Investigation of the Unusual Electronic Structure of *Pyrococcus furiosus* 4Fe Ferredoxin by EPR Spectroscopy of Protein Reduced at Ambient and Cryogenic Temperatures

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The hyperthermophilic archaeon *Pyrococcus furiosus* contains a novel ferredoxin (*Pf*-Fd) in which, in the native 4Fe form, three of the Fe ions are coordinated to the protein by cysteinyl thiolato ligands, but the fourth Fe is coordinated by an aspartyl carboxylato ligand ($[Fe_4S_4(cys)_3(asp)]^{2-,3-}$). Chemical reduction at ambient temperature of the oxidized 4Fe form (*Pf*-Fd 4Fe-ox, $S = 0$ ground state, with the cluster core indicated by $[Fe_4S_4]^{2+}_{ox}$) produces a reduced 4Fe form (*Pf*-Fd 4Fe-red, with the cluster core indicated by $[Fe_4S_4]^{+}_{red}$). *Pf*-Fd 4Fe-red, $[Fe_4S_4]^{+}_{red}$ core, in frozen solution exhibits $S = {}^1/_2$ and ${}^3/_2$ electronic states that are not in thermal equilibrium. The two spin states thus represent alternate ground states of the reduced cluster (cluster cores indicated by $[Fe_4S_4]^{+}_{red1}$ and $[Fe_4S_4]^{+}_{red2}$, respectively), rather than a ground and excited spin state. Low-temperature (77 K) reduction of 4Fe-ox in frozen solution by γ-irradiation produces in high yield the reduced state of the cluster that is trapped in the structure of the oxidized parent cluster, and thus has a cluster core denoted by $[Fe_4S_4]^{+}_{ox}$. The $[Fe_4S_4]^{+}_{ox}$ form also exhibits non thermally converting $S = {}^3/_2$ and ${}^1/_2$ components in the *same proportion* as seen for $[Fe_4S_4]^{+}_{red}$. The EPR signal of the $S = {}^3/_2$ component that results from cryoreduction ($[Fe_4S_4]^{+}_{ox2}$) is indistinguishable, within experimental variability, from that seen in the ambient-temperature, chemically reduced protein ($[Fe_4S_4]^{+}_{red2}$), and the signals of the two $S = {}^1/_2$ components ($[Fe_4S_4]^{+}_{ox1}$ and $[Fe_4S_4]^{+}_{red1}$, respectively) closely resemble each other, although they are not identical. Previous NMR studies at ambient temperature showed evidence for only one species in fluid solution for both *Pf*-Fd 4Fe-ox and 4Fe-red. Taken together, the NMR and EPR results indicate that *fluid* solutions of either oxidized or reduced *Pf*-Fd contain only one conformer, but that *frozen* solutions of each contain two distinct conformers, with each one of the pair of oxidized protein forms having a corresponding reduced form. A shift in the coordination mode of the aspartyl carboxylato ligand is proposed to account for this conformational flexibility.

Introduction

The 4Fe ferredoxin isolated from the hyperthermophilic archaeon *Pyrococcus furiosus* (*Pf*-Fd)[1] lacks the fourth cysteinyl ligand found in conventional Fd's (cluster coordination denoted by $[Fe_4S_4(cys)_4]^{3-,2-}$).[2,3] *Pf*-Fd instead exhibits an unusual structural feature: an aspartate residue (Asp-14) ligates one Fe (cluster coordination thus denoted: $[Fe_4S_4(cys)_3(asp)]^{2-,3-}$).[4] *Pf*-Fd also exhibits an unusual electronic feature: frozen solutions of the ambient temperature, dithionite-reduced 4Fe form (*Pf*-Fd 4Fe-red, with the cluster core denoted by $[Fe_4S_4]^{+}_{red}$) exhibit both $S = {}^1/_2$ and $S = {}^3/_2$ electronic ground states, as shown by EPR and MCD.[5] The relative amount of $S = {}^1/_2$ versus $S = {}^3/_2$ components of *Pf*-Fd 4Fe-red in frozen solution is insensitive to temperature; thus the two components (cluster cores indicated by $[Fe_4S_4]^{+}_{red1}$ and $[Fe_4S_4]^{+}_{red2}$, respectively) do not represent different electronic spin levels of the same protein form. Protein-bound[6,7] and synthetic $[Fe_4S_4(SR)_4]^{3-}$ clusters[8−13] typically exhibit $S = {}^1/_2$ ground states, but the $S =$

