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Redox Chemistry of the *Schizosaccharomyces pombe* Ferredoxin Electron-Transfer Domain and Influence of Cys to Ser Substitutions

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Abstract

Schizosaccharomyces pombe (*Sp*) ferredoxin contains a C-terminal electron transfer protein ferredoxin domain (etp^{Fd}) that is homologous to adrenodoxin. The ferredoxin has been characterized by spectroelectrochemical methods, and Mössbauer, UV-Vis and circular dichroism spectroscopies. The Mössbauer spectrum is consistent with a standard diferric [2Fe-2S]²⁺ cluster. While showing sequence homology to vertebrate ferredoxins, the E°' and the reduction thermodynamics for etp^{Fd} (−0.392 V) are similar to plant-type ferredoxins. Relatively stable Cys to Ser derivatives were made for each of the four bound Cys residues and variations in the visible spectrum in the 380 – 450 nm range were observed that are characteristic of oxygen ligated clusters, including members of the [2Fe-2S] cluster IscU/ISU scaffold proteins. Circular dichroism spectra were similar and consistent with no significant structural change accompanying these mutations. All derivatives were active in an NADPH-Fd reductase cytochrome c assay. The binding affinity of Fd to the reductase was similar, however, V_{max} reflecting rate limiting electron transfer was found to decrease ~ 13-fold. The data are consistent with relatively minor perturbation of both the electronic properties of the cluster following substitution of the Fe-bond S atom with O, and the electronic coupling of the cluster to the protein.

Keywords

ETP^{Fd}, ferredoxin; *Schizosaccharomyces pombe*; Ser mutants; reduction potential; electron-transfer; Fd reductase

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1. Introduction

The [2Fe-2S] family of ferredoxins (Fds) function as electron transfer agents in many biological electron-transfer chains, including photosynthesis, and respiration [1–5], with the cluster iron centers cycling between Fe(II)/Fe(III) and Fe(III)/Fe(III) oxidation states [6, 7]. They are classified into three groups, based on cluster-binding patterns and sequence similarities and include plant-type ($-CX_4CX_2CX_nC-$), adrenodoxin type ($-CX_5CX_2CX_nC-$), and those with no clear relationship to the former two [7–9]. The adrenodoxin-type Fds show five amino acids between the first and second cysteines, while the plant-type Fds exhibit only four. In addition, the reduction potential of the plant type Fds lies at ~ -0.4 V, while potentials for the adrenodoxin-type Fds are ~ -0.25 V [7].

Schizosaccharomyces pombe (*Sp*) Fd is an adrenodoxin-type Fd according to its amino sequence pattern, consisting of 631 amino acid residues that are organized into two domains. The first 504 amino acids constitute a transmembrane-spanning portion of the protein, and the remaining 127 amino acids constitute the soluble domain. The latter, named etp^{Fd} (electron transfer protein ferredoxin domain), contains a [2Fe-2S] cluster and serves an electron transfer role [10]. While the physiological function of etp^{Fd} in *S. pombe* is not yet fully understood, it is most likely involved in heme *a* biosynthesis and iron–sulfur protein biogenesis. Etp^{Fd} is homologous to human ferredoxin (46.8% identity) and to bovine adrenodoxin (45.0% identity) (Fig. 1).

Schiffler and coworkers [10] have considered the possibility of replacing bovine adrenodoxin (Adx) with etp^{Fd}. The small but significant differences of the EPR, visible and near UV CD, oxidized and reduced UV-visible (UV-Vis) spectra of etp^{Fd} with the corresponding spectra of adrenodoxin point to a distinct protein environment around the iron–sulfur cluster, which is evidenced also by the differing redox potential of the two ferredoxins. Schiffler *et al.* measured the redox potential of etp^{Fd} to be -0.353 V vs. standard hydrogen electrode (SHE) using the dye photoreduction method [10], which is considerably lower than that of Adx (-0.250 V vs. SHE) [11]. However, the results of the functional characterization of etp^{Fd} clearly demonstrate a close relationship to Adx, since etp^{Fd} could fully replace Adx in the interaction with Adx redox partners, consistent with the high sequence homology of the two ferredoxins in the interaction domain (Fig. 1).

