Spectroscopic Evidence for a New Type of $[Fe_3S_4]$ Cluster in a Mutant Form of *Pyrococcus furiosus* Ferredoxin

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In addition to the more established electron transfer and catalytic roles,[1] there is increasing evidence that Fe−S clusters are intimately involved in regulation of enzyme activity[2] or gene expression[3] by virtue of their ability to control protein structure in response to intracellular Fe, O_2, O_2^-, or NO levels. This has led to renewed interest in the mechanisms of cluster degradation/assembly and structural interconversions, since these are the primary processes by which Fe−S clusters can exert control over protein structure. Although $[Fe_3S_4]$ clusters have yet to be implicated in any regulatory processes, they have been found to exhibit a rich cluster conversion chemistry.[4−6] Here we report on a new type of medium-dependent reversible cluster interconversion involving the cuboidal $[Fe_3S_4]^+$ cluster in the A33C variant of *Pyrococcus furiosus* ferredoxin (*Pf* Fd).

Pf Fd is a small monomeric protein (7.5 kDa) containing a single cubane $[Fe_4S_4]^{2+,+}$ cluster that readily undergoes oxidative loss of the aspartyl-coordinated Fe to yield a cuboidal $[Fe_3S_4]^+$ cluster.[7] The 3D solution structure,[8] as deduced by NMR studies of the D14C mutant which contains an all cysteinyl ligated $[Fe_4S_4]^{2+}$ cluster,[9] indicates that the C_γ of A33 is 3.8 Å from the Fe coordinated by C17. Hence the A33C variant of *Pf* Fd was constructed, expressed, and purified to homogeneity according to published procedures,[10] in order to assess the consequences of positioning an additional free cysteine in close proximity to the cluster. The anaerobically purified A33C *Pf* Fd contained 3.1 ± 0.2 Fe/molecule,[11] and air oxidation after removal of excess dithionite via anaerobic gel filtration gave the characteristic "$g = 2.02$" resonance of the $S = {}^1/_2$ cuboidal $[Fe_3S_4]^+$ cluster (see Figure 1b). The resonance is identical to that induced by ferricyanide oxidation of wild-type *Pf* Fd (Figure 1a) and corresponds to 1.0 ± 0.1 spins/molecule, indicating the presence of one $[Fe_3S_4]^+$ cluster/molecule. However, the addition of poly(ethylene glycol) (PEG) in the range 10%−60% (v/v) decreased the intensity of the $g = 2.02$ resonance by ∼80% (spin quantitation = 0.2 ± 0.3 spins/molecule) with concomitant increase of an almost isotropic resonance centered at $g = 4.3$ and a much weaker absorption-shaped feature at $g = 9.7$ (Figure 1c), both of which are indicative of a rhombic $S = {}^5/_2$ species.[12] While "$g = 4.3$" resonances are commonly encountered in Fe proteins and generally attributed to adventitiously bound high spin Fe(III) ion, the additional intensity at $g = 4.3$ in the A33C variant is *reversibly* induced by PEG, i.e., removal of PEG by ultrafiltration quantitatively restores the original $g = 2.02$ (see Figure 1d).

The VTMCD spectrum of the $[Fe_3S_4]^+$ cluster in *Pf* Fd (Figure 2a, left) has been found to be remarkably invariant to mutations of residues in close proximity to the cluster,[13] and magnetization studies indicate that all transitions originate from a $S = {}^1/_2$ ground state. In contrast, a completely different pattern of VTMCD bands is observed for air-oxidized A33C *Pf* Fd frozen in the presence of 55% (v/v) PEG and magnetization data collected for the dominant band at 375 nm is readily rationalized in terms of the rhombic $S = {}^5/_2$ ground state observed in the EPR spectrum (see Supporting Information, Figure 1a). The contribution from the residual $S = {}^1/_2$ cuboidal $[Fe_3S_4]^+$ clusters with VTMCD characteristics analogous to those of wild-type *Pf* Fd is readily apparent and these bands can be differentiated on the basis of differences in temperature dependence and/or magnetization studies, e.g. the band at 470 nm is almost exclusively from the $S = {}^1/_2$ component based on magnetization studies (Supporting Information, Figure 1b). The VTMCD spectrum of the $S = {}^5/_2$ component in isolation (Figure 2c, left) was obtained by quantitative subtraction of 20% of the spectrum of ferricyanide-treated wild-type *Pf* Fd. The resulting VTMCD spectrum bears no resemblance to those reported for rubredoxin-type $S = {}^5/_2$ Fe^{3+} centers with complete or partial cysteinyl coordination[14] or to the linear-type $S = {}^5/_2$ $[Fe_3S_4]^+$ clusters in the structurally defined synthetic complex, $(Et_4N)_3Fe_3S_4(SEt)_4$, or formed irreversibly at alkaline pH in purple aconitase.[15]