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(1) Abbreviations and terminology used: *Av, Azotobacter vinelandii*; CW, continuous wave; EPR, electron paramagnetic resonance; ENDOR, electron nuclear double resonance; Fd, ferredoxin; hwhm, half-width at half-maximum; MCD, magnetic circular dichroism; *Pf, Pyrococcus furiosus*; *Pf*-Fd 3Fe-ox, form of the protein containing the $[Fe_3S_4]^{+}$ cluster; *Pf*-Fd 3Fe-red, form of the protein containing the $[Fe_3S_4]^{0}$ cluster; *Pf*-Fd 4Fe-ox, form of the protein containing the $[Fe_4S_4]^{2+}_{ox}$ cluster core, wherein the "ox" subscript indicates that the structure is that of the oxidized protein; *Pf*-Fd 4Fe-red, form of the protein containing the $[Fe_4S_4]^{+}_{red}$ cluster generated by chemical reduction at ambient temperature, wherein the "red" subscript indicates that the structure is that of the reduced protein; the $[Fe_4S_4]^{+}_{ox}$ cluster is generated by γ-irradiation at 77 K and has the charge of the reduced protein and the structure of the oxidized protein.

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$^3/_2$ state has also been found in both protein[7,14] and synthetic[9,15−17] systems. Subtle structural changes in the cluster environment favor one spin ground state over another in ways that are not well understood, despite the significant body of theoretical work on $[Fe_4S_4]$ systems.[10,18−26] These structural effects can involve the steric requirements of the thiolato ligands in model compounds[16] and analogous protein-imposed distortions, as well as solvation effects in both natural and synthetic systems. As an example of the latter, the DMF solvate of $(Et_4N)_3[Fe_4S_4(SCH_2Ph)_4]$ has $S = {}^1/_2$,[13] while the solvate-free compound has $S = {}^3/_2$.[17] In *Pf*-Fd 4Fe-red, the relative amounts of the two spin states are essentially insensitive to solution composition (e.g., amount of glassing agent)[5] in contrast to the above protein and model systems.

Both oxidized and reduced forms of *Pf*-Fd 4Fe have been investigated in fluid solution at ambient temperature by NMR.[4,27] Concerning the 4Fe-ox form ($[Fe_4S_4]^{2+}_{ox}$ cluster core), NMR supported a single protein species in solution with an $S = 0$ ground state and thermally populated $S = 1$, 2 excited states. For the 4Fe-red form ($[Fe_4S_4]^{+}_{red}$ cluster core), in contrast to the EPR/MCD data, NMR gave no evidence to support significant population of an $S = {}^3/_2$ ground state; only a single species with an $S = {}^1/_2$ ground state was found.[4] A more recent NMR study[27] made comparisons between wild-type and site-directed mutant *Pf*-Fd's, such as D14C, which has EPR behavior characteristic of tetracysteinyl 4Fe Fd's.[28,29] NMR showed wild-type *Pf*-Fd to have a ∼10% larger spin expectation value, $\langle S_z \rangle$, than that for the D14C mutant.[27] This could be due either to somewhat more extensive population of $S = {}^3/_2$, $^5/_2$, etc. excited states or to population of an $S = {}^3/_2$ ground state along with the $S = {}^1/_2$ ground state; however, the *maximum* proportion of an $S = {}^3/_2$ ground state is only ∼5%, regardless of the exchange rate between the $S = {}^1/_2$ and higher spin states.[30]

