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Potentiometric and Electron Nuclear Double Resonance Properties of the Two Spin Forms of the [4Fe-4S]$^+$ Cluster in the Novel Ferredoxin from the Hyperthermophilic Archaebacterium *Pyrococcus furiosus**

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Pyrococcus furiosus **ferredoxin contains a single [4Fe-4S] that exists in both $S = 1/2$ (20%) and $S = 3/2$ (80%) ground states in the reduced protein. We report here on the temperature-dependent potentiometric properties of the two spin forms, their stability, and on the structural features that differenciate them. The midpoint potential (E_m) of the cluster in either spin state was determined at −365 mV (30 °C, pH 8.0). By rapidly freezing samples for EPR analyses, it was shown that the E_m values of both spin states appear to change by −1.7 mV/ °C over the range 20° −80 °C, and by −6 mV/ °C between 80 and 89 °C. The E_m values and the relative amounts of the $S = 1/2$ and $S = 3/2$ forms of the cluster were unaffected by pH (6.8 − 10.5), even at 85 °C, and were unchanged by the presence of NaCl (1.0 M), sodium dodecyl sulfate (10%, w/v) or ethylene glycol (50%, v/v), even at 80 °C. The $S = 1/2$ form of the [4Fe-4S]$^+$ cluster was found to exhibit a strongly coupled ^{1}H ENDOR resonance ($A = 22$ MHz) that was exchangeable with the solvent. Such a large coupling has not been observed in any other iron-sulfur protein. Since a unique feature of this 4Fe-ferredoxin is that only 3 cysteinyl residues appear to be coordinated to the [4Fe-4S] cluster, the ENDOR data are consistent with an H_2O molecule being a ligand to the unique Fe site. The $S = 3/2$ form of the [4Fe-4S]$^+$ cluster exhibited a similar, strongly coupled ^{1}H ENDOR resonance, but in this spin state it was not exchangeable with the solvent. This suggests that the [4Fe-4S]$^+$ cluster exhibiting the $S = 3/2$, but not the $S = 1/2$ ground state, is "shielded" from the solvent, presumably by neighboring amino acid residues. In view of the pH dependence of the midpoint potential of the two spin states, the fourth ligand to the cluster and the source of the strongly coupled ^{1}H ENDOR resonance is probably an OH^- rather than H_2O molecule.**

Pyrococcus furiosus is a novel archaebacterium that has the remarkable property of growing optimally at 100 °C. The organism was isolated by Stetter and colleagues in 1986 from shallow marine hydrothermal vents (1). It grows by the fermentation of certain carbohydrates to produce organic acids, CO_2 and H_2, and is the only known archaebacterium that actively produces H_2 during its primary growth mode (see Ref. 2). We recently reported (3) on the purification and properties of the hydrogenase from *P. furiosus*, the enzyme responsible for catalyzing H_2 production. The hydrogenase has an optimum temperature for catalysis of above 95 °C in *in vitro* H_2 evolution assays and is one of the most thermostable enzymes yet known (3). The physiological electron donor for the hydrogenase is a ferredoxin, which has also been purified (4). Interestingly, the *in vitro* rates of H_2 production catalyzed by the hydrogenase using the ferredoxin as an electron carrier were significant only at the growth temperature of the organism (≥80 °C). *P. furiosus* ferredoxin also acts as an electron carrier for other *P. furiosus* enzymes, including pyruvate ferredoxin oxidoreductase[1] and a novel tungsten-iron containing oxidoreductase (5).

P. furiosus ferredoxin is a monomeric protein of molecular weight 7,500 and contains a single [4Fe-4S] cluster (6).[2] It has some unique properties in comparison with other bacterial ferredoxins containing 4Fe clusters, of which more than 30 examples are now known (7–12). The *P. furiosus* protein is stable at 95 °C for at least 12 h, in contrast to other "thermostable" ferredoxins which rapidly denature at 85 °C, and is also very resistant to protein denaturants such as SDS[3] (20%, w/v) (4). In further contrast to other ferredoxins, the [4Fe-4S]$^+$ cluster in *P. furiosus* ferredoxin exists in two different spin states. Only about 20% of the protein molecules contain the cluster in its conventional $S = 1/2$ form, as determined by EPR spectroscopy (4). In the remainder, the [4Fe-4S]$^+$ cluster has a well-defined $S = 3/2$ ground state and exhibits a unique EPR spectrum (6). Moreover, the amino acid sequence of the protein[2] shows that only 3 cysteinyl residues (Cys-11, -17, and -56) are in an arrangement typical of that found in other ferredoxins, while an aspartyl residue (Asp-14) replaces the cysteinyl residue that would customarily complete the cluster coordination. That the [4Fe-4S] cluster in this protein has anomalous coordination to a specific Fe

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[1] J. Blamey and M. W. W. Adams, manuscript in preparation.