A direct indication of the type of structural change responsible for the $S = {}^1/_2 \leftrightarrow S = {}^5/_2$ interconversion comes from resonance Raman (RR) studies in the Fe−S stretching region. In the absence of PEG, the RR spectrum of the air-oxidized A33C variant (Figure 2b, right) is very similar to that of ferricyanide-oxidized wild-type Fd (Figure 2a, right), thereby confirming the presence of a cuboidal $[Fe_3S_4]^+$ cluster. The latter has been assigned under effective C_{3v} symmetry for the $Fe_3S_4{}^bS_3{}^t$ unit on the basis of normal mode calculations and $^{34}S^b$ and ^{54}Fe isotope shifts using excitation wavelengths in the range 406−568 nm.[16] The dominant

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(11) Purified samples were in 50 mM Tris-HCl buffer, pH 7.8, containing 1 mM sodium dithionite. Metal analyses were carried out using inductively coupled plasma emission spectroscopy and protein concentrations were determined using a modified Lowry procedure.[10.]

(12) Spectral simulations indicate $g = 4.36, 4.28, 4.21$ for the near isotropic signal centered around $g = 4.3$. The resonance is readily interpreted in terms of a conventional $S = {}^5/_2$ spin Hamiltonian (Zeeman splitting ≪ zero field splitting and $g_0 = 2$) with $E/D = 0.32$ (predicts three doublets with $g_{x,y,z} = 0.65, 9.65, 0.93$, $g_{x,y,z} = 4.36, 4.21, 4.28$, $g_{x,y,z} = 9.70, 0.57, 0.79$, for the lower, middle, and upper zero field components assuming $D > 0$).

(13) This is best illustrated by recent VTMCD studies of the $S = {}^1/_2$ $[Fe_3S_4]^+$ clusters in the D14X series of mutants where D14 is the aspartyl residue that coordinates the removal Fe of the $[Fe_4S_4]$ cluster and X = N, S, C, H, V, and Y (unpublished results).

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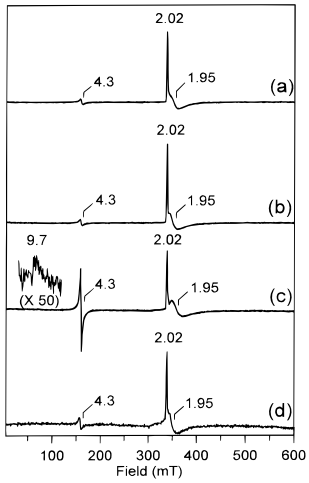


Figure 1. X-band EPR spectra ferricyanide-oxidized wild-type and air-oxidized A33C *Pf* Fd: (a) wild-type (0.53 mM); (b) A33C variant (0.45 mM); (c) A33C variant after addition of 55% (v/v) PEG (0.22 mM); (d) A33C variant sample used in (c) after removal of PEG (0.04 mM). Conditions: microwave power, 1 mW; temperature, 5 K; microwave frequency, 9.60 GHz; modulation amplitude, 0.64 mT.

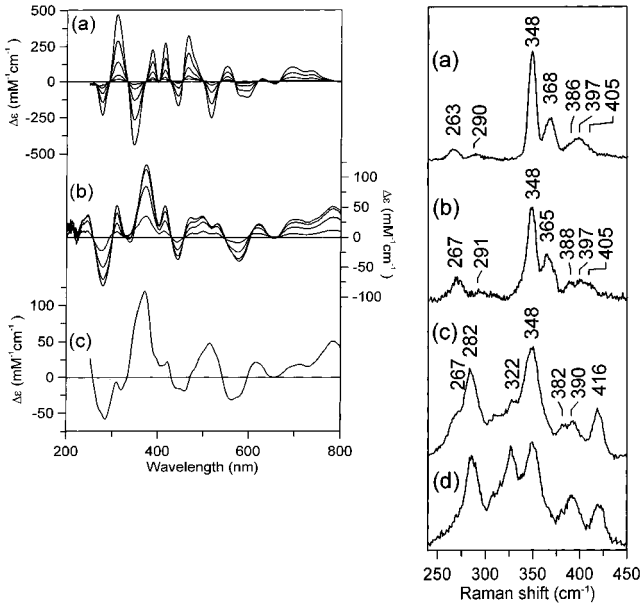
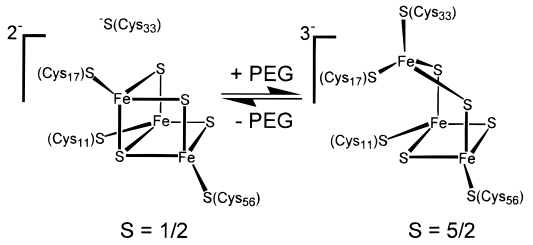