To shed light on the unusual nature of the 4Fe cluster in *Pf*-Fd, we have radiolytically reduced *Pf*-Fd 4Fe-ox in frozen solution at 77 K. This cryoreduction technique has been applied to many other metalloprotein systems[31−35] and generates a reduced protein form that retains the conformation of the original, oxidized protein. Of greatest relevance here, a very recent study of the 4Fe cluster of *Av* Fe protein showed that chemical reduction at ambient temperature and radiolytic cryoreduction gave the same novel $[Fe_4S_4]^0$ cluster.[36] We thus denote the species generated radiolytically as *Pf*-Fd with the $[Fe_4S_4]^{+}_{ox}$ cluster core, wherein the subscript indicates that the structure is that of the oxidized cluster, as opposed to the oxidized, parent protein, $[Fe_4S_4]^{2+}_{ox}$. These studies demonstrate that the $[Fe_4S_4]^{2+}_{ox}$ cluster core exists in two alternate conformers in frozen solution, each of which gives rise to a reduced form quite similar to that produced by ambient temperature reduction.

Experimental Section

Protein Preparation. *Pf*-Fd was isolated under anaerobic conditions in the presence of 2 mM sodium dithionite as described previously.[37] Anaerobic isolation and purification yields pure protein with the intact 4Fe cluster that is not degraded to 3Fe even upon lengthy exposure to air.[5] In contrast, aerobic purification leads to cluster degradation. The reasons for this difference are not understood; however, the consequence is that air oxidation of anaerobically purified, reduced *Pf*-Fd is a convenient method for generating the intact $[Fe_4S_4]^{2+}_{ox}$ cluster. In contrast, oxidation with $[Fe(CN)_6]^{3-}$ leads to formation of the $[Fe_3S_4]^+$ cluster.[5]

EPR Sample Preparation. Samples for EPR spectroscopy contained anaerobically purified, air-oxidized protein (1 mM) in D_2O with 10 mM phosphate buffer, pH 7.0, with ∼20% v/v ethylene glycol-d_6 added as a glassing agent and to improve the radiolytic reduction process. Perdeuterated solvents are employed so as to minimize the line width of the large solvent-derived free radical signal in irradiated samples.[31,34,35] Chemical reduction used 4 mM dithionite. Sample concentrations were based on the molar absorption coefficient at 390 nm, $\epsilon_{390} = 17\,000$ M^{-1} cm^{-1}, for air-oxidized samples.[5]

Irradiation Procedure. Frozen protein solutions in fused silica ENDOR tubes at 77 K were exposed to γ-irradiation from a ^{60}Co source (dose rate 0.45 Mr/h) for 6−7 h.

EPR Spectroscopy. "Q"-band (35 GHz) EPR spectra were recorded on a modified Varian E-109 spectrometer at 2 K in the dispersion mode using 100 kHz field modulation. Under these "rapid-passage" conditions, the EPR spectra represent the actual absorption envelope,[38] as seen in the Supporting Information (Figure S2). For ease of comparison with conventional EPR, digital-derivative spectra are also presented. X-band (∼9.5 GHz) EPR spectra were recorded on a Bruker ER300 spectrometer at 8−10 K in the absorption mode. Computer simulation of EPR spectra employed the program QPOWA.[39]

Quantitation of EPR spectra was carried out at 10 K under nonsaturating conditions using Cu/EDTA as the standard. For the reduced $[Fe_4S_4]^+$ cluster, the double integral of the $S = {}^1/_2$ region was determined and then subtracted from the total double integral ($S = {}^3/_2$ and $^1/_2$) to estimate the amount of $S = {}^3/_2$ species. The ratio of $S = {}^1/_2$ to $S = {}^3/_2$ signals was 5:95 in all cases. The amount of 3Fe cluster present, which in the ambient temperature reduced sample gave rise to the $g \sim 12$ resonance (see Figure S1B), was estimated to be ≤0.05 spin/mol by double integration of the $S = {}^1/_2$ EPR signal from the oxidized sample. From previous analyses of EPR signals seen from reduced *Pf*-Fd,[5] the uncertainty in spin quantitations is 10%.

