Site-Directed Mutations of the 4Fe-Ferredoxin from the Hyperthermophilic Archaeon *Pyrococcus furiosus*: Role of the Cluster-Coordinating Aspartate in Physiological Electron Transfer Reactions†

Zhi Hao Zhou and Michael W. W. Adams*

Department of Biochemistry and Molecular Biology and Center for Metalloenzyme Studies, University of Georgia, Athens, Georgia 30602

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ABSTRACT: Ferredoxin from the hyperthermophilic archaeon *Pyrococcus furiosus* is a monomeric protein (7.5 kDa) that contains a single $[4Fe\text{-}4S]^{1+,2+}$ cluster. The protein is unusual in that its cluster is coordinated by three Cys and one Asp residue, rather than by the typical four Cys residues. Site-directed mutagenesis has been used to obtain mutant forms in which the cluster-coordinating Asp was replaced by Cys (D14C) and also by Ser (D14S), together with a third mutant (A1K) which contained N-Met-Lys at the N-terminus instead of N-Ala. Analyses using UV−visible absorption, far-UV circular dichroism, and EPR spectroscopy showed that there were no gross structural differences between the native and the three mutant forms and that they each contained a [4Fe-4S] cluster. The reduction potentials, determined by direct electrochemistry (at 23 °C, pH 8.0), of the D14S, D14C, and A1K mutants were −490, −422, and −382 mV, respectively, which compare with values of −375 mV for native [4Fe-4S]-containing ferredoxin and −160 mV for the [3Fe-4S]-containing form. The native, D14C, and A1K proteins functioned as electron acceptors *in vitro* at 80 °C for pyruvate ferredoxin oxidoreductase (POR) and aldehyde ferredoxin oxidoreductase (AOR) from *P. furiosus* using pyruvate and crotonaldehyde as substrates, respectively. The calculated k_{cat}/K_m values were similar for the three proteins when ferredoxin reduction was measured either directly by visible absorption or indirectly by coupling ferredoxin reoxidation to the reduction of metronidazole. In contrast, using the D14S mutant and the 3Fe-form of the native ferredoxin as electron acceptors, the activity with AOR was virtually undetectable, and with POR the calculated k_{cat}/K_m values were at least 3-fold lower than those obtained with the native (4Fe-), D14C, and A1K proteins. The ability of this 4Fe-ferredoxin to accept electrons from two oxidoreductases of the same organism is therefore not absolutely dependent upon Asp14, as this residue can be effectively replaced by Cys. However, the efficiency of electron transfer is compromised if Asp14 is replaced by Ser, or if the 4Fe-cluster is converted to the 3Fe-form, but Asp14 does not appear to offer any kinetic advantage over the expected Cys.

Iron−sulfur clusters of the cubane [4Fe-4S] type are one of the, if not the, most common electron transfer groups in biological systems. They are present in all known membrane-bound respiratory systems, in an array of both simple and complex enzymes, and in small redox proteins known as ferredoxins [for reviews, see Howard and Rees (1991), Cammack (1992), Matsubara and Saeki (1992), and Johnson (1994)]. In most cases, the [4Fe-4S] cluster undergoes a one-electron redox couple ($[4Fe\text{-}4S]^{2+/1+}$) at low potential, typically near −400 mV, although high-potential [4Fe-4S]$^{3+/2+}$ clusters ($E_m \sim +350$ mV) are present in some small redox proteins known as high-potential iron proteins (Hipip). In addition, some proteins contain a $[3Fe\text{-}4S]^{+/0}$ cluster. This is a derivative of the cubane-type, but it has a higher potential (typically ∼−100 mV) and is often formed by degradation of a [4Fe-4S] cluster (Beinert & Thomson, 1983).

In both simple and complex iron−sulfur proteins, the iron atoms of the [4Fe-4S] cluster are coordinated typically by the S atoms of four cysteinyl residues, although there are some notable exceptions. The best studied example is aconitase in which the unique Fe site of its 4Fe-cluster has a solvent hydroxyl as a fourth ligand in the absence of substrate, while substrate binding results in a six-coordinate Fe site with two ligands from the substrate carboxylate (Kennedy & Stout, 1992; Lauble et al., 1994). Aconitase is representative of a large class of hydratase-type enzymes that contain a [4Fe-4S] cluster, a class which includes serine dehydratase, fumarase, and isopropylmalate isomerase (Flint et al., 1993ab; Hofmeister et al., 1994; Rodriguez et al., 1996; Muh et al., 1996). Similarly, one of the [4Fe-4S] centers in a NiFe-hydrogenase, an enzyme which reversibly activates H_2 gas, has a coordinating His residue replacing one of the expected Cys, and this is proposed to facilitate electron transfer from the cluster to an external electron carrier (Volbeda et al., 1995). In addition, the so-called "P" clusters, which structurally resemble two cubane-type clusters, in the MoFe protein of the N_2-fixing enzyme nitrogenase, contain incomplete Cys coordination with Ser replacing at least one of the expected Cys ligands (Kim & Rees, 1994).

The consequences of substituting a Cys ligand in a biological cubane cluster on the electronic properties, redox potential, and function of the protein that contains it are not understood, and ambiguous results have been obtained when

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* Address correspondence to this author at the Department of Biochemistry, Life Sciences Building, University of Georgia, Athens, GA 30602-7229. Telephone: 706 542-2060. FAX: 706 542-0229. E-MAIL: adamsm@bscr.uga.edu.

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Cys residues are replaced using site-directed mutagenesis approaches. So far, such investigations have been restricted to complex multisubunit systems, and elucidating the effects of Cys substitution on a particular cluster have been hampered by the presence of other redox active groups within the protein. In fact, the usual objective of such studies has been to determine which of a number of Cys residues are involved in coordinating a particular cluster, for example, in nitrogenase iron protein (Howard et al., 1989), nitrate reductase (Augier et al., 1993a), and fumarate reductase (Manodori et al., 1992). In other examples, replacement of a coordinating Cys (Cys^{II}, the second Cys in the consensus sequence motif: -$Cys^{I}X_2Cys^{II}X_2Cys^{III}X_nCys^{IV}$P-, where X and *n* vary) by either Asp or Ser led to the production of an enzyme with a [3Fe-4S] rather than a [4Fe-4S] cluster, as with DMSO reductase (Rothery & Weiner, 1991) and clusters F_A and F_B of PscC of photosystem I (Zhao et al., 1992). So far there are three examples where replacement of a single Cys (Cys^{II}) by Ser appears to lead to intact [4Fe-4S] clusters. These are cluster F_X of PscB (Warren et al., 1993), cluster II in nitrate reductase (Augier et al., 1993b), and the 4Fe-cluster in subunit FRD of fumarate reductase (Kowal et al., 1995). However, none of these proteins were obtained in a pure form, and the mutant forms of the clusters were not extensively characterized. Moreover, while cluster F_X of PscB was functional when Cys^{II} was replaced by Ser, this was not the case with His or Asp substitution for the same Cys (Smart et al., 1993).