Herein we report a more complete characterization of wild type and Cys-to-Ser derivative *Sp* etp^{Fd}, including Mössbauer, UV-Vis, and CD spectra, as well as functional studies in an NADPH-Fe reductase cytochrome *c* assay to evaluate the electron transfer activity for native and Cys-Ser substituted etp^{Fd} s. These studies further define the similarity of *Sp* etp^{Fd} to the plant class, and suggest limits for cluster-bound Cys in defining both the electronic properties of the cluster and the electronic coupling of the cluster to the protein.

2. Materials and Methods

General Chemicals

Solutions were argon purged and handled under positive Ar(g) pressure using standard Schlenk line techniques. Iron-57 was obtained from Pennwood Chemicals. Bpy, cytochrome *c*, NADPH, and tiron were all purchased from Sigma (St. Louis, MO). Hepes was purchased from Fisher (Fair Lawn, New Jersey), and Tris-HCl was purchased from Acros (New Jersey). All restriction enzymes and buffers were from Life Technologies (Rockville, MD). Pfu polymerase was obtained from Stratagene (La Jolla, CA), and gel extraction kits and PCR purification kits were purchased from QIAGEN (Valencia, CA). The *Schizosaccharomyces pombe* MATCHMAKER cDNA library was obtained from Clontech (Palo Alto, CA). The protein expression strain *E. coli* BL21-CodonPlus-RIL was purchased

from Stratagene (La Jolla, CA). Expression vectors for human ferredoxin (*Hs* Fd) and NADPH Fd reductase were kindly provided by J. L. Markley and G. Schulz, respectively, and purified as previously described [7, 12]. Methyl viologen, indigo disulfonate, phenazine methosulfate, and methylene blue were purchased from Sigma-Aldrich Co. and lumiflavin 3-acetate was purchased from Fluka Co. All chemicals were reagent grade and used without further purification. Nanopure water was used throughout.

Spectroelectrochemical Measurements

All UV-Vis spectroelectrochemical experiments were carried out in a homemade Optically Transparent Thin Layer Electrochemical (OTTLE) cell. The three-electrode configuration consisted of a gold minigrid working electrode (Buckbee-Mears, Chicago, IL), a homemade Ag/AgCl/KCl_{sat} microreference electrode, separated from the working solution by a Vycor set, and a platinum wire as the counter electrode. The reference electrode was calibrated against a saturated calomel electrode before each set of measurements. Potentials were applied across the OTTLE cell with an Amel mod. 2053 potentiostat/galvanostat. UV-Vis spectra were recorded using a Cary C50 spectrophotometer. All experiments were carried out under argon using 1 ml samples containing between 0.05 and 0.08 mM protein made up in 40 mM phosphate buffer plus 0.1 M NaCl at pH 7, in the presence of methyl viologen (1:15 molar ratio with the protein) and lumiflavine-3-acetate, indigo disulfonate, phenazine methosulfate and methylene blue (each 1:500 molar ratio with the protein) used as mediators [13, 14]. All reduction potentials reported in this paper are referred to SHE and were found to be reproducible within ± 3 mV. Nernst plots consisted of at least five points and were invariably linear, with a slope consistent with a one electron reduction process [13–15]. For each species, the experiments were performed at least two times.

The temperature dependence of the reduction potential was determined using a “non-isothermal” cell configuration [16]. The temperature of the reference electrode and the counter electrode was kept constant whereas that of the working electrode was varied from 5 to 30 °C with etp^{Fd}. Parameterization of enthalpic and entropic components of $E^{\circ'}$ was possible by calculating $\Delta S^{\circ'}_{rc}$ from the slope of the plot of $E^{\circ'}$ versus temperature, whereas $\Delta H^{\circ'}_{rc}$ could be obtained directly from the Gibbs-Helmholtz equation from the plot of $E^{\circ'}/T$ versus $1/T$. The nonisothermal behavior of the cell was carefully checked by determining the $\Delta H^{\circ'}_{rc}$ and $\Delta S^{\circ'}_{rc}$ values of the ferricyanide/ferrocyanide couple [16, 17].

