# Role of Cluster-Ligated Aspartate in Gating Electron Transfer in the Four-Iron Ferredoxin from the Hyperthermophilic Archaeon *Pyrococcus furiosus*

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The "bacterial"-type or cubane ferredoxins (Fds)[1−4] are small electron-transfer proteins containing one or two $[Fe_4S_4]^{+,2+}$ and/or $[Fe_3S_4]^{0,+}$ clusters, with the overwhelming majority possessing complete Cys ligation via the consensus sequence $Cys^{I}$-$X_2$-$Cys^{II}$-$X_2$-$Cys^{III}$•••$Cys^{IV}$. The rate of electron transfer between proteins depends on the driving force (the difference in reduction potentials, $E°$, of the reactants), the reorganization energy, $\lambda$, that results from structural changes accompanying loss or gain of an electron, and the "conductivity" of the intervening protein medium.[5,6] $E°$ values for Cys-only ligated cubane Fds range from +80 to −700 mV but are usually near −400 mV.[4] $E°$ is modulated by H-bonds to the cluster, polarity of the cluster environment, and water access to the cluster, among others, but precise mechanisms are incompletely understood.[7] There are two Fds known that contain a single 4Fe cluster where $Cys^{II}$ in the consensus sequence is occupied by another amino acid, Ala[8] or Asp.[9] Of particular interest here is the latter example, found in the 4Fe Fd[10] from the hyperthermophilc archaeon, *Pyrococcus furiosus* (*Pf*), an organism that thrives in sulfide-rich marine environments at temperatures near 100 °C.[11] While this 4Fe *Pf* Fd possesses several other unusual properties (beside its extraordinary thermostability), such as facile interconversion between 3Fe and 4Fe forms,[9] ease of formation of mixed metal $[MFe_3S_4]$ clusters,[12−15] and *in vitro* ligation of the $[Fe_4S_4]^+$ cluster by cyanide,[16,17] its reduction potential (−350 mV)[18] is well within the range spanned by Cys-only ligated Fds. Hence, a relevant question is, what, if any, is the role of Asp ligation of a Fd cluster in the context of its electron transfer? The recent cloning and heterologous expression of the gene for *Pf* Fd[19] has allowed us to address this issue.

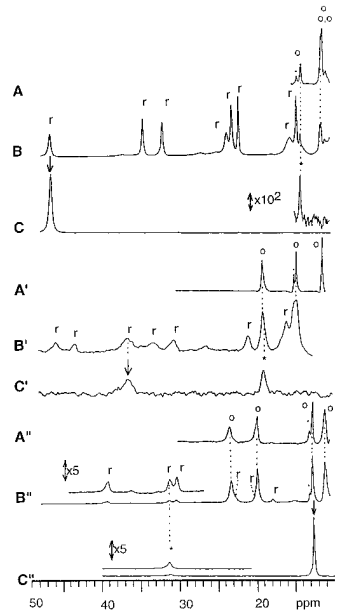


**Figure 1.** Resolved low-field portion of the 500 MHz spectra at 30 °C for oxidized ($[Fe_4S_4]^{2+}$) (A) 7 mM WT Fd, (A′) 7 mM Asp14 → Cys *Pf* Fd, and (A″) 5 mM Asp14 → Ser *Pf* Fd, with ligand peaks labeled o (for oxidized). Partial reduction of the oxidized Fd[21] with sodium dithionite yields resolved peaks for both $Fd^{ox}$ ($[Fe_4S_4]^{2+}$) and $Fd^{red}$ ($[Fe_4S_4]^{+}$) (peaks labeled r for reduced) in the 10−50 ppm spectral window for (B) 7 mM WT *Pf* Fd at 30 °C, (B′) 0.7 mM Asp14 → Cys *Pf* Fd at 10 °C, and (B″) 5 mM Asp14 → Ser *Pf* Fd at 10 °C. The difference spectra obtained upon saturating one resonance (indicated by vertical arrow) for each protein sample with spectra in B, B′, and B″ leads to saturation transfer to the same proton (indicated by asterisk) in the alternate cluster redox state, as shown for (C) WT *Pf* Fd at 30 °C, (C′) Asp14 → Cys *Pf* Fd at 10 °C, and (C″) Asp14 → Ser *Pf* Fds at 10 °C, respectively. All solutions are in 90% $^1H_2O$, 10% $^2H_2O$, 0.4 M KCl at pH 7.6. Minor component resonances marked by • arise from partial conversion of the Cys21, Cys48 free sulfhydryl groups to a disulfide bridge.[22]