To investigate the structural, electronic, and functional effects of Cys substitution on a biological cubane cluster, we have chosen as a model system the ferredoxin (Fd)[1] from the hyperthermophilic archaeon *Pyrococcus furiosus* (Pf), an organism that grows optimally at 100 °C. Pf Fd is a monomeric protein of 66 amino acids (7.5 kDa) and contains a single [4Fe-4S] cluster (Aono et al., 1989; Conover et al., 1990a). It is a remarkably stable protein, being unaffected after a 12 h incubation at 95 °C (Aono et al., 1989), and is also unusual in that in its consensus sequence of Cys residues, Cys^{II} (position 14), is replaced by an Asp residue. However, the 4Fe-form is stable, and it does not lose iron during purification, although it can be quantitatively converted to the 3Fe-form by ferricyanide treatment (Conover et al., 1990a). NMR analyses have demonstrated that the unique Fe atom of the [4Fe-4S] cluster is coordinated by the carboxylate of Asp14 in both the oxidized and reduced forms (Calzolai et al., 1995). The 4Fe-center of Pf Fd binds exogenous ligands such as cyanide (Conover et al., 1991), apparently by displacement of the Asp residue at the unique Fe site (Telser et al., 1995). Obviously, this has implications for substrate binding by complex cubane cluster containing enzymes. The reduced 4Fe-cluster in Pf 4Fe-Fd also exhibits anomalous spectroscopic properties as it has a predominant $S = {}^3/_2$ ground state as determined by low-temperature EPR (although it is $S = {}^1/_2$ at ambient temperature as measured by NMR: Calzolai et al., 1995). This is in contrast to the pure $S = {}^1/_2$ ground state for reduced 4Fe-clusters in most other proteins with complete Cys ligation (Conover et al., 1990a). Significant structural information is also available on Pf Fd. Its secondary structure has been determined (Teng et al., 1994), a disulfide bridge formed by two additional Cys residues (21 and 48) remote from the cluster has been shown to be redox active (Gorst et al., 1995a), and sequence-specific assignments of the ligated Cys residues have been determined for both the 3Fe-form (Gorst et al., 1995b) and 4Fe-form (Calzolai et al., 1995).

Pf Fd also provides a model system with which to investigate features of intermolecular electron transfer since this protein plays a central role in the primary metabolism of Pf [see Adams and Kletzin (1996)]. The Fd serves as the electron acceptor for oxidoreductases involved in carbohydrate fermentation, including those that utilize pyruvate (Blamey & Adams, 1993) and glyceraldehyde 3-phosphate (Mukund & Adams, 1995) as substrates, and for those involved in peptide fermentation, including those that utilize aldehydes (Mukund & Adams, 1991), formaldehyde (Mukund & Adams, 1993), indolepyruvate (Mai & Adams, 1994), 2-ketoglutarate (Mai & Adams, 1996), and 2-ketoisovalerate (Heider et al., 1996) as substrates. Reduced Fd is oxidized by ferredoxin:NADP oxidoreductase (Ma & Adams, 1994), and NADP serves as the electron donor to the H_2-evolving hydrogenase of Pf (Ma et al., 1994). Hence, there are a variety of enzymes available which utilize Pf Fd as their physiological electron carrier.

The gene encoding Pf Fd was recently cloned and expressed in *Escherichia coli* to give a recombinant form that was indistinguishable from the native protein (Heltzel et al., 1994). Herein we report on the spectroscopic, redox, and functional properties of three site-specific mutants of Pf Fd, two of which involve the cluster-liganded residue Asp14. We provide the first evidence for a serine-ligated [4Fe-4S] cluster in a pure iron−sulfur protein, and show that Asp14 is not obligatory for efficient electron transfer between the Fd and two physiological-relevant enzymes from Pf.

MATERIALS AND METHODS

Enzymes, Chemicals, and E. coli Strains. Restriction enzymes, oligonucleotides, T4 DNA ligase, *E. coli* strain JM105, and isopropyl β-D-thiogalactopyranoside (IPTG) were purchased from Stratagene (La Jolla, CA); Sequenase (version 2.0) was from US Biochemical Corp. (Cleveland, OH); [α-^{35}S]dATP was from Amersham Corp. (Arlington Heights, IL); the expression vector pTrc99A was from Pharmacia-LKB (Piscataway, NJ); and Taq DNA polymerase was from Perkin Elmer (Norwalk, CT). All enzymes were used according to the specifications of manufactures.

Plasmid Constructions and Site-Directed Mutagenesis. Plasmid pAH1993 (Heltzel et al., 1994) contains the entire gene for Pf Fd, and this was used as a template to construct mutants. Three synthetic oligonucleotides (Stratagene) were designed which started 6 bases upstream of the ATG start codon to change (a) Ala1 to Lys (AAA), 37 bases; (b) Asp14 to Ser (TCT), 57 bases; and (c) Asp14 to Cys (TGT), 57 bases. Each of these oligonucleotides was used as a primer together with an oligonucleotide (36 bases) which included the 2 C-terminal amino acids and was complementary to the downstream region of the Pf Fd gene. All PCR reactions gave a band of the expected size (220 bp) as the sole product of the reactions. The PCR fragments were purified, digested

[1] Abbreviations: Fd, ferredoxin; Pf, *Pyrococcus furiosus*; Tl, *Thermococcus litoralis*; EPPS, *N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; POR, pyruvate ferredoxin oxidoreductase; AOR, aldehyde ferredoxin oxidoreductase.

with *Nco*I and *Sma*I, and ligated into the *Nco*I and *Sma*I sites of the expression vector p*Trc*99A. The *Nco*I site of the vector is next to the *lacZ* ribosome-binding site and the IPTG-inducible *trc* promoter. The constructs were used to transform *E. coli* JM105. To confirm the desired mutations, Sanger dideoxy sequencing (Sanger et al., 1977) on double-stranded plasmid, isolated by Magic column (Promega, Madison, WI) extraction, was carried out using a Sequenase kit with [α-^{35}S]dATP.