Mössbauer Spectroscopy

The ⁵⁷Fe (III) solution was prepared by dissolving ⁵⁷Fe metal (Pennwood Chemicals) in a 1:1 mixture of concentrated hydrochloric and nitric acids. This stock was adjusted to pH 7.5 by 1.0 M Tris base and diluted to 50 mM of Fe³⁺ with 50 mM Tris-HCl buffer (pH 7.5) before use. Samples for Mössbauer analysis were prepared by use of ⁵⁷Fe (III) instead of regular FeCl₃. The Mössbauer cells were filled with 500 μ L of protein solution and frozen at –80 °C. Mössbauer spectra were recorded on a constant acceleration spectrometer, model MS-1200D from Ranger Scientific, using a Janis SuperVaritemp cryostat (model 8DT), a Lakeshore temperature controller (model 340), and a ⁵⁷Co source in a rhodium foil purchased from Isotope Products Laboratory. All isomer shifts are quoted relative to iron metal at room temperature.

Circular Dichroism

Circular dichroism spectra were measured on an Aviv model 202 circular dichroism spectrometer. Protein concentrations were 0.07 mM *Sp* etp^{Fd} and *Hs* Fd in 10 mM potassium phosphate, pH 7.4. Near-UV-Vis CD spectra were recorded by use of a 10-mm path-length cuvette/1 nm at 25 °C. Buffer spectra were always subtracted.

Cytochrome c Assay

Reaction mixtures contained 80 μ M cytochrome c and 200 nM Fd reductase in 1 mL of potassium phosphate buffer (10 mM, pH 7.5). The reaction was initiated by addition of holo Fd and NADPH (400 μ M) under anaerobic conditions and monitored by measuring the increase in absorbance at 550 nm from reduced cytochrome c ($\Delta\epsilon_{550} = 20 \text{ mM}^{-1} \text{ cm}^{-1}$) [2]. The values of K_m and V_{max} were obtained from the intercepts of the plots of $1/V$ (nmol cyt. c reduced/min) $^{-1}$ versus $1/[Fd]$.

3. Results and Discussion

3.1 Purification and Reconstitution of Wild Type and Cys-to-Ser *Sp etp*^{Fd} Derivatives

Fd is one of the most ancient proteins in nature. As seen by comparison of the primary sequences of the Fd protein family (Fig. 1), *Sp etp*^{Fd} exhibits a high degree of amino acid sequence similarity to bovine (66 %) and human (69 %) Fds. All three Fds have four conserved cysteine residues, Cys46, Cys52, Cys55, and Cys92 (Fig. 1, *Hs* Fd numbering), with an adrenodoxin-type cluster-binding motif (CX₅CX₂CX_nC). The DNA sequence corresponding to mature *Sp etp*^{Fd} lacking the transmembrane region was predicted according to NCBI database (PID: g1723522, ACCESSION: Q10361; GI:1723522) and by comparison with other eukaryotic and prokaryotic homologues, and the translation start site is indicated in Fig. 1. All the Cys-to-Ser mutants were made by the QuickChange method, and the mutations were confirmed by DNA sequencing. After purification, wild type *Sp etp*^{Fd} was red-brown (holo form) and all the Cys-to-Ser mutants were isolated colorless (apo form), which could be reconstituted by the addition of DTT, Fe³⁺, and S²⁻. The molecular weight of the homogeneous preparation of the gene product was estimated to be ~15 kDa, in good agreement with the value predicted from the amino acid sequence (15,223 Da, with his-tag). From the elution profile of holo *Sp etp*^{Fd} and standard proteins on Superose 12 (FPLC), the red-brown *Sp etp*^{Fd} was found to be a monomer.