[2] E. Eccleston, J.-B. Park, M. W. W. Adams, and J. B. Howard, manuscript in preparation.

[3] The abbreviations used are: SDS, sodium dodecyl sulfate; EPR, electron paramagnetic resonance; ENDOR, electron nuclear double resonance; HEPPS, *N*-2-hydroxyethylpiperazine-*N*′-3-propanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; CAPS, 3-(cyclohexylamino) propanesulfonic acid; E_m, midpoint potential.

atom is also suggested by resonance Raman data from the oxidized protein (6), the relative ease with which one Fe atom can be removed to generate a $[3Fe\text{-}4S]^{0,+}$ cluster (6), and the facile reconstitution of the 3Fe form with other metals (M) to produce mixed metal clusters, $[MFe_3S_4]$ (13).[4]

The objective of the present study was to further characterize the $S = 1/2$ and $S = 3/2$ forms of the $[4Fe\text{-}4S]^+$ cluster in *P. furiosus* ferredoxin with respect to their potentiometric, structural, and stability properties. Specifically, do they have the same redox properties, can they be interconverted, and what structural features differenciate the two spin states? Our results show that both states have the same redox properties and cannot be interconverted, even in the presence of potential denaturants, at extremes of pH, and at temperatures up to 89 °C. In addition, ^1H ENDOR data show that a strongly coupled proton, not previously seen in other iron-sulfur proteins, is associated with the cluster, and that this is solvent exchangeable in one spin state but not the other. These data are clearly consistent with the cluster being coordinated by only 3 cysteinyl residues, and suggest that an OH^- molecule is the fourth ligand to the unique Fe site, the solvent accessibility of which determines at least in part the spin state of the cluster.

MATERIALS AND METHODS

Protein Purification—*P. furiosus* cells were grown in a 400-liter culture, and the ferredoxin was purified under anaerobic conditions from the harvested cells as described previously (4).

Redox Potentiometry and X Band EPR Spectroscopy—Redox potentiometry was carried out in a water-jacketed cell under anaerobic conditions as described by Dutton (14). Where indicated, the following mediators were used at a final concentration of 40 μM (their midpoint potentials at pH 7.0 and 25 °C, were taken from Ref. 15): methyl viologen (−440 mV), benzyl viologen (−350 mV), neutral red (−325 mV), and safranine O (−250 mV). It should be noted that the midpoint potential of methyl viologen changes by −0.6 mV per 1 °C rise in temperature (16) and is calculated at −486 mV at 85 °C (see "Results"). The buffers used were either MOPS (pH 6.8), HEPPS (pH 8.5), or CAPS (pH 10.5), each at a final concentration of 100 mM and each containing 100 mM KCl. Each buffer was adjusted to the required pH at the temperature of the titration. In a typical titration, the ferredoxin was used at a concentration of 3 mg/ml and the initial volume was ~5 ml. The protein was first reduced by adding 2-μl aliquots of sodium dithionite (10 mM, in 50 mM Tris-HCl buffer, pH 8.0) until there was no further change in the redox potential (≤ −500 mV). Potassium ferricyanide (20 mM, in 50 mM Tris-HCl buffer, pH 8.0) was then added in microliter aliquots to raise the potential in increments of 20–30 mV up to ~−250 mV. Sodium dithionite was then added to re-reduce the protein in a similar fashion. At each stage, when the potential had stabilized, samples (250 μl) were removed from the titration cell by syringe, rapidly injected into Ar-flushed EPR tubes, and frozen in a liquid N_2/heptane bath, a procedure that took less than 5 s. EPR spectra were recorded on an IBM-Bruker ER 200D spectrometer interfaced to an IBM 9001 microcomputer and equipped with an Oxford Instruments ESR-9 flow cryostat. No differences were observed in the potentiometric data using samples from titrations performed in the oxidative or reductive directions, and all results include both measurements. All potentials are quoted relative to the standard hydrogen electrode, but were measured using a saturated calomel standard electrode calibrated at the appropriate temperature (see Ref. 5).