$^1$H NMR studies on WT 4Fe *Pf* Fd have shown that a disulfide bridge formed by two additional Cys (21 and 48) remote from the cluster is redox active[20] and that Asp14 (replacing $Cys^{II}$) is ligated to the cluster in all four redox states of the protein.[21,22] However, while the electronic structures for the alternate cluster redox states of *Pf* Fd appeared to resemble those of Cys-only ligated Fds,[23,24] the *rate of electron self-exchange was significantly slower*[21] than those for other Fds. We report herein on the NMR properties of two *Pf* 4Fe Fd mutants, Asp14 → Cys (D14C) and Asp14 → Ser (D14S), which demonstrate that the strongly retarded electron transfer rate in the wild-type (WT) *Pf* 4Fe Fd relative to those of other Fds is a direct result of Asp versus Cys ligation to the cluster. Construction, purification, and biochemical properties of the mutants will be described elsewhere;[25] the extreme thermostability[11] is retained in both mutants.[26] Preliminary spectroscopic

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(26) The two mutants exhibit thermal stability very similar to that of WT *Pf* Fd, i.e., undetectable denaturation over 15 min at 130 °C and detectable denaturation over 15 min at 140 °C.[25]

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characterization shows that the folding topology of the WT Fd, as reflected in the $^1$H NMR spectra and 2D NMR maps over the diamagnetic $^1$H NMR spectral region (Supporting Information), is retained in the mutants. The D14C and D14S Fds near neutral pH exhibit in their reduced state EPR spectra, characteristic of $S = {}^1/_2$ ground states for $[Fe_4S_4]^+$, and the absence of EPR signals for the oxidized Fd is consistent with the expected diamagnetic $[Fe_4S_4]^{2+}$ cluster.[3,4,23,24] The reduction potentials of D14C and D14S Fd are similar (−397 and −405 mV, respectively) and slightly more negative than that (−350 mV) of the WT.[18]

The low-field resolved portions of the 500 MHz $^1$H NMR spectra for ∼5−7 mM solutions of anaerobically-prepared[22,25] WT, D14C, and D14S oxidized ($[Fe_4S_4]^+$) Fds are shown in Figure 1A, A′, and A″, respectively. Each spectrum exhibits three or four resolved, strongly relaxed, and contact-shifted ligand signals (labeled o for oxidized) with uniform anti-Curie behavior that is characteristic of the diamagnetic $[Fe_4S_4]^{2+}$ ground state.[23,24] The ligation of Cys14 in the mutant is confirmed by the detection of four sets of contact-shifted resonances in each case[27] (data not shown), although the individual assignment remains to be determined. Partial reduction with dithionite of the 7 mM WT $Fd^{ox}$ sample[22] (Figure 1B) leads to the appearance of a set of narrow, low-field resonances (labeled r for reduced), previously shown[21] to arise from $Fd^{red}$. Similar partial reduction of the 5 mM D14C and 7 mM D14S $Fd^{ox}$ solutions at 30 °C led to loss of the $Fd^{ox}$ signals *without* appearance of $Fd^{red}$ signals for D14C Fd and to broadening of the $Fd^{ox}$ signal and appearance of broadened peaks for $Fd^{red}$ for D14S Fd (not shown). However, lowering the temperature to 10 °C for the 5 mM D14S $Fd^{ox}/Fd^{red}$ mixture (Figure 1B″), and diluting by a factor of 10 (to 0.7 mM), as well as lowering the temperature to 10 °C for D14C $Fd^{ox}/Fd^{red}$ (Figure 1B′), leads to spectra with the expected new peaks in the 10−50 ppm window (labeled r) for each mutant $Fd^{red}$, with both Curie and anti-Curie behavior characteristic of the $[Fe_4S_4]^+$ cluster.[23,24]

The observed temperature and concentrations effects on line width of the oxidized (o) and reduced (r) peaks are characteristic of rapid electron self-exchange for the mutants, with qualitative differences in rates at high salt concentration in the order WT << D14S < D14C Fd for the reaction

$$[Fe_4S_4]^+ + [Fe_4S_4]^{2+} \rightleftarrows [Fe_4S_4]^{2+} + [Fe_4S_4]^+ \qquad (1)$$

