

# *Pyrococcus furiosus* ferredoxin is a functional dimer

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**Abstract** *Pyrococcus furiosus* ferredoxin is subject to a monomer/dimer equilibrium as a function of ionic strength. At physiological ionic strength, approximately 0.35 M NaCl, the protein is very predominantly homodimer. The monomeric form exhibits impaired electron transfer on glassy carbon; it also has a decreased  $S=3/2$  over  $S=1/2$  ratio as shown by electron paramagnetic resonance spectroscopy. Even following sterilization at 121°C the dimer is stable in denaturing gel electrophoresis.

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**Key words:** Ferredoxin; Thermophile; Dimer; Electron transfer; *Pyrococcus*

## 1. Introduction

*Pyrococcus furiosus* is a strict anaerobic, hyperthermophilic marine archaeon with an optimal growth temperature of 100°C [1]. *P. furiosus* is a practical model organism, as it grows easily and rapidly, in the absence of elemental sulfur, on cheap substrates in up to several hundred liter batch cultures, and cell-free extract is readily obtained by osmotic shock in water. Furthermore, the complete genome of *P. furiosus* has been determined, as well as that of the related species *Pyrococcus abyssi* and *Pyrococcus horikoshii*. The biochemistry has now been studied for 13 years. When grown on carbohydrate, e.g. starch, *P. furiosus* uses a modified Embden-Meyerhoff pathway for glycolysis to the level of acetate [2].

A single ferredoxin (Fd) has been purified from *P. furiosus* and no putative additional Fds are indicated from our genome inspection. The 7.5 kDa *P. furiosus* Fd, *Pfu* Fd, is extremely thermostable. It contains a single [4Fe-4S]<sup>(2+;1+)</sup> cubane with Cys, Asp, Cys, Cys coordination [3]. Two additional Cys residues can reversibly form a disulfide bridge [4]. *Pfu* Fd is synthesized in considerable amounts reflecting its key role(s) in mainstream electron transfer. The protein acts as the electron-acceptor for the two glycolytic redox enzymes, glyceraldehyde-3-phosphate oxidoreductase and pyruvate oxidoreductase, and as the electron-donor for a transmembrane multi-subunit hydrogenase complex [5]. *Pfu* Fd is also the putative redox partner of several other oxidoreductases involved in oxoacid or aldehyde activation [6].

Structural studies of *Pfu* Fd have presented several difficul-

ties. It has thus far not been possible to obtain crystals suitable for X-ray crystallographic analysis. A crystallized one-to-one complex of the Fd with formaldehyde oxidoreductase afforded a high-resolution structure of the enzyme, however, the Fd was found to be disordered [7]. <sup>1</sup>H-NMR (nuclear magnetic resonance) studies have shown multiple structural heterogeneity, part of which has been interpreted to initiate at the disulfide bridge, which appears to occur in two mirror orientations [8]. Electron paramagnetic resonance (EPR) spectroscopy has established a spin mixture of  $S=1/2$  and  $S=3/2$  species for the [4Fe-4S]<sup>1+</sup> cluster. EPR also provided evidence for multiple  $S=1/2$  heterogeneity in the reduced cluster following aerobic purification [3].

The cumulative manifestations of heterogeneity have incited us to re-visit a long-standing, unsolved problem regarding the oligomeric state of the *Pfu* Fd. In the original paper on the purification of *Pfu* Fd, a molecular mass of 12–13 kDa was reported, and the possibility was raised that the Fd as isolated exists as a dimer [9]; the calculated mass of the gene product (ignoring the prosthetic group) is 7.26 kDa. However, in a subsequent 1990-paper by Conover et al. [3] *Pfu* Fd was re-defined as monomeric with reference to data to be published (E. Eccleston, J.B. Park, M.W.W. Adams, and J.B. Howard, manuscript in preparation). To our knowledge this paper has not been published. In all later literature *Pfu* Fd has been implicitly or explicitly taken to be monomeric with frequent reference to the paper by Conover et al. Also, Smith et al. claimed their MALDI-MS data on dried protein to indicate that *Pfu* Fd is monomeric [10]. During purifications of *Pfu* Fd over the years we noted chromatographic behavior suggestive of strong dimerization. In the present paper we describe experiments to determine the aggregation state of *Pfu* Fd and its relation to some physico-chemical properties of the protein.

