmid pLysS, which codes for a lysozyme that assists in breaking down the cell walls. The synthesis of T7 RNA polymerase was induced by the addition of IPTG, which, in turn, transcribed the target DNA in the plasmid and led to high-level production of the ferredoxin. The harvested cell pellet showed a red-brown color, indicating that the expression system produced measurable amounts of the holoferredoxin. The strongest protein band on the SDS/urea polyacrylamide gel corresponded to HuFd. Direct isolation of the holoprotein led to yields of about 10 mg of pure HuFd per liter of culture. The overall yield was increased to 30 mg per liter of culture by reconstituting the iron−sulfur cluster *in vitro* from the whole cell lysate. As described previously for the *Anabaena* ferredoxin (Cheng et al., 1995), the ferredoxin product was converted to apoprotein by the addition of urea; the cluster was reconstituted by addition of sulfide and Fe(III), and the resulting holoprotein was purified.

HuFd mutant C95A, which lacks the fifth (non-ligand) cysteine (Cupp & Vickery, 1988), was produced primarily as the holoprotein; its mobility on SDS/urea polyacrylamide gels was identical to that of wt HuFd. Judging from the pale color of the cell pellets, the four other Cys-to-Ser mutant proteins were produced in *E. coli* mostly as apoproteins. SDS/urea polyacrylamide gel electrophoresis of the cell extracts showed major bands with mobilities similar to that of wt apoferredoxin. *In vitro* reconstitution of the iron−sulfur cluster in these proteins was signaled by the generation of color: green-brown for C46S+C95A and red-brown for wt, C95A, and the other three double mutants (C52S+C95A, C55S+C95A, and C92S+C95A).

Although the C95A mutant had wild-type stability, each of the four ligand Cys-to-Ser mutants was much less stable than wt HuFd. The Cys-to-Ser mutants of HuFd were found to be less stable than the corresponding Cys-to-Ser mutants of VFd. Color bleaching from the ferredoxin was observed during the purification of all four mutants. The low stability of these mutant proteins made it impractical to carry out additional studies analogous to those conducted with the Cys-to-Ser mutants of VFd: reduction potential determinations (A. Weber-Main et al., in preparation), NMR spectroscopy (Cheng et al., 1994), structure determination by X-ray crystallography, and investigation of electron transport properties (Holden et al., 1994).

All four mutants could be reduced by sodium dithionite. The reduced samples bleached very quickly unless frozen. This was taken to indicate that the mutants are less stable in their reduced form than in their oxidized form. As indicated by the speed for color bleaching upon reduction, the mutant protein stability decreased in the following order: C55S+C95A (most stable) > C52S+C95A > C92S+C95A > C46S+C95A (least stable). Air reoxidization of the reduced mutants resulted in loss of the iron−sulfur cluster, as indicated by color-bleaching.

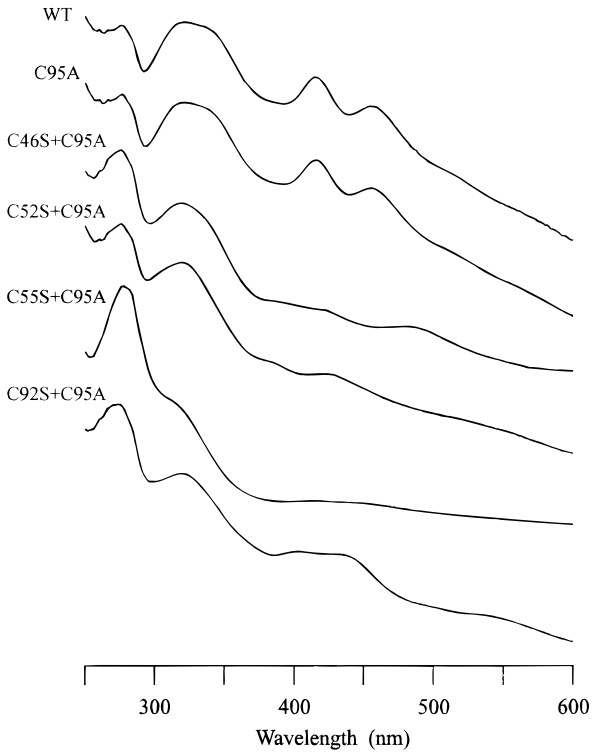

FIGURE 2: UV−visible spectra of wild-type (wt) human ferredoxin and mutants: C95A; C46S+C95A; C52S+C95A; C55S+C95A; C92S+C95A.