Quantitative description of the electron self-exchange is conveniently pursued by magnetization transfer.[28] Saturation of a peak for one oxidation state (species Y) leads to saturation transfer for the peak of the same proton in the alternate cluster oxidation state (species X), as shown for the three Fds in Figure 1C, C′, and C″. The fractional intensity change, $F_x$, for X upon saturating Y ($F_x = (X_0 - X_\infty)/X_0$, where $X_\infty$ and $X_0$ are the peak intensities with and without saturating Y, respectively) and the intrinsic relaxation rate for X, $\rho_x$, yield an observed rate, $k_{xy}$

$$k_{xy} = \rho_x F_x [1 - F_x]^{-1} \qquad (2)$$

and an electron self-exchange rate, $k_{se}$

$$k_{xy} = k_{se}[Y] \qquad (3)$$

The values of the parameters obtained from peak intensities and nonselective $T_1$ determination for several different pairs of resonances and at different ratios of [X],[Y], including the cases shown in Figure 1C, C′, and C″, yield $k_{se} = (4 \pm 1) \times 10^2$ $mol^{-1}$ $s^{-1}$ at 30 °C for WT *Pf* Fd and $(1.1 \pm 0.5) \times 10^6$ and $(3.0 \pm 0.8) \times 10^4$ $mol^{-1}$ $s^{-1}$ at 10 °C for D14C *Pf* Fd and D14S *Pf* Fd, respectively. Clearly, the ligation of an Asp rather than a Cys in the consensus ligating position II leads to a $>2 \times 10^3$-fold *reduction* of the electron transfer rate (rate data on D14C Fd are determined at 10 °C). The structure[27] and thermostability[26] of the three proteins are very similar, eliminating gross structural changes near the cluster as the origin of the very different electron exchange rates. Since electron self-exchange eliminates effects from the driving force for the reaction,[5,6] and the "conducting medium" is unlikely to change with the new conserved protein fold, the significant difference in $k_{se}$ must reflect a much larger reorganization energy, $\lambda$, in the vicinity of the cluster with ligated Asp rather than Cys. The slower $k_{se}$ by $>5 \times 10^3$ reflects a larger activation free energy, $\Delta G^* > 4.5$ kcal (and hence[5,6] larger $\lambda$, $\sim 4\Delta G^*$, by >19 kcal), in WT than in D14C Fd. The difference between ligated Cys and Ser is more modest.

A possible unique role for Asp vs Cys ligation to the Fe atom of a cluster is that the former, but not the latter, is capable of binding in either a monodentate or a bidentate fashion.[21,29] A cluster redox-state-dependent interconversion between these ligating modes of Asp could provide the trigger for a structural change that may involve portions of the protein structure outside the immediate vicinity of the cluster. A spectroscopic study of a 4Fe model complex with three thiolate ligands and one carboxylate ligand had concluded that the carboxylate binds in the bidentate fashion in the $[Fe_4S_4]^{2+}$ state.[29] The cluster ligand $H_\beta$ contact shift pattern, which reflects the orientation of the ligand relative to the cluster,[23,24,30] differed primarily for Asp14 for the alternate cluster redox states in WT *Pf* 4Fe Fd.[21] These NMR data are consistent with a role for the Asp ligation mode in gating the electron transfer rate.[31] The value for $k_{se}$ in D14S Fd is closer to that of D14C than WT Fd and suggests that even Ser vs Cys ligation results in an increase in the reorganization for Ser ligation.

It is noted that the retarded cluster self-exchange in *Pf* Fd relative to conventional Cys-ligated clusters in 4Fe Fd[32,33] is not a property common to all hyperthermophilic Fds. *Thermococcus litoralis* (*Tl*) 4Fe Fd, which, like Pf Fd, is also stable at 95 °C over 12 h without denaturation, has complete Cys ligation but exhibits rapid electron self-exchange[33] comparable to that observed with mesophilic 4Fe Fd.[32] Both *Pf* and *Tl* Fd serve as electron carriers to numerous oxidoreductive-type enzymes *in vivo*,[34] and it is not clear yet whether Asp rather than Cys ligation to the cluster in *Pf* Fd affords any advantage for mediating electron transfer at temperatures near 100 °C. The physiological role of the electron transfer "gating" notwithstanding, *Pf* 4Fe Fd and its ligation mutants provide an exceptional model for the detailed structural elucidation of the reorganization energy that accompanies cluster redox state changes. Although *Pf* Fd has so far failed to yield crystals amenable to high-resolution X-ray diffraction, preliminary 2D NMR studies[35] of the 3Fe form of *Pf* $Fd^{ox}$ indicate that the requisite molecular structures for both WT and mutants are obtainable on the basis of solution NMR; such studies are in progress.

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**Supporting Information Available:** Diamagnetic 0−10 ppm portion of the 500 MHz $^1$H NMR spectra for WT, D14C, and D14S $Fd^{ox}$ (1 page). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

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