## 2. Materials and methods

The cultivation of *P. furiosus* (DSM 3638) was carried out as previously described [11]. Purification of *Pfu* Fd was carried out as stated previously [12] except that lysozyme was omitted. The protein concentration was determined with Micro-Biuret reagents. Gel electrophoresis was carried out with the Phast System (Pharmacia) and NuPAGE (Invitrogen) Bis-Tris electrophoresis system according to the manufacturers' instructions. The Phast system is based on the Laemmli protocol except for Tricine in the running buffer. For the NuPAGE system Bis-Tris-HCl buffered (pH 6.4) 4–12% gradient or 10% homogeneous polyacrylamide gels were used with a running buffer (pH 7.3) comprised of 50 mM MES, 50 mM Tris base, 1% SDS, 1 mM EDTA, and an antioxidant. In contrast to the traditional Laemmli sample buffer [13], the sample buffer contained lithium dodecyl sulfate (LDS) and 50 mM DTT as denaturant.

Analytical gel filtration was carried out with a HR 10/30 (10 mm×30 cm, 24 ml) Superdex S-75 column (Pharmacia LKB). All the buffers used contained 50 mM Tris-HCl, pH 7.8, 10% (v/v) glycerol.

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erol, 2 mM DTT, 2 mM sodium dithionite, in addition to variable amounts of NaCl. The column was equilibrated with buffer containing the desired concentration of salt before every run. All the buffers and columns were thoroughly flushed with argon to make them anaerobic and all the experiments were carried out under a small over-pressure of argon to maintain strict anaerobicity. For molecular weight determination a mixture of 1.5  $\mu$ l cytochrome *c* (25 mg/ml), 10  $\mu$ l *Pfu* Fd (7.5 mg/ml) and 10  $\mu$ l myoglobin (3 mg/ml) was injected onto the column. In all other salt effect studies a mixture of 2.5  $\mu$ l *Pfu* Fd (7.5 mg/ml) and 4  $\mu$ l cytochrome *c* (2.5 mg/ml) was diluted to a total volume of 25  $\mu$ l with the running buffer at the desired salt concentration and briefly incubated at room temperature before loading onto the column.

Cyclic voltammetry experiments were carried out as described earlier [14]. All the EPR measurements were carried out as described earlier [15]. The conductivity of the cell lysate was measured with a Consort K720 conductivity sensor, calibrated with standard NaCl solutions (0–80 mM).

### 3. Results

#### 3.1. Size exclusion chromatography

To determine the physiological oligomeric form (monomer/dimer) of *Pfu* Fd size exclusion chromatography experiments were carried out at different salt concentrations from 0.15 M to 3.25 M NaCl. At 0.15 M–0.5 M NaCl the retention time for *Pfu* Fd was greater than that of myoglobin (Mw = 17 kDa) and less than that of cytochrome *c* (Mw = 13.7 kDa) (Fig. 1A), which suggests that at physiological salt concentration (approx. 0.35 M NaCl, see below) *Pfu* Fd exists as a dimer. With an increase of the salt concentration the retention time increased (Fig. 1B), indicating a change in apparent mass, which we attribute to a shift from dimer to monomer. Above 1 M NaCl the retention time for *Pfu* Fd became longer than that for cytochrome *c*. To exclude the possibility of hydrophobic interaction between the *Pfu* Fd and the gel matrix, we have also carried out an experiment adding 20% acetonitrile to the buffer at 3 M NaCl. No change in the retention time of *Pfu* Fd was observed compared to the run without acetonitrile (not shown).

#### 3.2. Gel electrophoresis

*Pfu* Fd as isolated appeared as a 12–13 kDa protein in denaturing polyacrylamide gel electrophoresis (Fig. 2). Several SDS–PAGE experiments were carried out to find conditions to monomerize the *Pfu* Fd. No change in apparent mass has