Protein Production and Purification. *E. coli* JM105 containing the mutated Fd gene within the p*Trc*99A vector was grown aerobically at 37 °C in Luria−Bertani broth containing ampicillin (100 μg/mL) and $FeCl_3$ (25 μM). A 100 L culture was grown until $OD_{600} \sim 0.5$, and IPTG (1.5 mM) was added to induce the *trc* promoter. The cells were harvested after a further 12 h, immediately frozen in liquid N_2, and stored at −80 °C until required. The purification of the mutant Fds was carried out under anaerobic conditions, whereby all solutions were degassed and flushed with Ar and contained sodium dithionite (2 mM) to remove trace O_2 contamination. The buffer used throughout the purification was 50 mM Tris-HCl, pH 8.0. Frozen *E. coli* cells (500 g) were suspended (2 mL/g) in buffer containing EDTA (1 mM), NaCl (0.1 M), phenylmethanesulfonyl fluoride (0.14 mM), and lysozyme (0.25 mg/mL), and were incubated at 25 °C for 1 h. DNase I (6.7 μg/mL) and RNase (1.7 μg/mL) were added, and the mixture was incubated for a further 30 min. After centrifugation for 45 min (23000*g* at 4 °C), the supernatant was diluted 3-fold with buffer and was applied to a column (5 × 27 cm) of Q-Sepharose Fast Flow (Pharmacia-LKB). The column was washed with 1 column volume of buffer, and the absorbed proteins were eluted with a gradient (3 L) from 0 to 0.5 M NaCl. The mutant Fds were all eluted as 0.39−0.46 M NaCl was applied to the column. Fractions with $A_{390}/A_{280} > 0.25$ were combined and concentrated by diluting them 3-fold with buffer and applying them to a column (1.6 × 13 cm) of Q-Sepharose. The ferredoxin was eluted with a gradient (20 mL) from 0 to 1.0 M NaCl. The concentrated pool (20 mL) was applied to a column (6 × 60 cm) of Superdex 75 and eluted with buffer containing 1 M NaCl. Fractions judged pure by SDS−PAGE analysis were concentrated to ∼20 mg/ml by ultrafiltration (Amicon, Beverly, MA; YM3 membrane) and were stored as pellets under liquid N_2 until required. The concentrations of the mutant Fds were determined using a modified Lowry method (Aono et al., 1989) after precipitation by trichloroacetic acid (10%, w/v).

N-Terminal Sequence Analysis. The N-terminal sequences of the mutant proteins were determined using an Applied Biosystems Model 477 sequencer.

Reduction Potential Determinations. Differential pulse voltammetry was carried out in a micro-cell with a glassy carbon electrode at 23 °C using a PARC 263A Polarographic Analyzer (Smith et al., 1995). Pf Fd and mutants thereof were at a final concentration of 0.33 mM in 50 mM Tris-HCl, pH 8.0, and they were examined in the presence of 1 mM neomycin. Reduction potentials are reported versus the standard hydrogen electrode. The experimental conditions were 50 mV pulse height and 2 mV/s scan rate. Reduction potentials were determined to be independent of scan direction at this scan rate.

Enzyme Assays. The ability of Pf Fd and mutants thereof to function as electron acceptors for pyruvate ferredoxin oxidoreductase (POR; Blamey & Adams, 1993) and aldehyde ferredoxin oxidoreductase (AOR; Mukund & Adams, 1991) was determined spectrophotometrically either by direct measurement of Fd reduction or by coupling the reoxidation of reduced Fd to the reduction of metronidazole [1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole; obtained from Sigma Chemical, St. Louis, MO]. For the direct assay, the reaction mixture for POR contained pyruvate (10 mM), coenzyme A (0.2 mM), Pf POR (5 μg), $MgCl_2$ (1 mM), and Fd (5−150 μM) in 50 mM *N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid (EPPS) buffer, pH 8.0. The reaction mixture for AOR contained crotonaldehyde (0.2 mM), Fd (5−250 μM), and AOR (10 μg) in the same buffer. The assays were carried out at 80 °C under anaerobic conditions. The reduction of the various Fds was measured at 390 nm, and results were calculated using the measured molar absorption coefficients (see Results). One unit of activity corresponds to the reduction of 1 μmol of Fd/min for both POR and AOR. Apparent K_m and V_m values were calculated using KaleidaGraph software (Synergy Software, Reading, PA). In the coupled assay system, the same reaction mixtures were used except that Fd was used at lower concentrations (1−30 μM for POR and 5−100 μM for AOR), metronidazole (100 μM) was added, and the reaction was measured by following metronidazole reduction at 320 nm. A molar absorption coefficient of 9300 M^{-1} cm^{-1} (Chen & Blanchard, 1979) was used for oxidized metronidazole, and bleaching of the chromophore was assumed to be a one-electron process (Whillans et al., 1975; Moreno et al., 1983). Metronidazole reduction occurred at an insignificant rate with both the POR and AOR assays in the absence of added Fd, and the absorption at 320 nm of a 100 μM solution (in 50 mM EPPS, pH 8.0) remained unchanged after a 20 min incubation at 80 °C, indicating that the compound does not undergo thermal degradation under assay conditions. The samples of POR and AOR used in these studies had specific activities of 31 units/mg (using 1 mM methyl viologen as the electron acceptor; Blamey & Adams, 1993) and 41 units/mg (using 3 mM benzyl viologen as the electron acceptor; Mukund & Adams, 1991), respectively. Fd (Aono et al., 1989), POR (Blamey & Adams, 1993), and AOR (Mukund & Adams, 1991) from Pf and ferredoxin from *Thermococcus litoralis* (Busse et al., 1992) were purified by the published procedures. The 3Fe-forms of Pf Fd and of the D14S mutant were prepared by ferricyanide treatment (Conover et al., 1990a).