Q Band EPR and ENDOR Spectroscopy—Samples of the reduced ferredoxin were prepared in H_2O (protein concentration, 10.7 mM) and D_2O (protein concentration, 13.3 mM) using 50 mM Tris-HCl, pH 8.0, as the buffer. Q-band EPR and ENDOR spectra were recorded as described elsewhere (17). The ENDOR transition frequency for a nucleus, J, of spin $I = 1/2$ is given by Equation 1 as follows.

$$\nu_\pm = |\nu_J \pm A^J/2| \quad (1)$$

where A^J is the orientation-dependent nuclear hyperfine-coupling constant, and $\nu_J = g_J\beta_n H_0/h$ is the nuclear Larmor frequency. For protons ($I = 1/2$) in biological systems, $\nu_H > A^*/2$, and the ENDOR spectrum is a hyperfine-split doublet centered about the free-proton Larmor frequency ν_H (42.6 MHz at $H_0 = 1.0$ T). The magnetic parameters of exchangeable proton and deuteron ($I = 1$) nuclei are related by fundamental nuclear parameters according to Equation 2. Thus, using the magnetic parameters obtained from the equation,

$$A_H/A_D = \nu_H/\nu_D = g_H/g_D = 6.5 \quad (2)$$

the resonances for one isotopic species can be used to predict those of the other. In addition to the $\nu_\pm$ lines, deuterons potentially show a further splitting from the quadrupole interaction. In a system with $S > 1/2$ (and $D > 0$), the observed hyperfine tensor, A_g, is highly anisotropic even when the intrinsic site hyperfine interaction is isotropic, $A_1 = A_2 = A_3 = A$. The effective coupling constant measured at a particular g-value can be taken as $A_g = A(g/2)$. For more details, see Ref. 18.

RESULTS

X Band EPR Spectroscopy and Redox Potentiometry—The EPR spectrum of the ferredoxin reduced by sodium dithionite is shown in Fig. 1. Only about 20% of the protein has a $[4Fe\text{-}4S]^+$ cluster in the conventional $S = 1/2$ form ($g_z = 2.10$, $g_y = 1.87$, $g_x = 1.80$ (4)). The remaining 80% have clusters with an $S = 3/2$ ground state ($g = 5.55$, 4.96, and 2.53 (6)). Together these two EPR signals account for approximately 1.0 spin/mol. EPR spectroscopy was used to determine the extent of cluster reduction and the midpoint potentials (E_m) of the two spin states of the cluster. The results from a typical titration are shown in Fig. 2. Within experimental error, the two spin forms exhibited Nernstian behavior with $n = 1$, and using samples poised at 30 °C in HEPPS buffer, pH 8.5, the apparent midpoint potentials were identical, with $E_m = -365$ mV. Such behavior might be explainable in terms of a single cluster which exists in two different spin states that are in thermal equilibrium at cyrogenic temperatures. This appears not to be the case, however, because the relative intensities of the EPR signals from the $S = 1/2$ and $S = 3/2$ states remain the same between 4 and 16K. (Note that these resonances undergo rapid relaxation and are not seen above 16K.) An alternative explanation, which is supported by the ENDOR data described below, is that the two spin states represent different conformations of a $[4Fe\text{-}4S]^+$ cluster, which are maintained by two different structures of the protein. Conditions were therefore sought to perturb the protein structure, and thus the properties of the two spin states.

The apparent E_m values of the two spin states were unchanged (−365 ± 10 mV) when titrations were performed at 30 °C in HEPPS buffer, pH 8.5, containing SDS (5%, w/v), NaCl (1.0 M), or ethylene glycol (50%, w/v), and the relative

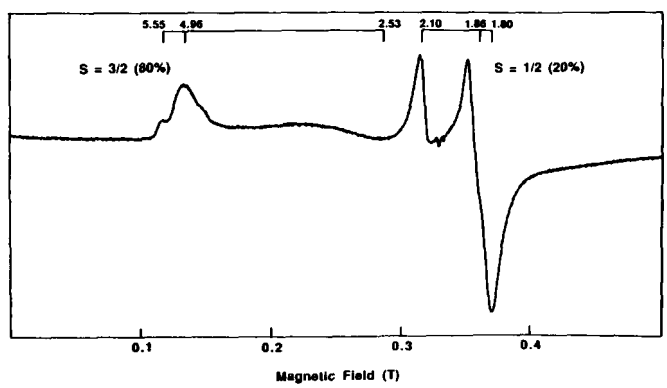

FIG. 1. **EPR spectrum of reduced *P. furiosus* ferredoxin.** The sample contained the ferredoxin (0.95 mM) and 2 mM sodium dithionite in 50 mM Tris-HCl, pH 8.0. Conditions of measurement: temperature, 7 K; microwave power, 10 milliwatts; modulation amplitude, 0.5 millitesla; microwave frequency, 9.41 GHz.

[4] Park, J.-B., and Adams, M. W. W., manuscript in preparation.