The iron content of *Sp etp*^{Fd} was determined by the method of Moulis et al. [18] by use of HCl treatment to facilitate cluster degradation and release of Fe that is then quantitatively complexed by bathophenanthrolinedisulfonate. By this method, *Sp etp*^{Fd} was found to have 2.0 ± 0.2 mol Fe/mol per subunit, consistent with one [2Fe-2S] cluster per subunit.

3.2 Reduction Potential and Thermodynamic Parameters for Native *Etp*^{Fd}

The reduction potential of *etp*^{Fd} (−0.392 V) obtained in this work by UV-Vis spectroelectrochemistry is in reasonable agreement with that obtained from Schiffler *et al.* (−0.353 V) by dye photoreduction method [10]. The difference can be ascribed to the different operating conditions. Oxidized *etp*^{Fd} exhibited electronic spectral details that were similar to those previously reported [7], indicative of the presence of a [2Fe-2S] cluster, and spectra recorded at various applied potentials at pH 7 in an OTTLE cell are reported in Fig. 2. The corresponding Nernst plots (Fig. 2, inserts) invariably show a linear behaviour with slopes close to the theoretical values of RT/F (0.059V), expected for the one-electron reduction of the iron-sulfur cluster from Fe(III)/Fe(III) to Fe(III)/Fe(II) [13–15].

The $E^{\circ'}$ value for *etp*^{Fd} measured at 25 °C and pH 7 is listed in Table 1. The $E^{\circ'}$ value obtained from the absorbance maximum at 415 nm is the same as that obtained from the absorbance maximum at 458 nm. The redox potential for vertebrate-type ferredoxins usually falls in the range from −0.235 to −0.275 mV, whereas that for plant-type ferredoxins is generally lower, namely from −0.325 to −0.425 V [19]. Unfortunately, the spectroelectrochemical measurements of the Cys-to-Ser derivatives were impractical due to marked protein instability in the reduced form, as observed previously for similar systems [19]. While many examples of relatively stable Cys-to-Ser derivatives have been reported

for ferredoxins, instability can arise if the cluster is either solvent accessible, or is located in a hydrophobic pocket with increased net charge following reduction. The latter case holds for *Sp* etp^{Fd} and impacts the relative stability of both oxidized and reduced states as noted later in the text.

The temperature dependences of the E° value and the E°/T vs $1/T$ plot at pH 7 is reported in Fig. 3. In all cases, the reduction potentials linearly decrease with increasing temperature from 5 to 30–35 °C. No experiments were carried out below 5 °C, because of the need of an excessively long time for the establishment of the equilibrium conditions. This is due to the lowering of the overall electron transfer rate between the protein and the electrode. At temperatures above 30 for etp^{Fd} protein denaturation prevented any successful spectroelectrochemical experiment.

The thermodynamic parameters for the reduction process, determined from these nonisothermal experiments are reported in Table 1. All the studied ferredoxins display positive ΔH°_{rc} values and negative ΔS°_{rc} values, in agreement with reduction thermodynamic parameters of several plant-type ferredoxins determined previously [20]. These values have been shown to originate from the intrinsic coordinative properties of the cluster, which favor the oxidized state, and to be subject to fine-tuning by the interactions between the cluster and the protein environment [20]. As noted elsewhere [21], the compensatory enthalpy and entropy changes within this protein family (evidenced by the significantly lower variations in ΔG°_{rc} relative to ΔH°_{rc} and ΔS°_{rc}) indicate that these terms are contributed mainly by reduction-induced solvent reorganization effects. Under these conditions it can be shown that, to a first approximation, the ΔG°_{rc} values correspond to the protein-based enthalpic changes, which include bonding and nonbonding interactions that result in the selective stabilization of one of the two oxidation states (the oxidized state in this case) [22]. Accordingly, the factors controlling ΔG°_{rc} , and therefore E° , are likely to be similar for *Sp* etp^{Fd} and *Hs* Fd, consistent with the similarity in CD spectral patterns observed for these two proteins in the near-UV and visible regions, which show only minor differences in the 300 nm to 440 nm range. The much lower reduction potentials for *Hs* Fd, relative to adrenodoxin has been ascribed, by analogy with plant-type ferredoxins, both to a less polar and less solvent exposed cluster environment and to the lack of a hydrogen bond between a labile sulfide of the cluster and the imidazole of a nearby histidine residue (His56 in *Hs* Fd) which in the case of adrenodoxin stabilizes the more negatively charged reduced $\text{Fe}_2\text{S}_2(\text{Cys})_4$ center [21, 23–25]. This argument most likely applies to *Sp* etp^{Fd} as well. The even lower E° observed for *Sp* etp^{Fd} presumably reflects more subtle variations in the polarity of the cluster binding pocket as defined by local residues as well as Langevin dipoles from backbone amides [26]. The apparent greater stability of the Ser derivatives toward degradation, relative to other modified Fd s, is also consistent with a deeper burying of the cluster within the protein and a lower dielectric, which would also tend to favor the oxidized (less charged) state.