Spectroscopic Studies. Purified, reconstituted, recombinant HuFd exhibited optical (Figure 2), EPR (Figure 3), and NMR spectra identical to those previously reported for HuFd isolated from other production systems (Coghlan & Vickery, 1989; Skjeldal et al., 1991). The UV−visible spectra had the usual absorption maxima at 456, 414, 322, and 276 nm, and the maximum A_{414}/A_{276} ratio (0.82) was close to the expected value. Reduced, recombinant HuFd exhibited an axial EPR signal with g values of 1.94 (g_1), 1.94 (g_2), and 2.02 (g_3). NMR spectra of the oxidized and reduced forms of HuFd showed the characteristic patterns of hyperfine-shifted peaks (Skjeldal et al., 1991). The single mutant, C95A, showed optical spectra (Figure 2 and Table 1) identical to those of wt HuFd. The reduced form of the C95A mutant exhibited an axial EPR signal with exactly the same principal g values as wt (Figure 3 and Table 1).

Figure 2 shows UV−visible spectra of the four ligand Cys-to-Ser mutants of HuFd. The spectra of the mutants are distinct from each other and from wt, indicating differences in ligand coordination to the iron−sulfur cluster. As with other ferredoxins, the absorption maxima observed in 300−600 nm range (summarized in Table 1) must arise from dipolar allowed sulfur (or oxygen) and iron charge transfer (Noodleman & Baerends, 1984). It is interesting that the UV−visible spectrum of C92S+C95A looks very similar to those of plant-type ferredoxins, except for blue shifts of absorption maxima in the 300−600 nm range (Table 1).

EPR spectra of the reduced mutants are shown in Figure 3. Their calculated g values are listed in Table 1. In contrast to the UV−visible spectra, which show large individual variations for each Cys-to-Ser mutant, the EPR spectra of these mutants show few differences. The g_{av} values of the

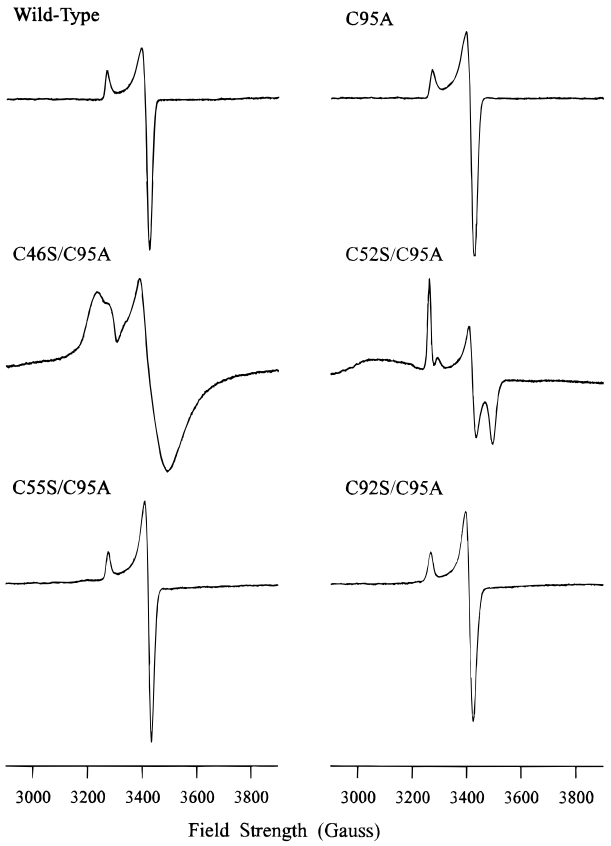

FIGURE 3: EPR spectra of reduced wild-type human ferredoxin and mutants at 10 K. Spectra were collected at varied conditions as listed below. wt: 9.237 GHz; modulation amplitude, 2 *g* at 1 mW power. C95A: 9.254 GHz; modulation amplitude, 2 G at 1 mW power. C46S+C95S: 9.234 GHz; modulation amplitude; 8 G at 1 mW power. C52S+C95S: 9.232 GHz; modulation amplitude, 2 G at 1 mW power. C55S+C95A: 9.254 GHz; modulation amplitude, 8 G at 1 mW power. C92S+C95A: 9.254 GHz; modulation amplitude, 8 G at 1 mW power.