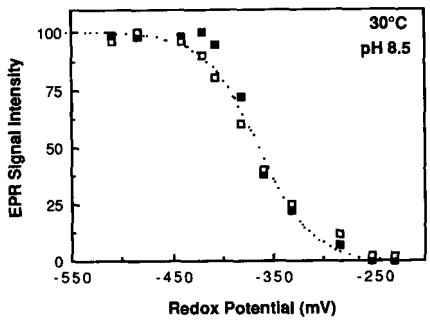

FIG. 2. **Redox titration of *P. furiosus* ferredoxin determined by EPR spectroscopy.** The titration was carried out as described under "Materials and Methods" at 30 °C using HEPPS buffer, pH 8.5. The conditions for EPR spectroscopy are in the legend to Fig. 1. The *open symbols* represent the relative amount of the $S = 1/2$ form of the $[4Fe\text{-}4S]^+$ cluster as measured by the amplitude of the $g = 1.86$ signal (peak to peak). The *closed symbols* represent the relative amount of the $S = 3/2$ form of the $[4Fe\text{-}4S]^+$ cluster as measured by the amplitude of the $g = 4.96$ signal. The curve shows $n = 1$ Nernstian behavior for $E_m = -365$ mV.

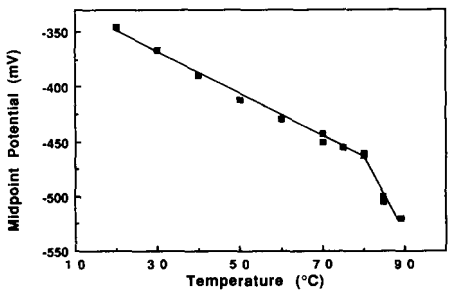

FIG. 3. **Effect of temperature on the midpoint potential of the [4Fe-4S] cluster in *P. furiosus* ferredoxin.** Redox titrations were carried out at the indicated temperature using HEPPS buffer, pH 8.5, as described under "Materials and Methods," and the midpoint potential was determined as in Fig. 2. Each point represents the average of two separate determinations.

amounts of the two spin forms also remained the same (data not shown). The effect of temperature on the midpoint potential of the ferredoxin is shown in Fig. 3. Again, there were no detectable differences between the E_m values of the $S = 1/2$ and the $S = 3/2$ forms of the [4Fe-4S] cluster, nor in their relative amounts, at all temperatures studied. The data in Fig. 3 were obtained from redox titrations carried out in the presence of redox mediators. However, identical results were obtained at temperatures ≥ 60 °C if the mediators were omitted.

In order to validate the apparent transition in the behavior of E_m value at ~80 °C (Fig. 3), additional titrations were performed between 70 and 89 °C, both in the presence and absence of redox mediators. The E_m values at and below 80 °C were consistently ≥ −470 mV, while those determined at and above 85 °C were ≤ −500 mV. These data correspond to $\Delta E_m/\Delta$ °C = −6 mV above 80 °C, which compares with $\Delta E_m/\Delta$ °C = −1.7 mV between 20 and 80 °C. Although the ferredoxin is stable for at least 12 h at 95 °C (4), attempts at redox titrations at 95 °C were unsuccessful because the potential was not stable at this temperature: it rapidly increased in the absence of added oxidant. The reason for this is not known and is currently under investigation. Below 90 °C, the oxidant-independent decrease in the potential was not significant, and in any event, would have the effect of raising the apparent midpoint potential of the protein. Thus, the observed $\Delta E_m/\Delta$ °C = −6 mV above 80 °C is at worst a minimum value. The data shown in Fig. 3 also allow an estimation of the E_m value of the ferredoxin at 100 °C, the optimum growth temperature of *P. furiosus*. It is lower than −500 mV, and from extrapolating the data obtained ≥ 80 °C, possibly as low as −600 mV.

To investigate the effect of potential denaturants on the midpoint potential of the ferredoxin and the relative amounts of the two spin forms of the $[4Fe\text{-}4S]^+$ cluster, redox titrations were also performed at 80 °C in HEPPS buffer, pH 8.5, containing SDS (5%, w/v), NaCl (1.0 M), or ethylene glycol (50%, w/v). The E_m values were −460 ± 10 mV for both spin states and the relative amounts of the two forms were unchanged under all conditions. The effects of changing pH (to 6.8 and 10.5) on the E_m value at 25, 70, and 85 °C are shown in Fig. 4. Only at pH 10.5 and ≥ 70 °C are there any significant differences from the values obtained at pH 8.5 and 6.8, but these are much less than 60 mV/pH unit. Moreover, one would expect a decrease in E_m value with pH if the reduced cluster were protonated at pH 8.5, so the observed pH effects probably arise from the deprotonation of an amino acid group adjacent to the cluster. No differences were observed at the pH extremes in the relative amounts of, or in the E_m values of, the $S = 1/2$ and $S = 3/2$ forms of the cluster.