3.3 Spectroscopic Characterization of [2Fe-2S] Cluster Cofactors in *Sp* Etp^{Fd}

The Mössbauer spectrum of *Sp* etp^{Fd} (Fig. 4) shows a single iron-containing species with an isomer shift, $\delta = 0.33 \pm 0.05$ mm/s, and quadrupole splitting, $\Delta E_Q = 0.59 \pm 0.05$ mm/s. These parameters are consistent with a diferric $[\text{2Fe-2S}]^{2+}$ cluster [27–31].

Both human and *Sp* etp^{Fd} display similar spectral patterns in the near-UV and visible regions (Fig. 5) and is also reflected in their circular dichroism spectra (Fig. 6), consistent with similar coordination and electronic structures for the iron-sulfur cluster. The UV-Vis absorption spectra of native *Sp* etp^{Fd} shows a characteristic spectrum with peak maxima at 276, 340, 414, and 458 nm (Fig. 5). The pattern of absorption features and relative intensities is very similar to that of *Hs* Fd, which also contains a $[\text{2Fe-2S}]^{2+}$ cluster per

monomer. Minor deviations revealed in the near UV domain of the CD spectrum (Fig. 6) are consistent with subtle structural and electronic effects in the cluster binding pocket that most likely are relevant to the unusually low potential of the *Sp etp*^{Fd}.

The Ser-derivatives display minor changes in the relative extinctions for peak maxima (Fig. 5 and Table 2). is typically less For native 2Fe Fd s the absorbance ratio of $A_{340\text{ nm}}/A_{414\text{ nm}}$ than 1.3. For the Cys to Ser derivatives studied here, and by others, the ratio exceeds 1.5, as is found for other [2Fe-2S]-cluster containing proteins where a natural O ligand is found [29, 32], including Fe/S assembly proteins of the IscU/ISU class (Table 3).

While more labile than the native protein, the Ser derivatives were found to be more stable than other reported Cys to Ser derivatives of high potential and low potential ferredoxins [7, 19, 33], a finding that is consistent with prior studies of the reactivity of these derivatives toward iron chelators [34].