Table 1: Optical Absorption Maxima and EPR *g* Values Of Wild-Type and Mutant Human Ferredoxin

variant	UV−visible spectroscopy absorption maxima (nm)[a]	EPR spectroscopy			
		g_x	g_y	g_z	g_{av} [a]
wild-type	276, 322, 414, 456	1.94	1.94	2.02	1.97
C95A	276, 322, 414, 456	1.94	1.94	2.02	1.97
C46S+C95A	276, 320, 378,[b] 414, 480	1.93	1.93	2.04	1.97
C52S+C95A	276, 320, 378,[b] 422	1.89	1.93	2.02	1.95
C55S+C95A	276, 324, 432	1.93	1.93	2.02	1.96
C92S+C95A	276, 320, 402, 428	1.94	1.94	2.04	1.97

[a] Calculated from $g_{av} = \sqrt{(g_x^2 + g_y^2 + g_z^2)}/3$. [b] *Shoulder*.

mutants, which varied between 1.95 and 1.97, were identical or nearly identical to that of the wild-type ($g_{av} = 1.97$) (Table 1). C55S+C95A and C92S+C95A exhibited axial type EPR spectra almost identical to that of wild-type HuFd.

DISCUSSION

Protein Overexpression and Purification. Because they suspected that the mammalian protein might be degraded by proteases when produced heterologously, the first group to express the cloned HuFd gene in *E. coli* (Coghlan & Vickery, 1989) chose to use a fusion protein. The rationale was that a fusion protein should be less susceptible to proteolysis. The disadvantages of this approach are that an expensive protease (factor Xa) is needed to cleave out the desired product and that the overall yield depends on the efficiency of this additional step. Our interest in incorporating stable isotopes from relatively expensive precursors (Xia et al., 1995) and in making a number of site-directed mutants motivated us to optimize the protein yield; for this, a direct protein expression strategy is preferred. Two groups, Palin et al. (1992) and Uhlmann et al. (1992), reported direct expression of bovine ferredoxin (adrenodoxin) in *E. coli*. At almost the same time, Ta and Vickery (1992) cloned a gene from *E. coli* that codes for a [2Fe-2S] ferredoxin that is a close homologue of HuFd. These results suggested to us that it should be possible to overproduce the mature form of HuFd in *E. coli*.

By following the approach used in overproducing VFd (Cheng et al., 1994), we obtained consistent yields of 30 mg of native HuFd per liter of *E. coli* culture. This simplified overproduction and purification scheme yielded a product that gave optical and EPR spectra identical to those previously published for HuFd.

High-level protein production required a long incubation time (16 h) following induction; by comparison, a much briefer incubation time (2 h) was needed to produce similar levels of VFd (Cheng et al., 1995). Similarly long incubation periods had been reported for optimal expression in *E. coli* of the HuFd fusion protein (Coghlan, & Vickery, 1989) and of bovine ferredoxin (Palin et al., 1992). The slow protein synthesis was attributed initially to differences in codon frequencies in the HuFd gene (Mittal et al., 1988) as compared to typical *E. coli* genes (Ikemura, 1981). Consequently, we mutated five of the 12 most unfavorable codons of HuFd into ones more optimal for protein expression in *E. coli* (Ile^{7} and Ile^{12}, ATA→ATC; Gly^{83}, GGA→GGT; Arg^{87}, AGA→AGC; Arg^{89}, CGG→CGT). However, these codon changes did not lead to a significant increase in the level of protein production. We conclude that the rate of protein synthesis was not limited by codon usage or that the usage of those codons that were mutated was not rate determining.

The red-brown color of the cell pellet indicated that human holoferredoxin is formed in *E. coli*. When HuFd was purified directly from the cell, the protein yield per liter culture was only 5−10 mg. As with VFd, it was found that the yield per liter of culture could be increased to ∼30 mg by *in vitro* [2Fe-2S] cluster reconstitution; these results suggest that more than 70% of the HuFd overproduced in *E. coli* failed to incorporate the iron−sulfur cluster. The cluster incorporation machinery of the cells apparently cannot keep up with the high rate of protein production.