Q Band EPR and ENDOR Spectroscopy—The above experiments failed to reveal any diffences in the redox properties of the $S = 1/2$ and $S = 3/2$ forms of the $[4Fe\text{-}4S]^+$ cluster in *P. furiosus* ferredoxin, even under extreme conditions of temperature, pH, and presence of potential denaturants, showing that the structures that stabilize the two forms are extremely stable. The protein was therefore examined by Q band ENDOR spectroscopy to determine if these structural differences were manifested by an inequivalence between the two spin forms of magnetically coupled protons. The EPR absorption envelope of the dithionite-reduced ferredoxin recorded at Q band (35.31 GHz) is shown in Fig. 5, together with the corresponding X band spectrum (9.41 GHz). The coincidence of the g-values serves to confirm that the observed resonances arise from a magnetically isolated $[4Fe\text{-}4S]^+$ cluster. The Q band proton ENDOR spectrum of the $S = 1/2$ form of the cluster was taken from the same sample by setting the magnetic field near $g = 1.84$, which corresponds to the maximum of the EPR absorption envelope (Fig. 6*A*). This spin state exhibits a distant ENDOR signal at the proton Larmor frequency ($\nu_H = 42.57$ MHz at $H_0 = 1.0$ T), and at least two weakly coupled doublets centered at ν_H. Each proton ENDOR doublet corresponds to a set of protons with a distinct hyperfine coupling, $A^H = \nu_\pm - \nu_H < 5$ MHz. Similar signals have been observed from other ferredoxins and from FeS-containing enzymes (19, 20), and are assigned to methylene protons of the cysteinyl residues that bind the clusters. However, in addition to these weakly coupled protons, there is a proton resonance at $\nu^H = 69$ MHz, which corresponds to a proton hyperfine coupling of $A^H = 22$ MHz. Such a large coupling

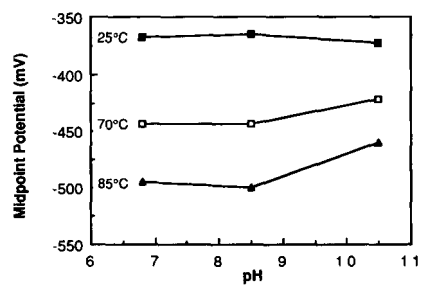

FIG. 4. **Effect of pH on the midpoint potential of the [4Fe-4S] cluster in *P. furiosus* ferredoxin.** Redox titrations were carried out at the indicated temperature using buffer at the indicated pH as described under "Materials and Methods." The midpoint potentials were determined as in Fig. 2.

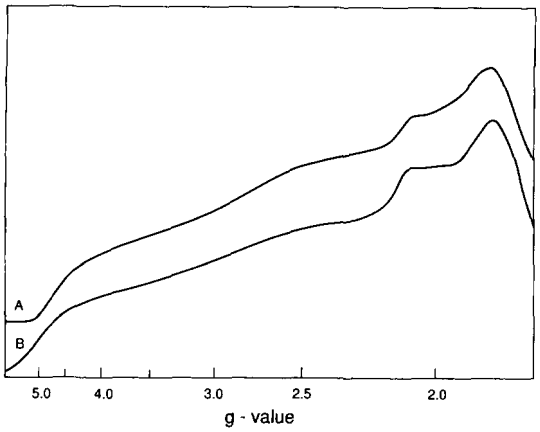

FIG. 5. **EPR absorption envelope of reduced *P. furiosus* ferredoxin at Q band and X band frequencies.** The conditions of measurement for Q band (*A*) were: temperature, 2 K; microwave power, 1 milliwatt; microwave frequency, 35.31 GHz, field modulation, 0.4 millitesla. The X band spectrum (*B*) was obtained by integration of the spectrum shown in Fig. 1.

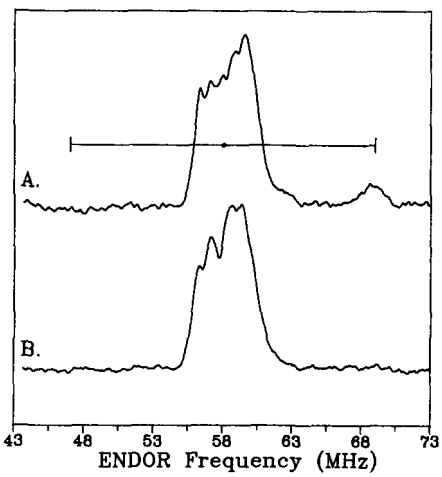

FIG. 6. **Proton ENDOR of the $S = 1/2$ form of the $[4Fe\text{-}4S]^+$ cluster in *P. furiosus* ferredoxin at Q band.** The samples were prepared in H_2O (*A*) and D_2O (*B*). Conditions of measurement: temperature, 2 K; microwave power, 0.05 milliwatt; microwave frequency, 35.39 GHz; magnetic field, 1.375 Teslas; 100 KHz field modulation, 0.16 millitesla; rf scan rate, 2 MHz/s; signal averaging, 100 scans.

has not been observed in any other iron-sulfur protein. The absence of a ν_-^H partner in proton ENDOR spectra taken at Q band has been seen previously (18). It reflects details of the spin relaxation at Q band microwave frequency and is of no importance here, except it leads us to focus on the ν_+^H features.