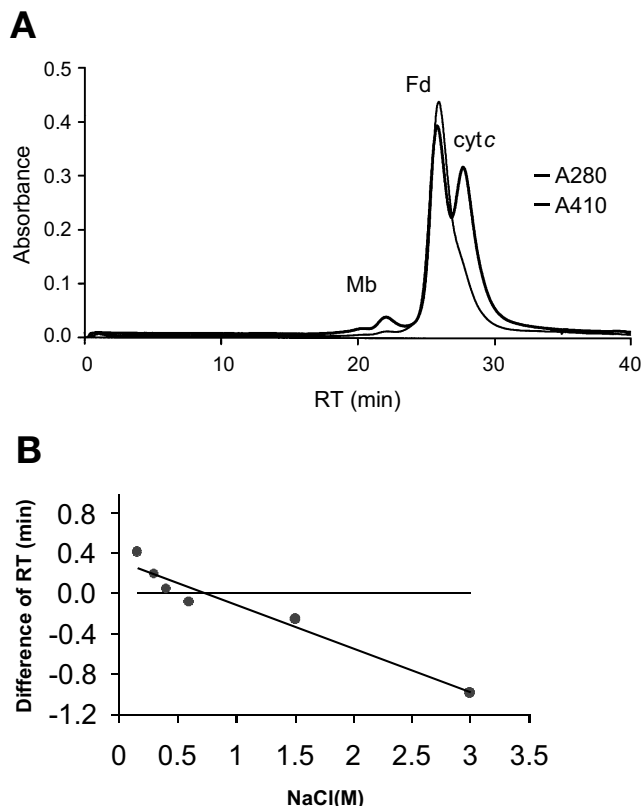


Fig. 1. A: Gel filtration of anaerobically purified *Pfu* Fd, horse heart cytochrome *c*, and horse myoglobin on a Superdex-75 column (10 mm  $\times$  30 cm). The buffer was 100 mM NaCl, 50 mM Tris–HCl (pH 7.9), 10% glycerol (v/v), 2 mM DTT, 2 mM sodium dithionite. The experiment was carried out with a flow rate of 0.5 ml/min, at room temperature ( $\sim$ 20°C) and anaerobic condition. Amounts of proteins loaded were *Pfu* Fd  $\sim$ 75  $\mu$ g, cytochrome *c*  $\sim$ 40  $\mu$ g and myoglobin 30  $\mu$ g. B: Dependence of the retention time (RT) of *Pfu* Fd on the salt concentration. The Y-axis shows the difference of the retention time between cytochrome *c* and *Pfu* Fd, with respect to the RT for cytochrome *c*. The experiments were carried out under the same conditions as in (A) except for a stepwise increment of salt concentration.

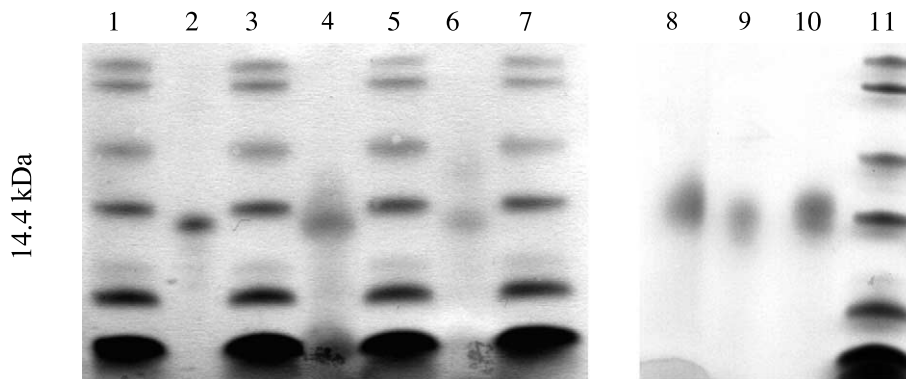


Fig. 2. SDS–PAGE of pure *Pfu* Fd at different conditions, a mixture of *Pfu* Fd plus *Pfu* Rb, and cytochrome *c*. Conditions: Bis–Tris buffered 10% polyacrylamide gel, 50 mM MES running buffer with antioxidant, LDS sample buffer with 50 mM DTT. Lanes 1, 3, 5, 7, 11 – Mark 12 MW standard kit (Invitrogen); sizes are from top to bottom 36.5, 31, 21.5, 14.4, 6, 3.5 kDa; Lane 2 – Cytochrome *c*; Lane 4 – Mixture of *Pfu* Fd and *Pfu* Rb; Lane 6 – Pure *Pfu* Fd; Lane 8 – Sterilized *Pfu* Fd in LDS sample buffer; Lane 9 – Sterilized *Pfu* Fd in LDS sample buffer+100 mM DTT; Lane 10 – Sterilized *Pfu* Fd in Laemmli sample buffer.

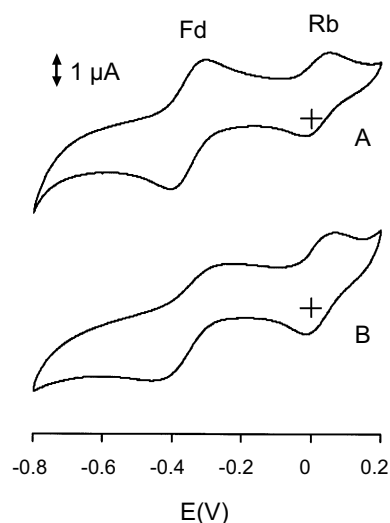


Fig. 3. Cyclic voltammograms of a mixture of *Pfu* Fd and *Pfu* Rb. Experimental conditions: Scan rate 10 mV/s, room temperature, hydrophobic carbon electrode surface. Trace A: Voltammogram of the mixture in 25 mM MOPS (pH 7.2) with 7 mM neomycin; trace B: voltammogram of the mixture in 3 M NaCl, 25 mM MOPS (pH 7.2), with 7 mM neomycin.