When one of the normal iron−sulfur ligand cysteines of *Azotobacter vinelandii* 7Fe ferredoxin was mutated to serine, a nearby free cysteine replaced it as a cluster ligand (Martin et al., 1990). Although this type of rescue was not observed with Cys-to-Ser mutants of bovine ferredoxin (Uhlmann, 1992), we decided to avoid the possibility of such a ligand replacement by the free cysteine of HuFd by mutating the fifth cysteine (Cys^{95}) to alanine. The subsequent four Cys-to-Ser mutations were created in the C95A background.

Each of these four Cys-to-Ser mutants retained spectral properties characteristic of [2Fe-2S] ferredoxins. Each could be reduced by sodium dithionite. These results indicate that the iron−sulfur cluster of vertebrate-type [2Fe-2S] ferredoxins, as was found for plant-type (Cheng et al., 1994) and bacterial-type (Meyer et al., 1994) ferredoxins, can tolerate

the replacement of at least one ligand cysteine by serine. The formation of [2Fe-2S] cluster in all four Cys-to-Ser mutants of human ferredoxin was also confirmed by MCD and RR studies of these mutants (L. A. Akin and M. K. Johnson, personal communication).

EPR Spectra of Cys-to-Ser Mutants. Bertrand and Gayda (1979, 1980) proposed a model to explain the variation in the *g* values of different $[2Fe\text{-}2S]^+$ clusters as a function of perturbation of the ligand-field (or its rhombicity) at the ferrous site. The Bertrand−Gayda model (Bertrand et al., 1985) puts $[2Fe\text{-}2S]^+$ clusters into two classifications according to their g_{av} values: ferredoxin-type and Rieske-type $[2Fe\text{-}2S]^+$ clusters ($g_{av} \approx 1.96$ and 1.91 for ferredoxin-type and Rieske-type, respectively). Within each class, the *g* values are a linear function of the rhombicity at the ferrous site, and the ligand-field energies of the Fe(II) ion should be similar. This model suggests that Cys-to-Ser modifications of ligands to the Fe(II) site should have a greater effect on EPR spectra than modifications of ligands to the Fe(III) site and that the former modifications should decrease the EPR g_{av} values (Werth et al., 1990).

NMR studies of plant-type ferredoxins have established which cysteines are ligated to Fe(II) (Cys^{41} and Cys^{46}) and which are ligated to Fe(III) (Cys^{49} and Cys^{79}) in the reduced protein (Dugad et al., 1990; Skjeldal et al., 1991). Furthermore, it was concluded from NMR studies of Cys-to-Ser mutants of VFd that these mutations do not alter which iron is reduced (Cheng et al., 1994). Assignments of which cysteines ligate Fe(II) and which cysteines ligate Fe(III) also have been made for a bacterial-type ferredoxin, [2Fe-2S] CpFd, on the basis of EPR results from Cys-to-Ser mutants (Meyer et al., 1994). It has not been possible yet to assign which cysteines are ligated to Fe(II) and which to Fe(III) in any vertebrate-type ferredoxin; the methods used previously have not proved applicable. The hyperfine-shifted signals of vertebrate-type ferredoxins relax too rapidly for the kind of NOE measurements that were critical to the assignments in plant-type ferredoxins, and, as discussed below, the EPR data from the Cys-to-Ser mutants of both VFd and HuFd do not fit the theoretical model (Bertrand & Gayda, 1979, 1980) that was used in the assignments in [2Fe-2S] CpFd (Meyer et al., 1994).

In VFd, replacement of Fe(II) ligand Cys^{41} by Ser led to a decrease in g_{av} from 1.96 of the wild-type to 1.91; by contrast, individual Cys-to-Ser substitutions at the other three cysteines, including Cys^{46} which is the other ligand to the Fe(II) ion, produced very little change in g_{av} (Cheng et al., 1994). The four ligand Cys-to-Ser mutants of HuFd all show g_{av} values (between 1.95 and 1.97) that differ very little from that of wt HuFd (1.97) (Table 1). One of the Fe(III) ligand Cys-to-Ser mutations of VFd (C49S) exhibited a nearly axial EPR signal which was different from the rhombic EPR signal of wt; the other Fe(III) ligand mutant (C79S) showed an essentially rhombic EPR signal (Cheng, et al., 1994). This result indicated that the ferric ion in the $[2Fe\text{-}2S]^+$ cluster can contribute a large rhombic component to the EPR spectrum (Hagen, 1992). Taken together, the results discussed above imply the following for the $[2Fe\text{-}2S]^+$ cluster: (i) Cys-to-Ser replacement of a ligand to Fe(II) does not necessarily lead to a decrease in the EPR g_{av}; (ii) Cys-to-Ser replacement of a ligand to Fe(III) can alter the anisotropy of the EPR spectrum. These results do not fit the expectations of the Bertrand−Gayda model (Bertrand et al., 1985).