Fig. 6*B* shows the proton ENDOR spectrum of the $S = 1/2$ spin state of the cluster taken from an analogous ferredoxin sample prepared in D_2O. The signal at ν_+^H ~69 MHz clearly has been eliminated. In addition, there is a loss of distant ENDOR signal intensity at the proton Larmor frequency. Thus, the difference of proton ENDOR spectra upon *H/D* exchange discloses exchangeable proton(s) associated with the $S = 1/2$ $[4Fe\text{-}4S]^+$ cluster, one with hyperfine couplings of $A^H = 22$ MHz.

The strongly coupled proton associated with the $S = 1/2$ form of the reduced cluster is exchangeable with the solvent, being absent in the spectrum recorded from the ferredoxin in D_2O (Fig. 6). The presence of exchangeable protons is confirmed by the observation of deuteron ENDOR signals from the sample prepared in D_2O (Fig. 7). A distant deuteron

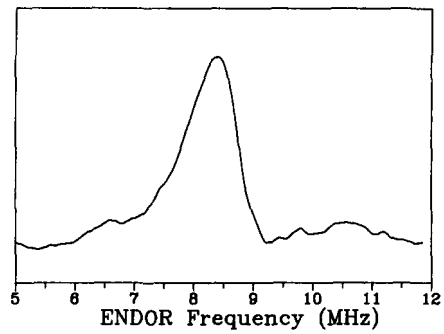

FIG. 7. **Deuterium ENDOR of $S = 1/2$ form of the $[4Fe\text{-}4S]^+$ cluster in *P. furiosus* ferredoxin at Q band.** Conditions of measurement: temperature, 2 K; microwave power, 0.1 milliwatt; microwave frequency, 35.31 GHz; magnetic field, 1.35 Teslas; 100 KHz field modulation, 0.2 millitesla; rf scan rate, 3 MHz/s; signal averaging, 900 scans.

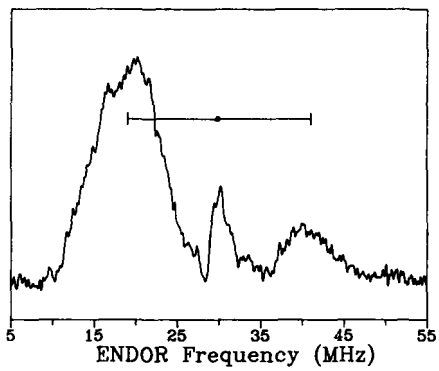

FIG. 8. **Proton ENDOR of the $S = 3/2$ form of the $[4Fe\text{-}4S]^+$ cluster in *P. furiosus* ferredoxin at Q band.** Conditions of measurement: temperature, 2 K; microwave power, 0.8 milliwatt; microwave frequency, 35.24 GHz; magnetic field, 0.7 Tesla; 100 KHz field modulation, 0.32 millitesla; rf scan rate, 2 MHz/s; signal averaging, 300 scans.

ENDOR feature that arises from solvent deuterons appears at the deuteron Larmor frequency ($\nu_D = 8.4$ MHz at 1.35 *T*). In addition, there appears to be a weak and broad feature at ν_- ~10.4 MHz that would correspond to a deuteron resonance with $\nu_+ \sim \nu_D + 2$ MHz. This matches the ν_+ proton peak lost from the proton spectrum upon deuteration when the frequency axes are scaled by the ratio of the nuclear *g*-factors ($g_H/g_D = 6.514$). The observation of an exchangeable proton with a coupling of A^H ~22 MHz most likely indicates that a H_2O or OH^- molecule is in intimate contact with the reduced $S = 1/2$ $[4Fe\text{-}4S]^+$ cluster and that it is solvent accessible.

A similar series of experiments focusing on the $S = 3/2$ form of the $[4Fe\text{-}4S]^+$ cluster gave a surprizing result. A strongly coupled resonance pattern of ^1H doublets was also exhibited by the ferredoxin in H_2O with the magnetic field set at $g = 3.60$; the observed coupling constants are in the range $5 \leq A_g \leq 35$ MHz (Fig. 8), which correspond to $2.5 \leq A \leq 20$ MHz. However in this case, the resonance remained unchanged when the solvent was D_2O (data not shown). The solvent accessibility of the $[4Fe\text{-}4S]^+$ cluster therefore appears to be quite different in the $S = 1/2$ and $S = 3/2$ ground states.