Spectroscopy. Optical absorption spectra were obtained with a Hewlett-Packard 8452A diode array spectrophotometer. Electron paramagnetic resonance (EPR) spectra were recorded at X-band on an IBM-Bruker ER 300D spectrometer interfaced to an ESP 3220 data system and equipped with an Oxford Instruments ITC-4 flow cryostat. Spin quantitations of the $S = {}^1/_2$ type EPR signals were determined by double integration of the first-derivative spectra and comparing them with spectra of Cu(1 mM)/EDTA(100 mM) recorded under the nonsaturating conditions. Far- and near-UV circular dichroism spectra were acquired using a JASCO 700 spectrophotometer with a N_2-purged, 1 mm quartz cell containing the Fd (14 μM) in 10 mM phosphate buffer at pH 8.0.

RESULTS AND DISCUSSION

Expression of Mutant Fds. In the expression system for the Pf Fd gene previously reported (Heltzel et al., 1994),

the amount of pure recombinant Fd obtained represented ca. 0.15% of the total soluble protein in induced cell extracts of *E. coli*. We therefore undertook an initial study to try and increase the Fd yield by varying parameters such as induction time, aerobic *vs* anaerobic growth, Fe content of the medium, and minimal *vs* complex media. Of these, only variation in induction time made a significant difference; namely, increasing the time period before harvesting cells from 2.5 h (Heltzel et al., 1994) to 12 h resulted in a 10-fold increase in the yield of Fd. Approximately 250 mg of pure Fd was obtained from ~350 g (wet weight) of *E. coli* cells (or ~3 mg/L of culture), which compares with a previously reported value of ~25 mg from a similar quantity of cells (Heltzel et al., 1994).

It was previously shown that although the gene for Pf Fd encodes an N-terminal Met residue, this is removed *in vivo* when the gene is expressed in *E. coli* to give a recombinant protein with an N-terminal Ala, like native Pf Fd (Heltzel et al., 1994). The efficiency of this process is largely dependent upon the nature of the second translated amino acid, the radius of gyration of which inversely correlates with the cleavage activity of methionine-aminopeptidase (MAP) in *E. coli* (Hirel et al., 1989). To investigate whether MAP activity limited expression of the Pf Fd gene, a mutant (A1K) was designed in which the second codon was changed from Ala to Lys. This change prevented modification by MAP as the N-terminus of the recombinant A1K protein was N-Met-Lys- instead of N-Ala. However, lack of MAP activity did not increase the production of recombinant protein, as the yields of the A1K mutant and the wild-type protein from *E. coli* were similar. Changing the cluster-coordinating Asp14 to either Ser (D14S) or Cys (D14C) also gave amounts of recombinant proteins comparable to that of the wild type. Moreover, all three mutant proteins (A1K, D14S, and D14C) contained an iron−sulfur chromophore and could be purified by following their visible absorption.

Spectroscopic Characterization of Mutant Fds. The UV−visible absorption spectra of the three mutant Fds were very similar to that of the native protein, which shows a broad absorption band near 400 nm (Aono et al., 1989). The molar absorption coefficients for native Fd and for the D14S, D14C, and A1K mutants at 25 °C were 17.0, 17.0, 20.2, and 17.1 mM^{-1} cm^{-1}, respectively. The A_{390}/A_{280} ratios for native Fd and for the D14S, D14C, and A1K mutants were 0.56, 0.54, 0.64, and 0.56, respectively. A comparison of the far-UV CD spectra of the native and three mutant Fds is shown in Figure 1. The high similarity in the spectral features suggested that there are no significant differences between the four proteins in their secondary and tertiary structures. In addition, the similarity in their A_{390}/A_{280} ratios indicated that all four proteins contain the same cluster type. Since the absorption of Fe−S bonds in the visible region is greatly increased compared to Fe−O/N, the higher ratio for the D14C mutant is consistent with only this protein containing an iron−sulfur cluster with complete cysteinyl coordination.

That the three mutant Fds did, in fact, each contain a [4Fe-4S] cluster was confirmed by EPR spectroscopy. As shown in Figure 2, the spectra of the dithionite-reduced forms of the native and A1K proteins are very similar. Although they are dominated by a broad rhombic signal centered at $g \sim 1.94$, which originates from the $S = {}^1/_2$ ground state of the $[4Fe\text{-}4S]^{1+}$ cluster, this represents only ~0.2 spin/mol. The cluster is predominantly in the $S = {}^3/_2$ state which gives rise

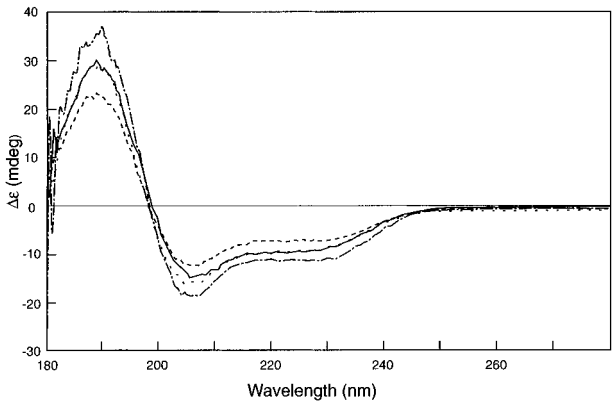

FIGURE 1: Far-UV circular dichroism spectra of native and mutant forms of *P. furiosus* Fd. The spectra were recorded as described under Materials and Methods. They represent native Fd (− · −), the D14S mutant (- - -), the D14C mutant (—), and the A1K mutant (•••).