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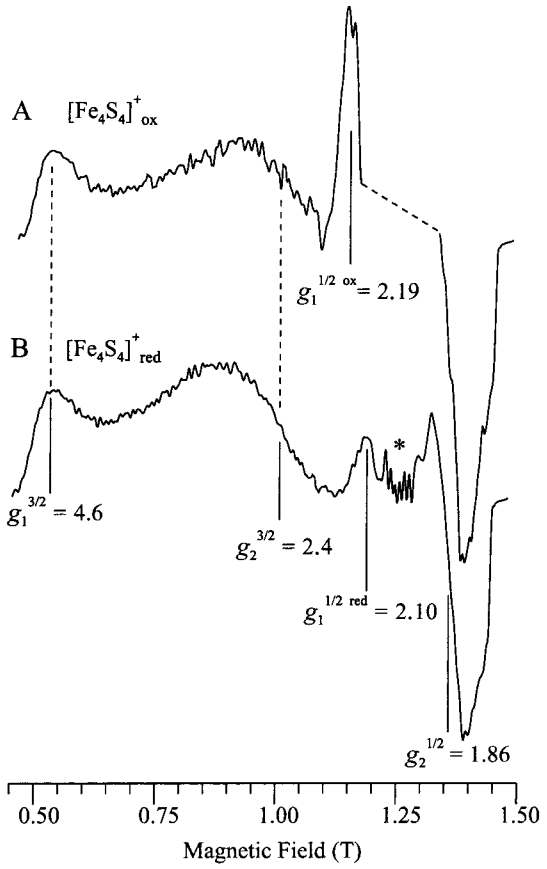

Figure 1. EPR spectra at 35 GHz of *Pf*-Fd 4Fe-red presented as digital derivatives of experimental dispersion mode EPR data. (A) Protein radiolytically reduced at 77 K ($[Fe_4S_4]^+_{ox}$ core), which reduced cluster maintains the structure of *Pf*-Fd 4Fe-ox. (B) Protein chemically reduced at ambient temperature ($[Fe_4S_4]^+_{red}$ core), which reduced cluster achieves the structure of *Pf*-Fd 4Fe-red. Sample conditions: Both samples are anaerobically purified *Pf*-Fd[5] (1 mM) in D_2O, 10 mM phosphate buffer, pH 7.0, with 20% v/v ethylene glycol-d_6; (A) sample exposed to γ-irradiation from a ^{60}Co source (dose rate 0.45 Mr/h) for 6−7 h; (B) sample reduced using 4 mM dithionite. Spectrometer conditions: (A) temperature, 2 K; microwave frequency, 35.125 GHz; microwave power, 2 mW (20 dBm); 100 kHz field modulation amplitude, 0.08 mT; time constant, 64 ms; scan time, 480 s; (B) as in part A except microwave frequency, 35.054 GHz; 100 kHz field modulation amplitude, 0.10 mT; time constant, 32 ms; scan time, 240 s. The large signal from radiolytically generated free radicals in part A is numerically truncated, as indicated by the dashed line. A weak signal from adventitious $Mn^{2+}_{(aq)}$ is seen at 1.25 T ($g = 2.00$) in part B and is indicated by an asterisk. Both numerically calculated derivative spectra have been smoothed by a Fourier transform routine; original experimental spectra are given in the Supporting Information. Canonical g values are indicated with the superscript identifying the spin state; the subscripts are assigned as follows: $g_1 \equiv g_{max}$, $g_2 \equiv g_{mid}$, and $g_3 \equiv g_{min}$ (g_3 not shown for $S = {}^3/_2$ due to magnetic field limitations; for $S = {}^1/_2$, see the Supporting Information). The g values indicated in parts A and B are almost identical, with the exception of g_1 in the $S = {}^1/_2$ component; these $g_1^{1/2}$ values are thus further identified by the superscripts "ox" (A) and "red" (B).