Table 2: Comparison of Properties of Cys-to-Ser Mutants of Human Ferredoxin with Those from *Anabaena* 7120 Vegetative Ferredoxin

(A) Anisotropy of EPR Spectra

	human ferredoxin		vegetative ferredoxin[a]	
mutation site[b]	mutation	EPR	mutation	EPR
wt	wt	axial	wt	rhombic
c1	C46S	axial	C41S	rhombic
c2	C52S	rhombic	C46S	rhombic
c3	C55S	axial	C49S	axial
c4	C92S	axial	C79S	rhombic

(B) Relative Stabilities of the Mutant Proteins

	human ferredoxin		vegetative ferredoxin[a]	
stability[c]	mutation	mutation site[b]	mutation	mutation site[b]
1	C55S	*c3*	C46S	*c2*
2	C52S	*c2*	C49S	*c3*
3	C92S	*c4*	C41S	*c1*
4	C46S	*c1*	C79S	*c4*

[a] Data from Cheng et al. (1994). [b] The symbols *c1*, *c2*, *c3*, and *c4* refer to the ligand cysteines in their order in the protein sequence (Figure 1). [c] Stability decreases from 1 to 4.

Why is the Bertrand−Gayda model, which explains differences in the EPR spectra of ferredoxin-type and Rieske-type clusters, inappropriate for single-site Cys-to-Ser mutations in [2Fe-2S] ferredoxins? The answer may concern the magnitude of the change being considered. Rieske-type [2Fe-2S] clusters differ from ferredoxin-type [2Fe-2S] clusters by the substitution of both cysteines ligated to one iron by histidines; the electronic properties of these ligands are quite different. It may be that the much more conservative substitution of a single S (from Cys) by O (from Ser) perturbs the EPR g_{av} value to a much smaller extent. Alternatively, the mutation may alter hydrogen bonding to the cluster. An example of the latter effect is provided by studies of cytochrome b_6f, which contains a Rieske-type cluster. The EPR g_{av} of the [2Fe-2S] cluster was found to shift to 1.95 upon binding of inhibitor DBMIB (Malkin, 1981). Electron spin echo envelope modulation (ESEEM) results indicated that DBMIB treatment of this protein causes no change in iron−sulfur cluster ligands and little change in the magnitude of magnetic couplings between the unpaired electron and the nitrogen nuclei of the ligated histidine imidazoles (Britt et al., 1991). The ESEEM study also revealed that DBMIB inhibition may induce a change of conformation in the vicinity of [2Fe-2S] cluster which generates new hydrogen bonds between the cluster and nearby peptide nitrogens. This suggests that the pattern of hydrogen bonds about the cluster are important determinants of EPR *g* values in iron−sulfur clusters (Britt et al., 1991).

Comparison of Cys-to-Ser Mutants of HuFd and VFd. Interesting correlations can be drawn from comparing the present results from HuFd with those from the analogous studies of Cys-to-Ser mutants of VFd (Cheng et al., 1994). Human ferredoxin and VFd are representatives of the two simplest classes of [2Fe-2S] ferredoxins: vertebrate ferredoxins and plant-type ferredoxins, respectively. Both are monomeric proteins that contain only one iron−sulfur cluster. The four cluster ligand cysteines in each of these two ferredoxins can be designated as *c1*, *c2*, *c3*, and *c4* according to their position in the alignment (Figure 1) of their amino acid sequences (VFd, *c1* = Cys^{41}, *c2* = Cys^{46}, *c3* = Cys^{49}, and *c4* = Cys^{79}; HuFd, *c1* = Cys^{46}, *c2* = Cys^{52}, *c3* = Cys^{55}, *c4* = Cys^{92}). Table 2A summarizes the reduced-state EPR