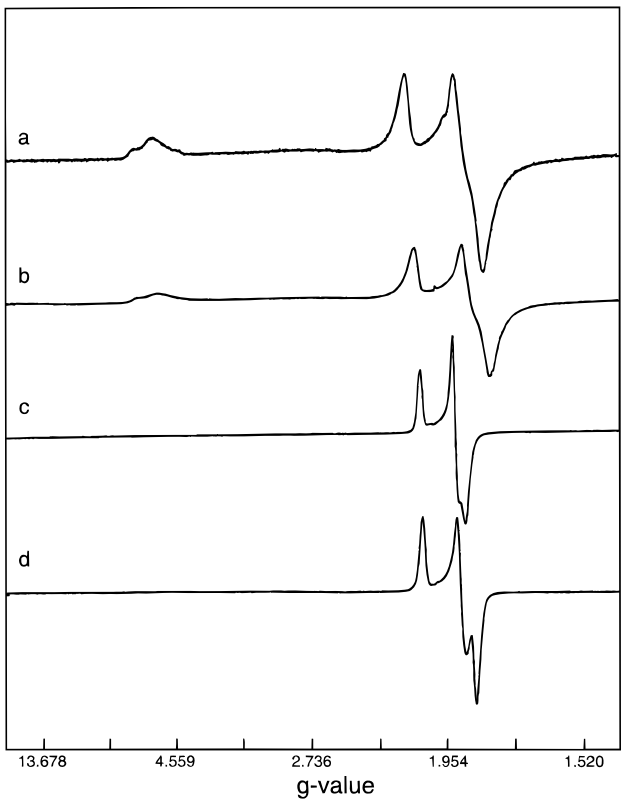

FIGURE 2: EPR spectra of the reduced forms of native and mutant forms of *P. furiosus* ferredoxin. The protein concentrations were 8 mg/mL in 50 mM Tris-HCl, pH 8.0, buffer containing sodium dithionite (2 mM). The samples were (a) native Pf Fd, (b) A1K mutant, (c) D14C mutant, and (d) D14S mutant. The spectrometer settings were as follows: microwave power, 10 mW; modulation amplitude, 0.2 mT; microwave frequency, 9.57 GHz; gain, 8×10^3. The spectra were recorded at 8 K.

to the low-field resonances near $g \sim 5$ (Conover et al., 1990a). In contrast, such low-field resonances were not detectable in spectra recorded under identical conditions (8 K, 10 mW microwave power) with the dithionite-reduced forms of the D14C and D14S mutants (Figure 2). Instead, both gave rise to much sharper rhombic spectra at $g \sim 1.94$ with similar, although not identical, g-values. These $S = {}^1/_2$ signals represented 0.42 ± 0.05 and 0.90 ± 0.05 spin/mol for the D14S and D14C mutants, respectively. Under conditions of lower temperature (4 K) and higher microwave power (50 mW), the D14S mutant exhibited very weak

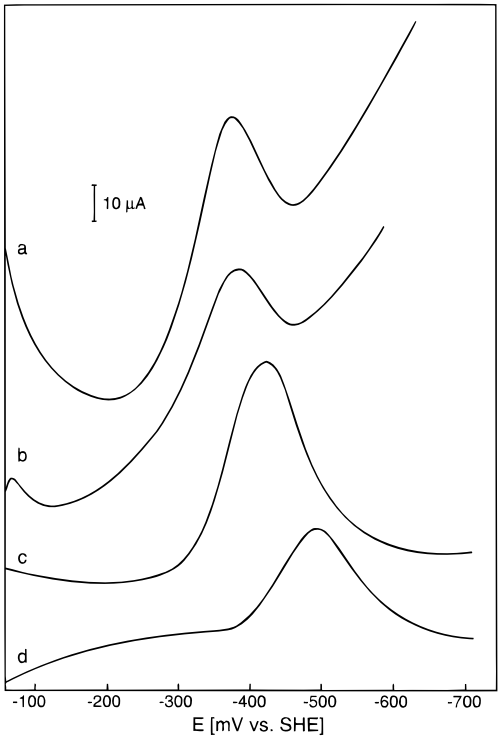

FIGURE 3: Differential pulse voltammograms of native and mutant forms of *P. furiosus* ferredoxin. Each sample contained the protein (0.33 mM) in 50 mM Tris-HCl, pH 8.0, and 1 mM neomycin, and the data were collected at 23 °C with a 50 mV pulse height and a scan rate of 2 mV/s. The samples were as follows: (a) native Pf Fd; (b) A1K mutant; (c) D14C mutant; and (d) D14S mutant.

resonances near $g \sim 5$ (data not shown), showing that the cluster ground state spin is not completely $S = {}^1/_2$. Such resonances were not observed with the D14C mutant, indicating the spin state of its cluster is homogeneous. Like the native Fd, the oxidized forms of the mutants were EPR-silent.

Hence, substitution of the cluster-coordinating Asp14 residue of Pf Fd by either Ser or Cys causes spin conversion of the [4Fe-4S]$^{1+}$ cluster from the $S = {}^3/_2$ to the $S = {}^1/_2$ spin state (Figure 2). This was anticipated for the D14C mutant as virtually all known biological [4Fe-4S]$^+$ clusters with complete cysteinyl coordination have a $S = {}^1/_2$ ground state [see Johnson (1994)]. On the other hand, in the few examples where the coordinating Cys (Cys^{II}) residue has been replaced by Ser, the results are inconsistent. Thus, an EPR signal was not assigned to the 4Fe-cluster in nitrate reductase (Augier et al., 1993b), the 4Fe-cluster F_X of PSI remained low spin albeit with a change in the *g*-values (Warren et al., 1993), and in fumarate reductase (FrdB), Cys to Ser mutagenesis caused conversion of the $S = {}^1/_2$ to $S = {}^3/_2$ state (Kowal et al., 1995). The D14S mutant of Pf Fd therefore represents the first example of a $S = {}^3/_2$ to ${}^1/_2$ spin state conversion, albeit incomplete. The biological significance of the spin state is arguable, however, in light of a recent NMR study which showed that the reduced 4Fe-cluster in Pf Fd has a ground state with $S = {}^1/_2$ at ambient temperature (Calzolai et al., 1995).