DISCUSSION

The high thermal stability of *P. furiosus* ferredoxin has allowed us to carry out the first investigation of the redox properties of a $[4Fe\text{-}4S]^{+,2+}$ cluster at temperatures up to 89 °C. Interestingly, redox mediators were superfluous at temperatures ≥ 60 °C, indicating that a mechanism exists for

direct electron transfer between the [4Fe-4S] cluster of this relatively small protein and a platinum electrode. This result merits some emphasis since there has been a considerable effort in recent years to achieve direct and preparative electrochemistry of iron sulfur proteins using electrodes such as gold, mercury, platinum, and graphite, *e.g.* Refs. 21 and 22. However, this has not been realized without the use of "promoters," such as viologen derivatives or various bifunctional compounds, which modify the electrode surface (21). For example, direct electrochemistry of a ferredoxin at an elaborately treated graphite electrode was found to occur only in the presence of an aminoglycoside (1 mM), which presumably mediates the protein-electrode interaction (22). Significant insight into mechanisms of electron transfer might therefore be obtained using *P. furiosus* ferredoxin at moderate temperature (60 °C) in the absence of exogenous reagents. It should also be noted that this is not purely a temperature effect. We have recently conducted analogous potentiometric titrations with *P. furiosus* rubredoxin between 20 and 90 °C, and in contrast to the ferredoxin, redox mediators were obligatory even at 90 °C.[4]

Like the 4Fe clusters in the prototypical ferredoxin of the mesophile *Clostridium pasteurianum* (23), there was no formal pH dependence of the E_m value of the 4Fe cluster in *P. furiosus* ferredoxin in the range 6.8–10.5 (25–85 °C). The $S = 1/2$ and $S = 3/2$ spin states of the cluster showed identical redox behavior, and their relative amounts remained constant under all conditions, even in the presence of high salt concentrations, SDS, or ethylene glycol at 80 °C. These results are remarkable for a biological system, since the redox potential and spin state of a [4Fe-4S] cluster are extremely sensitive monitors of the cluster environment, yet this equilibrium appears to be unperturbed when this protein is subject to extreme conditions. The temperature dependence of the E_m value of the ferredoxin (−1.7 mV/ °C over the range of 20–80 °C) is similar to that found with the metal clusters of a novel WFe-protein from *P. furiosus* (approximately −2 mV/ °C over the range 20–70 °C (5)), but less than that found with *P. furiosus* rubredoxin (−3.5 mV/ °C over the range of 50–90 °C).[4] All of these values are greater than those reported for the mesophilic protein, cytochrome *c* (−0.55 to −1.4 mV/ °C, 5–60 °C (24)).

Hysteresis was observed at 80 °C in the temperature dependence of the midpoint potential of *P. furiosus* ferredoxin, with an apparent change of approximately −6 mV/ °C above 80 °C. Although accurate determinations were not possible at temperatures > 90 °C, a similar hysteresis was not observed with the WFe-protein or rubredoxin from *P. furiosus* (5).[4] In addition, the fact that the redox potentials of the metal centers in these three proteins show very different responses to temperature argues against the observed hystersis being an experimental artifact. Moreover, the results shown in Fig. 3 are consistent with the tremendous increase in the rate of H_2 evolution above 80 °C catalyzed by *P. furiosus* hydrogenase when the ferredoxin is the electron carrier (3). In other words, the redox potential of the ferredoxin appears to limit the rate of electron transfer (and H_2 production) below 80 °C. In support of this, a similar temperature effect on the rates of H_2 evolution was not observed with artificial electron donors such as reduced methyl viologen (3).

We now turn to what structural features differenciate the two spin forms of the $[4Fe\text{-}4S]^+$ cluster in *P. furiosus* ferredoxin. The ENDOR data show that this is related, at least in part, to the exchangeability (in the $S = 1/2$ form) or nonexchangeability (in the $S = 3/2$ form) of a strongly coupled and water-derived hydrogen atom(s), that must be in intimate contact with the cluster. The EPR and redox properties of the ferredoxin were essentially unchanged over the pH range 6.8–10.5, so it would seem likely that this hydrogen atom is derived from OH^- rather than H_2O, and that this OH^- molecule remains unprotonated over this pH range. The solvent exchangeability and the pH data effectively rule out protein-derived hydrogen atoms as a source of the strongly coupled ENDOR resonance. Since the amino acid sequence of this protein shows that one of the four consensus sequence of cysteinyl residues that usually coordinate the [4Fe-4S] cluster in ferredoxins is replaced by an aspartyl residue,[2] the most obvious conclusion is that this OH^- molecule is a ligand to the unique Fe site. The replacement of one coordinating cysteinyl group by an aspartyl residue is found in only four other ferredoxins (25–28), but these are of the 8Fe-type, leaving the *P. furiosus* protein as the only example of a 4Fe-ferredoxin with incomplete cysteinyl coordination. That there is a unique Fe atom in the [4Fe-4S] cluster of this protein that has noncysteinyl coordination is also suggested by (*a*) the unusual spectroscopic properties (EPR and resonance Raman) of the ferredoxin (4, 6), (*b*) the relative ease, compared with other ferredoxins, with which one Fe atom can be removed to form a $[3Fe\text{-}4S]^{0,+}$ cluster (6), (*c*) the ease with which other metals (M), *e.g.* Zn, Co, and, in particular, Ni, can reconstitute the 3Fe form to generate a mixed metal $[MFe_3S_4]$ cluster (13), and (*d*) the ability of exogenous ligands such as cyanide to bind to the $[4Fe\text{-}4S]^+$ cluster (29), presumably by displacing the proposed OH^- ligand.