Results and Discussion

EPR spectra were recorded at low temperature for *Pf*-Fd 4Fe in the $[Fe_4S_4]^{2+}_{ox}$, $[Fe_4S_4]^+_{ox}$, and $[Fe_4S_4]^+_{red}$ cluster core forms at both X- and Q-band microwave frequencies. Figure 1 presents numerical derivative 35 GHz EPR spectra of *Pf*-Fd 4Fe cryoreduced at 77 K (denoted by $[Fe_4S_4]^+_{ox}$, Figure 1A) and chemically reduced at ambient temperature (denoted by $[Fe_4S_4]^+_{red}$, Figure 1B). The Supporting Information presents corresponding X-band spectra (Figure S1) and the original, absorption line shape 35 GHz spectra (Figure S2). Both reduction methods give a quantitative yield of $[Fe_4S_4]^+$ cluster species: 1.0 ± 0.1 spins/mol, based on quantitation of the X-band spectra (see Experimental Section). The spectral match between the two samples also is quite close, both in terms of the relative amounts of the $S = {}^3/_2$ and ${}^1/_2$ components and in their individual parameters. This can be seen, for example, by comparison of the feature at the low magnetic field edge, which is due to g_{max} of the $S = {}^3/_2$ signal (denoted in Figure 1 as $g_1^{3/2}$).

The presence of two spin states for $[Fe_4S_4]^+_{ox}$ demonstrates that the cluster core of the parent, oxidized protein, $[Fe_4S_4]^{2+}_{ox}$, itself exists in two alternate, major conformers (tier 0[40] states) in frozen solution, $[Fe_4S_4]^{2+}_{ox1}$ and $[Fe_4S_4]^{2+}_{ox2}$, with these two conformers producing the reduced cluster cores indicated by $[Fe_4S_4]^+_{ox1}$ ($S = {}^1/_2$) and $[Fe_4S_4]^+_{ox2}$ ($S = {}^3/_2$), respectively. We find that the EPR spectrum of $[Fe_4S_4]^+_{red}$ is completely independent of the rate of freezing the solution and that annealing a frozen solution of $[Fe_4S_4]^+_{ox}$ to 270 K does not change the EPR parameters of the two spin states and leads only to a slight change in the relative amounts of the $S = {}^3/_2$ versus $S = {}^1/_2$ components.