The midpoint potentials of the Pf Fd mutants were determined by differential pulse voltammetry at 25 °C (Figure 3). The values were calculated from the voltage of peak current (Bard et al., 1984), and these are summarized in Table 1. Not surprisingly, that of the A1K mutant was almost the same as that of the native protein, but the midpoint potentials of the D14C and D14S mutants were 47 and 115 mV, respectively, more negative. The very low value for the D14S mutant suggested that its cluster may not be completely reduced by sodium dithionite at pH 8.0 ($E_m \sim -500$ mV; Mayhew et al., 1978). This was confirmed by visible absorption spectroscopy. As shown in Figure 4, increasing the pH from 8.0 to 10.0 resulted in a further ~25% decrease in the visible absorption of the D14S mutant upon dithionite addition ($E_m < -700$ mV at pH 10; Mayhew et al., 1978), indicating that the protein is ~50% reduced at pH 8.0. This is consistent with the less than integral value (~0.4) for the spin content of the $S = {}^1/_2$ EPR signal of the protein reduced by dithionite at pH 8.0. Notably, the difference (68 mV) in the midpoint potentials of the D14S and D14C mutants is almost exactly the same as that (72 mV) reported for the mutant form (Cys^{II} to Ser) of the [4Fe-4S] cluster in subunit FRD of fumarate reductase (Kowal et al., 1995). In the latter case, it was proposed that the decrease in midpoint potential upon Cys to Ser replacement was consistent with serinate coordination to the cluster, as serinate would preferentially stabilize the oxidized form of the cluster relative to cysteinate. With the native form of Pf Fd, its cluster has been shown to be coordinated by the carboxylate of Asp14 (Calzolai et al., 1995), and this has a higher E_m value than the D14C mutant. These data are in accordance with the relative pK_a values of the relevant residues, namely, ~4, 8, and 16 for Asp, Cys, and Ser, respectively. In any event, from their spectroscopic and redox properties, we conclude that all three mutant Fds contain a [4Fe-4S] cluster which, in the cases of D14S and D14C proteins, have significantly lower redox potentials than the native Fd.

Functional Characterization of Mutant Fds. An important question we wished to address was the following: Why, in contrast to virtually all other iron−sulfur proteins that contain a [4Fe-4S] cluster, does Pf Fd contain such a cluster with one coordinating Asp rather than complete Cys coordination? Does this have any biological significance? To investigate this issue, we determined the abilities of the mutant forms of Pf Fd to function as an electron acceptor for the enzyme pyruvate ferredoxin oxidoreductase (POR) from the same organism (Blamey & Adams, 1993). POR catalyzes the oxidative decarboxylation of pyruvate to acetyl-CoA (eq 1).

$$\text{pyruvate} + \text{CoASH} + 2\text{Fd}_{ox} \rightarrow \text{acetyl-CoA} + \text{CO}_2 + \text{H}^+ + 2\text{Fd}_{red} \quad (1)$$

As shown in Table 1, the enzyme exhibited similar kinetic parameters (determined at 80 °C) with the D14C mutant as it did with the native form. The same was true with the A1K mutant, which differs from the native protein mainly in charge (with one additional Lys residue in the mutant). On the other hand, while the K_m value for the D14S mutant was comparable to the value for the native protein, it supported much lower rates of pyruvate oxidation, with a V_m value that was less than 50% of that obtained with the other 4Fe-Fds. This was somewhat unexpected as the pyruvate/acetyl-CoA reaction has a very low reduction potential (−560 mV; Thauer et al., 1977) and, therefore, POR should readily reduce both the D14C and D14S mutants. To further investigate the role of the reduction potential of the cluster, similar kinetic analyses were performed with the 3Fe-

Table 1: Reduction Potentials and Kinetic Parameters for the Native and Mutant Forms of *P. furiosus* Ferredoxin

		POR[c]			AOR		
Fd type[a]	E_m[b] (mV)	K_m (μM)	V_m (units/mg)	k_{cat}/K_m[d] (μM^{-1} s^{-1})	K_m (μM)	V_m (units/mg)	k_{cat}/K_m[d] (μM^{-1} s^{-1})
Pf native (D14)	−375	27.6 (9.8)	20.2 (2.6)	1.40	108 (41)	15.1 (2.8)	0.158
Pf A1K	−382	16.7 (7.0)	17.7 (2.4)	2.03	115 (38)	17.9 (2.8)	0.176
Pf D14C	−422	11.2 (3.9)	13.0 (1.2)	2.22	290 (176)	17.3 (6.5)	0.068
Pf D14S	−490	20.4 (5.4)	6.4 (0.6)	0.60	nd[e]	<0.3	nd
Pf native 3Fe (D14)	−160	38.2 (15.0)	14.6 (2.8)	0.73	nd	<0.3	nd
Tl[f] Fd	−400	11.7 (2.7)	13.0 (0.8)	2.12	149 (97)	23.7 (8.6)	0.181

[a] All Fds are the 4Fe-forms, except where indicated. [b] Measured at 23 °C, pH 8.0. [c] Kinetic parameters with pyruvate ferredoxin oxidoreductase (POR) and aldehyde ferredoxin oxidoreductase (AOR) were measured at 80 °C, pH 8.0, where 1 unit/mg is the reduction of 1 μmol of Fd min^{-1} (mg of POR or AOR)$^{-1}$. Standard deviations are given in parentheses. [d] k_{cat} values were calculated on the assumption that 1 mol of POR (230 kDa) and 1 mol of AOR (136 kDa) each reduce 4 mol of Fd [see Adams and Kletzin (1996)]. [e] Not determined. [f] Tl, *Thermococcus litoralis.*

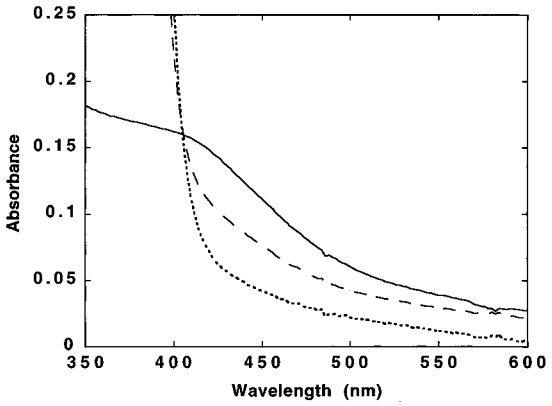

FIGURE 4: Visible spectra of D14S mutant of *P. furiosus* ferredoxin. Each sample contained the protein (16 μM) under the following conditions: (a) in 50 mM Tris-HCl, pH 8.0 (solid line); (b) in 50 mM Tris-HCl, pH 8.0, containing 1 mM sodium dithionite (dashed line); and (c) in 50 mM CAPS, pH 10.0, containing 1 mM sodium dithionite (dotted line). Spectra were recorded at 25 °C.