3.4 Cytochrome c assay

A standard cytochrome c assay was used to analyze the activity of the electron transfer between Fd and Fd reductase [2]. NADPH is used as an electron source with redox reactions involving NADPH/Fd reductase and Fd reductase/Fd ultimately resulting in cytochrome c reduction via a Fd/Cytc couple. While the latter reaction does not occur physiologically, it is non-rate-limiting and provides a facile method of monitoring Fd reductase/Fd electron exchange. Kinetic results obtained in this assay are plotted in Fig. 7 and are summarized in Table 4. In Fig. 7, Cys-to-Ser Fd mutants yield Michaelis K_m values that are similar to those of wild type Fds, but activity V_{max} values that are almost an order of magnitude lower than those of wild type Fds (Table 4), indicative that while electron transfer activity is substantially perturbed the binding affinity between Fd and Fd reductase does not change much from wild type Fd to Cys-to-Ser Fd mutants. In fact the K_m values are in the range 17 to 32 nM, which are similar to the results (17.2 nM, wild type Hs Fd) obtained by Vickery, where K_m values (1.9–2.4 M) for surface D76N, D79A and D79N substitutions in the putative binding interface were found to be more than 100-fold greater than that obtained for wild type Fd, while the V_{max} values of wild type and mutants were similar [35, 36]. The reduced activities of mutants D76N, D79A and D79N were due to decreased binding affinity and not to defects in electron transfer. In this study, four Cys-to-Ser mutants have similar K_m values with wild type Fd, but the V_{max} values of mutants are more than 10-fold lower than wild type Fd. These results indicate that mutation of any one of the four conserved cysteines to serine does not affect the binding affinity, but greatly influence the electron transfer efficiency in Fds. The latter result also suggests that the critical parameter that is influenced is not the directional coupling of any specific Cys to the cluster, but rather the intrinsic electronic structure (and presumably activation energy barriers) of the cluster itself.

4. Conclusions

Sp etp^{Fd} is an adrenodoxin-type [2Fe-2S] ferredoxin according to its amino acid sequence, iron quantitation, and Mössbauer spectroscopy, however, the reduction potential is more in line with plant-type ferredoxins. The circular dichroism spectra have shown that *Hs* Fd and *Sp etp*^{Fd} proteins have similar patterns of spectra in near-UV and visible regions, with only a minor difference in the range 300 nm to 440 nm, consistent with the moderate difference in E° for the two species. In line with prior studies where systematic substitutions of amino acid residues have been made around cluster binding sites [26, 37–39], the potential must be sensitive to subtle variations in the polarity of the cluster binding pocket as defined by local residues as well as Langevin dipoles from backbone amides [26]. The apparent greater stability of the Ser derivatives toward degradation, relative to other modified Fd s, is also consistent with a deeper burying of the cluster within the protein and a lower dielectric,

which in the case of the native Fd would also tend to lower the potential to the more negative value (as observed) in line with plant-type Fd s relative to the adrenodoxin class [40].

Along similar lines, the change in absorbance ratio (from $A_{340\text{ nm}}/A_{414\text{ nm}} < 1.3$ for native to > 1.5 for Ser derivatives) is also consistent with oxygen ligation, by comparison to both substituted ferredoxins and also natural IscU/ISU proteins that are likely to coordinate water as a non-cysteiny l cluster ligand. The greater stability of the derivatives described here stems from the fact that IscU/ISU clusters are surface-bound and solvent accessible, which facilitates cluster hydrolysis.

The cytochrome c assay data demonstrate that the K_m values of four Cys-to-Ser mutants are similar to wild type Fd, but the V_{max} values of mutants are more than 10-fold lower than wild type Fd. As expected, substitution of any one of the four conserved cysteines to serine does not affect the binding affinity, but greatly decreases electron transfer efficiency. In turn, the similar effect observed for each Cys-to-Ser point derivative supports perturbation of the electronic structure of the cluster with an increase in the activation barrier for electron exchange rather than the directional coupling of any specific Cys to the cluster. These results are consistent with prior findings that electron transfer from the flavin in Fd reductase to the iron sulfur center in Fd, rather than electron transfer from Fd to cytochrome c is rate-limiting in the transfer of electrons from NADPH to cytochrome c [41].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

<i>Sp</i>	Schizosaccharomyces pombe
<i>Hs</i>	Homo sapiens
Fd	ferredoxin
$\Delta H'^{\circ}_{\text{rc}}$ and $\Delta S'^{\circ}_{\text{rc}}$	enthalpy and entropy changes for reduction (the subscript rc stands for the iron “redox couple”)
OTTLE	Optically Transparent Thin Layer Electrochemical cell
SHE	standard hydrogen electrode