There are only slight differences between the EPR signals of the two $S = {}^3/_2$ species, $[Fe_4S_4]^+_{ox2}$ and $[Fe_4S_4]^+_{red2}$. In a highly rhombic $S = {}^3/_2$ system (here $D = 3.3$ cm^{-1}, $E = 0.73$ cm^{-1} [5]), the EPR spectrum is quite sensitive to electronic parameters, particularly E. For example, exact calculations show that a change in $|E/D|$ of only 10% yields $\Delta g \approx 0.1$ for both $g_1^{3/2}$ and $g_2^{3/2}$, which correspond at 35 GHz to shifts in resonant field of ~10 and ~40 mT, respectively. The difference here between $[Fe_4S_4]^+_{ox2}$ and $[Fe_4S_4]^+_{red2}$ is no greater than this and, moreover, is within the range seen among different *Pf*-Fd 4Fe-red samples.[41,42] Thus, the $S = {}^3/_2$ $[Fe_4S_4]^+_{ox2}$ and $[Fe_4S_4]^+_{red2}$ cluster cores are electronically, and presumably structurally, the same. Close comparison of the $S = {}^1/_2$ $[Fe_4S_4]^+_{ox1}$ and $[Fe_4S_4]^+_{red1}$ signals also shows only slight differences between the two cluster cores (see Figure 1 and, for more detail, the Supporting Information, Figure S3), but these differences do appear to be outside the range of sample variation. Thus the protein conformers with the $S = {}^1/_2$ cluster cores $[Fe_4S_4]^+_{ox1}$ and $[Fe_4S_4]^+_{red1}$ are similar, but not identical. Computer simulation[39] of these 35 GHz EPR spectra yielded the following parameters: $\boldsymbol{g}\{[Fe_4S_4]^+_{ox1}\} = [2.19(1), 1.860(5), 1.805(5)]$ and $\boldsymbol{g}\{[Fe_4S_4]^+_{red1}\} = [2.105(5), 1.855(5), 1.775(5)]$. These g values are also summarized in Table 1 in the Supporting Information together with those for relevant protein and model systems with $S = {}^1/_2$ $[Fe_4S_4]^+$ cluster cores. The differences between the g values of the $S = {}^1/_2$ *Pf*-Fd $[Fe_4S_4]^+_{ox1}$ and $[Fe_4S_4]^+_{red1}$ cores are similar to those seen for the multiple $S = {}^1/_2$ $[Fe_4S_4]^+$ species produced by irradiation of single-crystal $[Fe_4S_4]^{2+}$ model compounds.[10,11,17,43−46] The differences among the sites in these model compounds are attributed to slight structural effects peripheral to the cluster.[10,11,45,46] The small differences observed

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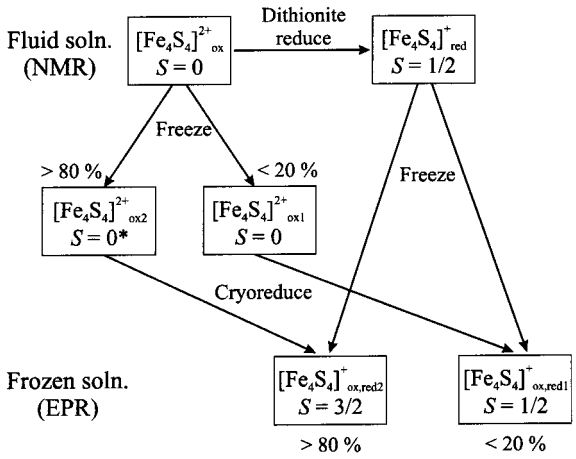
Scheme 1



here between the EPR properties of the *Pf*-Fd $S = {}^1/_2$ $[Fe_4S_4]^+_{ox1}$ and $[Fe_4S_4]^+_{red1}$ cores likewise indicate at most a modest conformational difference between oxidized and reduced 4Fe Fd forms in frozen solution. A similarly slight difference has been observed in a cryoreduction study of the Rieske protein ($[Fe_2S_2(cys)_2(his)_2]^{0,+}$ core).[31] The presence of additional (tier 1^{40}) conformational variation *within* the tier 0^{40} oxidized and reduced forms themselves is manifest by broad EPR line widths in both forms (see Table 1), indicating comparable minor conformational disorder in both the oxidized and reduced states of this Fd.

The fact that the same frozen state can be prepared by freezing the reduced protein as by cryoreducing the oxidized protein suggests that both oxidized and reduced *Pf*-Fd 4Fe exist as two conformers in fluid solution. NMR data,[4,27,30] however, provide no support that either oxidized or reduced *Pf*-Fd exists as two distinct conformers in fluid solution. To reconcile these observations, we suggest the situation as presented in Scheme 1. A fluid solution of either oxidized or reduced *Pf*-Fd contains only one conformer, and these species studied by NMR thus are indicated solely by the subscript "ox" or "red", respectively (these conformers are not necessarily identical either to ox1 or to red1). Frozen solutions of each contain two conformers that are distinguishable by EPR *and* formed in the same proportions. Scheme 1 incorporates the experimental finding that there is no distinction by EPR between the $[Fe_4S_4]^+_{ox2}$ and $[Fe_4S_4]^+_{red2}$ forms and for simplicity ignores the slight distinction between the $[Fe_4S_4]^+_{ox1}$ and $[Fe_4S_4]^+_{red1}$ forms.