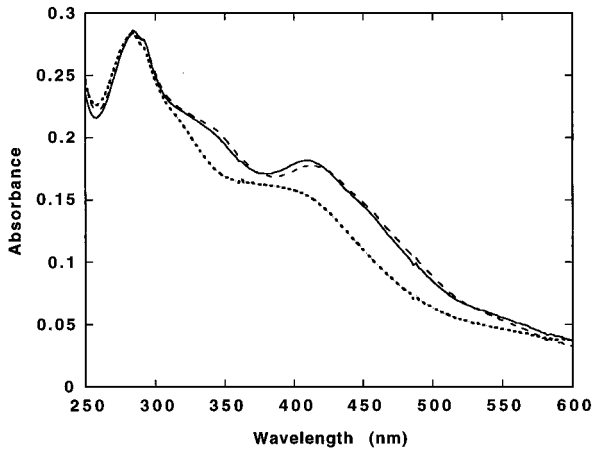

FIGURE 5: Visible spectra of the 3Fe- and 4Fe-forms of the D14S mutant of *P. furiosus* ferredoxin. The samples contained the indicated protein (16 μM) in 50 mM Tris-HCl, pH 8.0, and were: (a) the 3Fe-form of the native Fd (solid line), (b) the 3Fe-form of the D14S mutant (dashed line), and (c) the 4Fe-form of the D14S mutant (dotted line). Spectra were recorded at 25 °C.

form of native Pf Fd, which has a midpoint value of −160 mV (23 °C, pH 8.0; Aono et al., 1989). As shown in Table 1, this form exhibited a significantly higher K_m value and a lower V_m value compared to the native 4Fe-form, such that the k_{cat}/K_m value was similar to that measured with the D14S mutant. To confirm that the D14S mutant did, in fact, contain a 4Fe-cluster (rather than a 3Fe-cluster) under assay conditions, the 3Fe-form of the mutant was generated by chemical treatment (Conover et al., 1990a). As shown in Figure 5, the 3Fe- and 4Fe-forms have distinct visible spectra, and that of the 4Fe-form was unchanged after a 5 min incubation at 80 °C with no evidence for the 3Fe-form. Thus, the 4Fe-form of the D14S mutant did not undergo cluster conversion to the 3Fe-form during the enzyme assays. From the kinetic data, it therefore seems that the reduction potential of the electron carrier is not a predominant factor in determining the efficiency of the interaction of Fd with POR; rather, a more determining role might be played both by the nature of residue at position 14, at least in the case of serine, and by the cluster type.

The kinetic data shown in Table 1 were obtained by measuring direct reduction of Fd by POR, but significant variation was observed (typically ∼40%) in the apparent K_m value for a given Fd sample. To try and obtain more accurate comparisons between the different Fd forms, we also utilized a linked assay system in which the Fd that is reduced by POR is spontaneously reoxidized by metronidazole, the reduction of which is essentially irreversible (Chen & Blanchard, 1979). Hence, the concentration of oxidized Fd effectively remains constant over the assay period. As shown in Table 2, the V_m values obtained in this coupled system were less than half of those calculated in the direct assay using all five forms of Pf Fd, but they were much less variable (typically <5% deviation). Notably, the values with the 3Fe-form and the D14S mutant were significantly lower than those for the native and the A1K and D14C mutants. In addition, the calculated K_m values were much less variable (<20%) for each Fd form, and those for the 3Fe-form and the D14S mutant were significantly higher than the values for the other three proteins. These results confirm that Asp at position 14 is not essential for physiological electron transfer reactions involving Fd, as the residue can be replaced by Cys. These results also suggest that the 3Fe-form of the protein and the D14S mutant function as less efficient electron acceptors for POR. It should be noted that the K_m values obtained in the coupled assay were at least an order of magnitude lower than those from the direct assay. Theoretically they should be the same, and why this is not the case is not clear, although it may be related to the spontaneous regeneration of the substrate, oxidized Fd, in the coupled system.

To determine if the conclusions drawn with the mutant Fds and POR were unique to this system or were of a more general nature, similar kinetic analyses were performed with the enzyme aldehyde ferredoxin oxidoreductase (AOR), also obtained from Pf (Mukund & Adams, 1991). AOR catalyzes the oxidation of a range of both aliphatic and aromatic aldehydes (eq 2). The results with the three mutants and

Table 2: Kinetic Parameters for the Native and Mutant Forms of *P. furiosus* Ferredoxin Using Metronidazole-Linked Assays

	POR[b]			AOR			
Fd type[a]	K_m (μM)	V_m (units/mg)	k_{cat}/K_m (μM^{-1} s^{-1})	K_m (μM)	V_m (units/mg)	k_{cat}/K_m (μM^{-1} s^{-1})	k_{sd}[c] (mol^{-1} s^{-1})
Pf native (D14)	1.59 (0.38)	5.05 (0.27)	6.05	54.5 (17.9)	6.95 (1.14)	0.15	1.9×10^2
Pf A1K	1.00 (0.15)	4.75 (0.16)	9.10	39.5 (4.4)	6.25 (0.31)	0.18	nd[d]
Pf D14C	1.54 (0.18)	3.78 (0.10)	4.73	54.3 (9.2)	7.28 (0.61)	0.15	1.1×10^6
Pf D14S	4.89 (0.95)	1.80 (0.12)	0.70	nd	<0.2	nd	3.0×10^4
Pf native 3Fe (D14)	2.36 (0.53)	2.25 (0.15)	1.83	nd	<0.2	nd	nd
Tl Fd	1.69 (0.38)	5.10 (0.27)	5.80	77.9 (13.7)	6.60 (0.31)	0.10	nd

[a] All Fds are the 4Fe-forms, except where indicated. [b] Kinetic parameters with POR and AOR were measured at 80 °C, pH 8.0, using metronidazole as the terminal electron carrier, where 1 unit/mg equals the reduction of 1 μmol of Fd min^{-1} (mg of POR or AOR)$^{-1}$. Standard deviations are given in parentheses. [c] The self-exchange rates (k_{se}) were measured at pH 7.6 either at 30 °C (native Fd) or at 10 °C (D14C and D14S mutants). The data were taken from Calzolai et al. (1996). [d] Not determined.

$$R{-}CHO + H_2O + 2Fd_{ox} \rightarrow R{-}COO^- + 3H^+ + 2Fd_{red} \quad (2)$$

with the native 3Fe-form, using both the direct and coupled assays, are summarized in Tables 1 and 2. As with POR, the kinetic behavior of the D14C mutant, and also the A1K mutant, was similar to that of native Fd, and the V_m values obtained for each Fd type were about 50% lower in the direct assay system. Similarly, the K_m values measured in the direct assay were higher than in the coupled system, in this case by 2−4-fold. Overall, however, Fd is a much less efficient electron carrier for AOR than it is for POR, with at least an order of magnitude lower k_{cat}/K_m values. Moreover, the activities with the D14S mutant and the 3Fe-form were barely measurable using either of the assay systems, and K_m values could not be calculated. The reaction catalyzed by AOR is also of very low reduction potential (−580 mV; Thauer et al., 1977), so it is not clear why this enzyme exhibits much lower activities (in both assay systems) than POR with the D14S mutant, relative to the native Fd. Clearly, there is a difference in the electron acceptor requirements for these two enzymes. In any event, using both direct and coupled assays, the presence of Asp at position 14 of the Fd appears to offer no kinetic advantage compared to Cys, at least with these two Pf enzymes.