parameters for wild-type and mutant VFd and HuFd. Wild-type VFd shows a rhombic EPR spectrum with *g* values of 2.05, 1.96, and 1.88. Of the four Cys-to-Ser mutations of VFd, only that at site *c3* resulted in a change of the EPR signal, from rhombic to near-axial (Cheng et al., 1994). In HuFd, a change in the EPR spectral anisotropy is also seen to result from substitution at only one of the four sites; in this case the anisotropy changes from near-axial to rhombic, and the sensitive site is at *c2*. Thus, in both classes of ferredoxin, the substitution of a single S atom of one ligating residue by O suffices to switch the electron distribution within the cluster, resulting in a change in the anisotropy of the EPR spectrum. The anisotropy change is inverse in each class, and the sensitive position is at a different cysteine.

The relative stabilities of the Cys-to-Ser mutants of the two ferredoxins upon reduction are compared in Table 2B. For both ferredoxins, mutations at the *c2* or *c3* positions are less destabilizing than mutations at the other two positions. The *c3* mutant of HuFd is the least destabilizing, whereas the *c2* mutant of VFd is the least destabilizing. Cysteines *c2* and *c3* are those in the -Cys-X-X-Cys- sequence which are conserved in all known members of these families of ferredoxins. The *c1* mutant of HuFd is the most destabilizing, whereas the *c4* mutant of VFd is the most destabilizing.

Several different effects are expected to contribute to the comparative stabilities of these wild-type and mutant ferredoxins. Because of their differences in pK_a values, 10.8 for the sulfhydryl of Cys and around 16 for the hydroxyl of Ser, it is energetically more costly to produce a serinate than a cysteinate ligand; the energy difference is about 7 kcal mol^{-1} at 298 K. This factor should be constant for substitutions at any of the four cysteines in either type of ferredoxin (VFd or HuFd). The Fe−O and Fe−S bonds themselves have inherently different bond energies which will depend on the oxidation state of the iron. The bond energy for diatomic Fe−O is 93 kcal mol^{-1}, and that for Fe-S is 77 kcal mol^{-1}. Additional factors that influence protein stability and may influence position-sensitive effects of S-to-O substitutions include changes in strain energy of the cluster itself, differences in flexibility of the protein, differential hydrogen bonding to the cluster, and alterations in solvent accessibility to the cluster. A high-resolution crystal structure has been solved for VFd (Rypniewski et al., 1991; Holden et al., 1995), but further analysis of these differences awaits determination of the structure of HuFd at high resolution. A low-resolution NMR solution structure is available for putidaredoxin (Pdx) (Pochapsky et al., 1994), a ferredoxin with vertebrate-type [2Fe-2S] cluster. Pdx contains cluster-ligating cysteines at equivalent positions as those of HuFd: 39, 45, 48, and 86. However, the significant difference in the secondary structures of HuFd and Pdx (Figure 1) suggests that Pdx may not serve as a reliable model for the structure of HuFd.

In both HuFd and VFd, the least destabilizing sites for Cys-to-Ser substitutions are at the cysteines central to the sequence (*c2* and *c3*). This is satisfying from the standpoint of chain entropy considerations, such as those invoked in estimating the stabilizing effect of disulfide bridges (Pace et al., 1988). The ligation pattern of the plant-type ferredoxins places the two most destabilizing Cys-to-Ser mutations of VFd (C41S and C79S) on different iron atoms. Cupp and Vickery (1988) pointed out that vertebrate ferredoxins could either adopt the ligation pattern observed in plant-type ferredoxins or an alternative, zinc-finger-type ligation pattern. The plant-type ligation pattern places the two central cysteines on different iron atoms, whereas the zinc-finger ligation pattern places the two internal cysteines on a single iron. Further studies will be needed to determine which ligation pattern HuFd uses and which cysteines ligate the reducible iron.

ACKNOWLEDGMENT

The authors thank Dr. Larry E. Vickery for providing the gene (pHFdx1) for human ferredoxin and for helpful discussions and Dr. Brian Fox for help in collecting the EPR spectra.

SUPPORTING INFORMATION AVAILABLE

One table which shows sequences of the oligonucleotides used in cloning and mutagenesis (one page). Ordering information is given on any masthead page.

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BI960467F