What factor could lead a single fluid-solution conformation to generate two conformers with different spin states upon freezing? The obvious source of the unusual electronic behavior of *Pf*-Fd is its atypical, carboxylato ligand (Asp-$14^{4,41,42}$). In contrast to the normal, thiolato ligands (from cysteinyl), a carboxylato ligand can exhibit variable coordination, ranging from monodentate to bidentate coordination, and can exhibit carboxylate shifts under varying conditions.[47] Freezing *Pf*-Fd, whether in the oxidized or reduced form, presumably induces a shift in coordination mode for Asp-14. This altered conformation changes the spin ground state from $S = {}^1/_2$ to ${}^3/_2$ for the reduced cluster. One could propose a corresponding change in the oxidized cluster, from $S = 0$ to $S = 1$, as $S = 1$ would be "EPR-silent" and a one-electron-oxidized parent to $S = {}^3/_2$; however, MCD studies of frozen-solution *Pf*-Fd 4Fe-ox can rule out an $S = 1$ spin ground state.[5,48] Nevertheless, spin-coupling models show that $S = 0$ and $S = 1$ states can be close in energy in $[Fe_4S_4]^{2+}$ clusters, depending on the individual coupling parameters.[19] It is thus reasonable to speculate that the integer-spin excited states ($S = 1, 2, ...$) are lower-lying in $[Fe_4S_4]^{2+}_{ox2}$ than in $[Fe_4S_4]^{2+}_{ox1}$. We indicate this in Scheme 1 by the use of "$S = 0^*$" for $[Fe_4S_4]^{2+}_{ox2}$. We plan to apply appropriate physical methods (e.g., high-field EPR spectroscopy and magnetization) to *Pf*-Fd 4Fe-ox forms to study this further. In parallel, we note that theoretical efforts thus far have emphasized the paramagnetic, reduced $[Fe_4S_4]^+$ core state;[10,18−26] however, the oxidized $[Fe_4S_4]^{2+}$ core may need further scrutiny, such as to determine the accessibility of states with $S > 0$.

Given that the $S = {}^1/_2$ state is predominant at ambient temperature (>95%[30]), while in frozen solution the $S = {}^3/_2$ state is the majority (>80%), most of the protein undergoes this conformational change. This change occurs equivalently for both oxidized and reduced protein, as indicated by the EPR proportion correspondence of $[Fe_4S_4]^+_{ox1}$ to $[Fe_4S_4]^+_{ox2}$ and of $[Fe_4S_4]^+_{red1}$ to $[Fe_4S_4]^+_{red2}$. Combined with the EPR parameter correspondence of $[Fe_4S_4]^+_{ox1}$ to $[Fe_4S_4]^+_{red1}$ and of $[Fe_4S_4]^+_{ox2}$ to $[Fe_4S_4]^+_{red2}$, there is clearly little conformational difference between oxidized and reduced forms of *Pf*-Fd. Many factors are important in electron transfer, such as the site specificity of binding between electron transfer partners,[49] which is not addressed in this unimolecular study. Another factor, however, is the rate of the electron transfer itself. For this process to be optimal, there ought to be little reorganization between oxidized and reduced forms, which is indeed the case for *Pf*-Fd.

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Supporting Information Available: One table listing EPR data for *Pf*-Fd and other proteins and model compounds containing $S = {}^1/_2$ $[Fe_4S_4]^+$ cluster cores and three figures (S1−3) showing additional EPR spectra for *Pf*-Fd. This material is available free of charge via the Internet at http://pubs.acs.org.

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