Figure 2. (left) VTMCD spectra of ferricyanide-oxidized wild-type and air-oxidized A33C *Pf* Fd with 55% (v/v) PEG: (a) wild-type (0.25 mM) MCD at 4.5 T, and 1.66 K, 4.22 K, 10.9 K, 18.6 K; (b) A33C variant (0.22 mM) MCD at 6.0 T, and 1.66 K, 4.22 K, 10.4 K, 32.6 K. (c) Difference spectrum of (b) − 0.2(a) at 1.86 K. (right) Low-temperature (30 K) resonance Raman spectra of ferricyanide-oxidized wild-type and air-oxidized A33C *Pf* Fd: (a) wild-type with 457.9-nm excitation; (b) A33C variant with 457.9-nm excitation; (c) A33C variant containing 15% (v/v) PEG with 457.9-nm excitation; (d) As in (c) except for 488.0-nm excitation. All samples were between 0.25 and 0.45 mM in Fd and each spectrum is the sum of 25 scans. Each scan involved photon counting for 1 s every 0.5 cm^{-1} with a 6-cm^{-1} spectral bandwidth.

band at 348 cm^{-1} is assigned primarily to the symmetric stretch involving the single μ_3-S^b. While it is probable that the residual cuboidal $[Fe_3S_4]^+$ cluster contributes in whole or in part to the bands at 267 and 348 cm^{-1}, it is clear that the $S = {}^5/_2$ species has a very different RR spectrum with dominant bands at 282, 322, 390, and 416 cm^{-1}. Overall the spectrum is not indicative of a cluster with μ_3-S^b units, such as cuboidal $[Fe_3S_4]$ or $[Fe_4S_4]$ clusters. Rather it corresponds closely to that observed for $[Fe_2S_2]^{2+}$ clusters in 2Fe Fds. An intense band between 280 and 305 cm^{-1} is a unifying and characteristic feature of all protein-bound and synthetic $[Fe_2S_2]^{2+}$ clusters investigated thus far.[17] By analogy with wild-type and variant forms of rubredoxin,[18] the 322-cm^{-1} band that is preferentially enhanced with 488-nm excitation is a good candidate for the symmetric stretch of an associated FeS_4 unit.

Scheme 1



The availability of an additional cysteinyl ligand, coupled with the $S = {}^5/_2$ ground state, the reversibility of the interconversion, and the structural properties deduced from the RR data, all point to a simple model for the medium-dependent structural equilibria that is observed in the A33C variant of *Pf* Fd (see Scheme 1). Conversion to the $S = {}^5/_2$ form is proposed to involve ligation of C33, coupled with cleavage of one of the Fe−(μ_3-S) bonds, to yield a $S = 0$ $[Fe_2S_2]^{2+}$ fragment with two terminal cysteines that is bridged via two doubling bridging sulfides to a $S = {}^5/_2$ Fe^{3+} site with two cysteinyl-S ligands completing the tetrahedral coordination. From an historical perspective, it is interesting to note that this type of structure was originally proposed as a plausible alternative to a cuboidal $[Fe_3S_4]$ cluster prior to definitive crystallographic identification.[19] Although deprotonation of C33 would provide a logical driving force for the conversion, there is as yet no evidence that the equilibrium is dependent on pH. No conversion to the $S = {}^5/_2$ form was observed at pH 10 in the absence of PEG and the extent of PEG-induced conversion was unaltered at pH 10. Hence the conversion appears to result from a PEG-induced protein conformational change. In addition to demonstrating the existence of an alternative type of cluster conversion involving cuboidal $[Fe_3S_4]^+$ clusters and characterizing a novel type of $S = {}^5/_2$ $[Fe_3S_4]^+$ cluster, this work raises the possibility that analogous clusters may have been overlooked in other proteins, since $g = 4.3$ resonances are ubiquitous in oxidized Fe−S proteins. The nature of the magnetic coupling in this $S = {}^5/_2$ $[Fe_3S_4]^+$ cluster are currently being investigated by Mössbauer studies and the structural, electronic, and magnetic properties of the one-electron reduced $[Fe_3S_4]^0$ cluster in the A33C variant are currently under investigation using the combination of parallel-mode EPR, VTMCD, RR, and Mössbauer spectroscopies.

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Supporting Information Available: Figure 1a,b giving MCD magnetization data for air-oxidized, PEG-treated A33C *Pf* Fd (2 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

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