Some support for the coordination of OH^- to the unique Fe site of the 4Fe cluster in *P. furiosus* ferredoxin comes from studies of the enzyme aconitase. This also contains a single [4Fe-4S] cluster coordinated by only 3 cysteines, and it also readily converts to the 3Fe form (rendering the enzyme inactive (30)). Crystallographic analyses show that the fourth Fe of the 4Fe cluster in the native enzyme has H_2O or OH^- ligation (31), and this exhibits a D_2O-exchangeble ^1H ENDOR resonance of 4 MHz (without substrate) or 8 MHz (in the presence of the substrate, citrate). This compares with a value of $A \sim 20$ MHz for the proposed H_2O/OH^- ligand of the 4Fe cluster in *P. furiosus* ferredoxin. As yet there is no information as to whether the aspartyl group at position 14, which replaces the expected cysteinyl residue in the ferredoxin, also coordinates the fourth Fe atom.

The ability of the proposed OH^- ligand to the $[4Fe\text{-}4S]^+$ cluster in *P. furiosus* ferredoxin to exchange with the solvent in the $S = 1/2$ spin state, but not in the $S = 3/2$ form, suggests that nonexchangeabilty of this noncysteinyl ligand is a prerequisite for the stabilization of the $S = 3/2$ spin state. This is supported by the fact that the reduced 4Fe cluster of aconitase, which has an exchangeable noncysteinyl ligand, exists only in the $S = 1/2$ state (30). However, this does not appear to be general rule, because the $[4Fe\text{-}4S]^+$ clusters in nitrogenase Fe protein (32) and phosphoribosyl amidotransferase from *Bacillus subtilis* (33) exist in mixed $S = 1/2$ and $S = 3/2$ spin states, yet analyses of the protein structures indicate that their clusters have complete cysteinyl coordination (33, 34). Like the *P. furiosus* protein, the two spin forms of the 4Fe cluster in nitrogenase Fe protein have the same midpoint potential (35), but in contrast to the ferredoxin, the two spin forms in the Fe protein are partially interconverted by treatment of the protein with ethylene glycol or denaturants (32).

The finding that a proton is exchangeable in the $S = 1/2$ but not in the $S = 3/2$ form of the cluster in *P. furiosus* ferredoxin suggests that significant structural differences exist between these two spin states. That is, the [4Fe-4S] cluster

appears to be shielded from the solvent in the $S = 3/2$ form, presumably by neighboring amino acid residues. The apparent inaccessibility of the $S = 3/2$ form of the cluster raises the question as to what aspects of the protein structure determines which of the two spin forms predominates. This is an intrinsic property of the protein, as the cluster in the reconstituted ferredoxin (after acid precipitation) has the same $S = 1/2$ to $S = 3/2$ ratio as the native protein (4, 6). Whatever they may be, the factors that favor one spin state or the other are very stable, as they are unaffected by high temperature and denaturants. It should be noted that an exhaustive examination of 13 different synthetic $[Fe_4S_4(SR)_4]$ compounds showed that all exist in mixtures of $S = 1/2$ and $S = 3/2$ spin states in frozen dimethylformamide solution, but this study provided no information on how proteins are able to completely stabilize one spin state or the other (36). The list of biological $S = 1/2$ and $S = 3/2$ $[4Fe\text{-}4S]^+$ clusters now includes those in hydrogenase I of *C. pasteurianum* (37), pyruvate ferredoxin oxidoreductase of *P. furiosus*,[1] and *D. africanus* ferredoxin III (38), in addition to *B. subtilis* amidotransferase (33) and nitrogenase Fe protein (32). The ferredoxin from *P. furiosus* may therefore prove to be a model system in which to investigate the protein-cluster interactions that contribute to the spin state of a $[4Fe\text{-}4S]^+$ cluster.

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