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Hs Fd      --DKITVHFINRDGETLTTKGKVGDSLDDVVVENNLDIDGFGACEGTLACSTCHLIFE-D 61
adrenodoxin --DKITVHFINRDGETLTTKGKIGDSLDDVVVQNNLDIDGFGACEGTLACSTCHLIFE-Q
S. pombe ETPFd PGTGIKVFFV-----TPEGR-----EIMIEGN-E-E--GACEGSVACSTCHVIVDPE
              *,*,*:          *.:*:          ::::.* : :  *****:*****:*. : :

Hs Fd      HIYEKLDAITDEENDMLDLAYGLTDRSRLGCQICLTKSMDNMTVRVPETVADARQSIDVG 121
adrenodoxin HIFEKLEAITDEENDMLDLAYGLTDRSRLGCQICLTKAMDNMTVRVPDAVSDARESIDMG
S. pombe ETPFd H-YELLDPPEDEEDMLDLAFGLEETSRLGCQVLLRKDLDGIRVRIP---AQTR-NIRLE
              * : * *:.  ::*:*****:*. :  *****: * * :*.: **:*  ::* .* :

Hs Fd      -KTS--- 124
adrenodoxin MNSSKIE 124
S. pombe ETPFd -RPKA-- 102
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Fig. 1.

Sequence alignment of *Sp etp*^{Fd}, human ferredoxin and bovine adrenodoxin. Residues conserved are indicated by ':', the same charged residues are indicated by '*'. The yellow region indicates the acidic interaction domain.

Fig. 2. Electronic spectra of *S. pombe etp^{Fd}* obtained at various applied potentials in spectroelectrochemical experiments carried out with an OTTLE cell. Spectra were recorded at 25 °C. The corresponding Nernst plots are reported in the inserts, where X stands for: $[A_{\lambda_{OX}}/(A_{\lambda_{OX}}^{\text{Max}} - A_{\lambda_{OX}})]$, and $\lambda_{OX}=415$ nm.

Fig. 3. Temperature dependence of the reduction potential of *S. pombe* etp^{Fd}. The ΔS°_{rc} and ΔH°_{rc} values are available from the slope in (A) and (B), respectively. Dashed lines are least-squares fits to the data points.

Fig. 4.
100 K Mössbauer spectrum of *Sp etp*^{Fd} in a 450 G applied field, protein was in 50 mM Tris-HCl (pH 7.5), 50 mM NaCl.

Fig. 5. (Top) UV-Vis absorption spectra of holo *Sp etp*^{Fd} (solid line) and apo *Sp etp*^{Fd} (dotted line). (Bottom) UV-Vis absorption spectra of mixture of apo and holo Cys-to-Ser *Sp etp*^{Fd} mutants. Spectra were taken in 50 mM Tris-HCl buffer, 50 mM NaCl, pH 7.5.

Fig. 6. Circular dichroism spectra of *Sp* etp^{Fd} and *Hs* Fd. Spectra were determined for 70 μ M proteins in 10 mM potassium phosphate, pH 7.4, and the signal intensity was adjusted to the same protein concentration. Spectra in the visible and near-UV regions were recorded by using 10-mm path length cells.

Fig. 7.

The plots, $1/V$ (nmol cyt. c reduced/min) $^{-1}$ versus $1/[Fd]$, of Cytochrome c assay. Reaction mixtures contained 80 μ M cytochrome c and 200 nM Fd reductase in 1 mL of potassium phosphate buffer (10 mM, pH 7.5). The reaction was initiated by the addition of holo Fd and NADPH (400 μ M) under anaerobic conditions and monitored by measuring the increase in absorbance at 550 nm from reduced cytochrome c. The values of K_m and V_{max} were available from the intercepts.

Table 1

Thermodynamic parameters of protein reduction for [2Fe-2S] ferredoxins^[a].