Further insight into what factors might influence the interaction of Pf Fd and the two Pf oxidoreductases was sought by performing the same kinetic analyses with the 4Fe-ferredoxin from the hyperthermophile *T. litoralis* (Tl; Adams, 1992; Busse et al., 1992). As shown in Tables 1 and 2, the kinetic constants obtained with Tl Fd and the two enzymes mirrored those obtained with Pf Fd. This included similar V_m values in the two different assay systems (direct and coupled), a 2-fold (with AOR) and a 10-fold (with POR) difference in the K_m values determined by the two assays, and an order of magnitude greater activity with POR than with AOR. In light of the data obtained with the Pf Fd mutants, these results are perhaps not too surprising as the Tl protein shares 61% sequence identity with Pf Fd, its cluster, which is coordinated by four Cys residues, has a similar redox potential (−400 mV, 25 °C, pH 8.0), and structurally the two proteins are highly similar. For example, from the molecular model for the solution structure of Tl Fd recently determined by ^{1}H NMR techniques (Donaire et al., 1996; Wang et al., 1996) and the secondary structure of the Pf protein determined by similar methods (Teng et al., 1994), each of them consists of two antiparallel β-sheets (one triple- and one double-stranded), two α-helices, and four turns. The main differences between these two Fds are that one of the α-helices is three residues longer in the Pf protein while one of the turns is two residues shorter, differences that appear not to affect their ability to interact with the two Pf oxidoreductases. Another feature shared by the two hyperthermophilic Fds is the incorporation of their N and C termini into the triple-stranded β-sheet. This interaction is presumably disrupted in the A1K mutant of Pf Fd, and from the data presented in Tables 1 and 2, the terminal regions do not appear to affect the efficiency of electron transfer from the Fd to the two Pf oxidoreductases.

The kinetic data therefore show that the ability of Pf Fd to accept electrons from Pf oxidoreductases is not dependent on Asp14, as it can be replaced by Cys with no significant difference in the kinetic values. However, this was not the case if Asp was replaced by Ser, or if the 4Fe-cluster is converted to the 3Fe-form. Interestingly, it was recently shown that the rates of the intermolecular electron self-exchange reaction (k_{se}, eq 3) for the D14C and D14S mutants

$$[Fe_4S_4]^{1+} + [Fe_4S_4]^{2+} \leftrightarrow [Fe_4S_4]^{2+} + [Fe_4S_4]^{1+} \quad (3)$$

were least 5000- and 100-fold faster, respectively, than for the native protein (see Table 2). It was therefore postulated that Asp coordination to an Fe atom of the 4Fe-cluster had a role in gating the electron transfer rate between Fd molecules, possibly by a cluster redox state-dependent interconversion between mono- and bidentate ligating modes of Asp to Fe (Calzolai et al., 1996). The results presented herein show that such a mechanism does not limit the efficiency of electron transfer from the Pf oxidoreductases to Fd, as the self-exchange reaction rates do not correlate with the V_m values (obtained in either assay system) for the D14C and D14S mutants and the native form of the protein. Note that this assumes that electron transfer to the oxidoreductases is rate-limiting, which has yet to be demonstrated experimentally. Moreover, it is possible that the gating mechanism would be apparent during the oxidation rather the reduction of Fd, e.g., when reduced Fd is the electron donor to Fd:NADP oxidoreductase (Ma & Adams, 1994), and studies to investigate this are underway. Substitution of Asp14 for Ser or Cys does not lead to gross structural changes in Pf Fd, as determined by far-UV CD (Figure 1) and 1D NMR spectroscopy (Calzolai et al., 1996), and the same is true for the 3Fe- and 4Fe-forms of the native protein (Busse et al., 1992; Teng et al., 1994). The inefficient electron-accepting abilities of the D14S mutant and the 3Fe-form, and particularly with Pf AOR, are therefore hard to rationalize at present.

Factors that affect the molecular interactions between 4Fe-Fds and the enzymes that function as their redox partners have not been previously investigated. In fact, for 8Fe-Fds (containing two [4Fe-4S] clusters) there has been only one study in this regard, and that focused on the 8Fe-Fd from the mesophile *Clostridium pasteurianum* (Moulis & Davasse, 1995). It was concluded that electrostatic interactions do not play a major role in its ability to transfer electrons from POR to hydrogenase of the same organism. In contrast, there is a wealth of information from similar studies with redox proteins such as 2Fe-type Fds and cytochrome *c*. The latter protein does form a specific, crystallographically-resolvable, electron transfer complex with cytochrome *c* peroxidase (Pelletier & Kraut, 1992), but in general cytochrome *c* appears to interact with a range of enzymes by a nonspecific mechanism [see McLendon and Hake (1992)]. This is not the case with 2Fe-Fds, at least with those from human [see Brandt and Vickery (1993)] and *Anabaena* (Hurley et al., 1994), as these interact with their respective reductases by pairwise-specific electrostatic interactions, and similar although less specific interactions appear to occur with spinach 2Fe-Fd and its redox partners, e.g., De Pascalis et al. (1994). There has been no study on the effects of the reduction potential of the redox active group ([2Fe-2S] or heme) on the apparent affinities for, or on the electron transfer rates between, these redox proteins and their redox enzymes. The results presented herein with Pf Fd provide the first information along these lines. Moreover, we demonstrate that one of the unusual properties of this protein, a cluster-coordinating Asp residue, is not obligatory for physiological electron transfer reactions, although why it can be efficiently replaced by Cys but not by Ser is not clear at present. Molecular structures for both the native and mutant proteins should soon be available from 2D NMR studies (Calzolai et al., 1996), and these will hopefully provide some insights into this problem.

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