Protein	$E^{\circ}/[b]_{(V)}$	$\Delta H^{\circ}_{rc} \text{ (kJ mol}^{-1}\text{)}$	$\Delta S^{\circ}_{rc} \text{ (J mol}^{-1}\text{K}^{-1}\text{)}$	$\Delta G^{\circ}_{rc} \text{ (kJ mol}^{-1}\text{)}$	$-\Delta H^{\circ}_{rc}/F \text{ (V)}$	$T\Delta S^{\circ}_{rc}/F \text{ (V)}$
<i>H. sapiens</i> Fd	-0.380	+26	-37	+36.7 ^[c]	-0.269	-0.114
<i>S. pombe</i> etp ^{Fd}	-0.392	+20	-61	+37.8	-0.207	-0.188
<i>S. platensis</i> Fd ^[d]	-0.454	+15	-97	+44	-0.155	-0.299
<i>P. umbilicalis</i> Fd ^[d]	-0.444	+8	-118	+43	-0.083	-0.365
<i>S. oleracea</i> Fd ^[d]	-0.459	+25	-63	+44	-0.259	-0.195

^[a] Average errors for E° , ΔH°_{rc} , ΔS°_{rc} and ΔG°_{rc} values are ± 3 (mV), ± 4 (kJ mol⁻¹), ± 8 (J mol⁻¹K⁻¹) and ± 0.3 (kJ mol⁻¹), respectively (expressed as the upper values of the standard deviations for the data points). The sum $-\Delta H^{\circ}_{rc}/F + T\Delta S^{\circ}_{rc}/F$ often does not closely match the experimental E° , as a result of the experimental errors in the ΔH°_{rc} and ΔS°_{rc} values, which are rounded to the closest integer.

^[b] The reduction potentials are measured at 25 °C and are referred to SHE.

^[c] From [21]

^[d] From [20].

Table 2

Summary of absorbance wavelengths and relative intensities for native and Ser-substituted *Sp etp*^{Fd}.

<i>etp</i> ^{Fd} derivative	A _{316 nm}	A _{340 nm}	A _{414 nm}	A _{440 nm}	A _{458 nm}	A _{340 nm} /A _{414 nm}
native	0.249	0.254	0.197	0.159	0.169	1.29
C46S	0.303	0.243	0.161	0.151	0.136	1.51
C52S	0.406	0.300	0.179	0.170	0.152	1.68
C55S	0.350	0.297	0.198	0.169	0.155	1.50
C92S	0.534	0.366	0.233	0.212	0.186	1.57

UV-Vis spectra were obtained in 10 mM potassium phosphate, pH 7.4. Absorbances are normalized to protein concentrations of 20 μM.

Table 3

Comparison of $A_{340\text{ nm}}/A_{414\text{ nm}}$ ratios for select Fd's, Ser derivatives of Fd's and IscU/ISU proteins.

protein	$A_{340\text{ nm}}/A_{414\text{ nm}}$	ref
native <i>Sp</i> etp ^{Fd}	1.29	this work
native <i>Anabena</i> Fd	1.28	[19]
native <i>Sp</i> etp ^{Fd} C92S	1.57	this work
native <i>Anabena</i> Fd C41S	1.64	[19]
<i>Tm</i> IscU	1.54	[29]
<i>Hs</i> ISU	1.7 – 1.96 [a]	[28]

[a] The relative intensities of peaks is distinct for human ISU. The ratio of 1.96 is obtained using the absorbance at 414 nm, while 1.7 reflects the absorbance at the peak maximum at 460 nm.

Table 4

Summary of kinetic parameters for Fd oxidation of NADPH Fd reductase monitored by the cytochrome c assay.

	K_m (nM)	V_{max} (nmole/min) ⁻¹
<i>Sp</i> etp ^{Fd}	21.1	40.3
C46S <i>Sp</i> etp ^{Fd}	19.5	3.3
C52S <i>Sp</i> etp ^{Fd}	17.5	3.0
C55S <i>Sp</i> etp ^{Fd}	22.8	3.9
C92S <i>Sp</i> etp ^{Fd}	20.9	3.3
<i>Hs</i> Fd	32.4	49.7

The reaction mixtures contained 80 μ M cytochrome c and 200 nM Fd reductase in 1 mL of potassium phosphate buffer (10 mM, pH 7.5). The reaction was initiated by addition of holo Fd (5–100 nM) and NADPH (400 μ M).