been observed on 4–12% gradient or on 10% homogeneous NuPAGE; nor on 8–25% gradient or on 20% PhastGel (Amersham Biosciences), after heating the protein for 1 h at 70°C or 95°C in a denaturing buffer containing 2.5% SDS or LDS, 1 mM EDTA, 5% 2-mercaptoethanol or 5–50 mM DTT (data not shown). We also did not observe any change in the apparent mass after treating the protein for 1 h under sterilizing conditions (121°C and ~90 kPa overpressure) either in 100 mM DTT+LDS sample buffer, or in LDS sample buffer, or in Laemmli sample buffer (Fig. 2).

### 3.3. Cyclic voltammetry

Direct electrochemistry was carried out with a mixture of *Pfu* rubredoxin (Rb) and *Pfu* Fd at pH 7.2, room temperature (approximately 20°C), and at 0 and 3 M NaCl concentration. To eliminate the artefacts of different salt concentration on the electrode response, *Pfu* Rb was used for comparison. It has similarity in charge and monomeric molar mass with *Pfu* Fd. Well defined, reversible and reproducible voltammograms were obtained at low potential scan rate of  $v = 10$  mV/s for both *Pfu* Fd and *Pfu* Rb (Fig. 3). The reduction potential for the [4Fe-4S] cluster of the *Pfu* Fd was found to be  $-365 \pm 5$  mV, while for *Pfu* Rb it was  $\sim 0$  mV, similar to the values reported earlier at low salt [16,17]. However, at high salt concentration the voltammogram for *Pfu* Fd broadened considerably, indicating a kinetically impaired heterogeneous electron transfer, while that of *Pfu* Rb was unchanged. Heterogeneous electron transfer rate constants of *Pfu* Fd were determined from the apparent cathodic-to-anodic peak potential separation at different potential scan rates, assuming a protein diffusion coefficient of  $D = 1 \times 10^{-6}$  cm<sup>2</sup>/s, according to the method of Nicholson [18]. From the estimated rate constants,  $k = 0.92 \pm 0.1$  cm/s at 0 M NaCl and  $k = 0.35 \pm 0.1$  cm/s at 3.25 M NaCl, it was apparent that at high salt the electron transfer rate of *Pfu* Fd was impaired by 70%, i.e. monomeric *Pfu* Fd was considerably less efficient in heterogeneous electron transfer than the dimeric *Pfu* Fd.

### 3.4. EPR spectroscopy

The EPR spectrum attributed to the [4Fe-4S]<sup>1+</sup> cubane cluster in reduced *Pfu* Fd is a mixture of a high-spin  $S = 3/2$  signal and an unusually broad  $S = 1/2$  signal [19]. This combination of spin states (sometimes called ‘physical spin mixtures’) to discriminate it from thermodynamic spin mixtures) is quite commonly found for [4Fe-4S]<sup>1+</sup> clusters especially in proteins from anaerobic microorganisms, however, their nature is not understood [20]. In the Fe-protein of nitrogenase, which is paradigmatic for cubane physical spin mixtures, the ratio of  $S = 3/2$  over  $S = 1/2$  can be changed discretely with addition of ethylene glycol or urea [21].

In *Pfu* Fd we found that the  $S = 3/2$  over  $S = 1/2$  ratio can be changed monotonously as a function of the ionic strength (NaCl concentration) as illustrated in Fig. 4. With increasing ionic strength the relative amount of  $S = 1/2$  signal increased. Spectral simulations (following [22]; not shown) indicated that the relative amount of  $S = 1/2$  increased from approximately 2% at 0.15 M NaCl to approximately 20% at 3.25 mM NaCl. For obvious sensitivity reasons the EPR experiments were done with protein concentrations that were several orders-of-magnitude higher than, e.g. those of the gel filtration experiments, and so we would expect an increased tendency towards dimerization under otherwise equal conditions. We therefore tentatively identify the  $S = 3/2$  to  $S = 1/2$  shift with a shift in *Pfu* Fd protein dimer to monomer equilibrium.

### 3.5. Intracellular ionic strength

Frozen cells were thawed and suspended in de-ionized water to make the cells lysate. 5× and 10× diluted cell lysates were used to estimate the conductivity. With a calibrated conductivity sensor the intracellular salt concentration was determined to be ~350 mM, which is similar to the ionic strength of the media used for cultivating the organism.

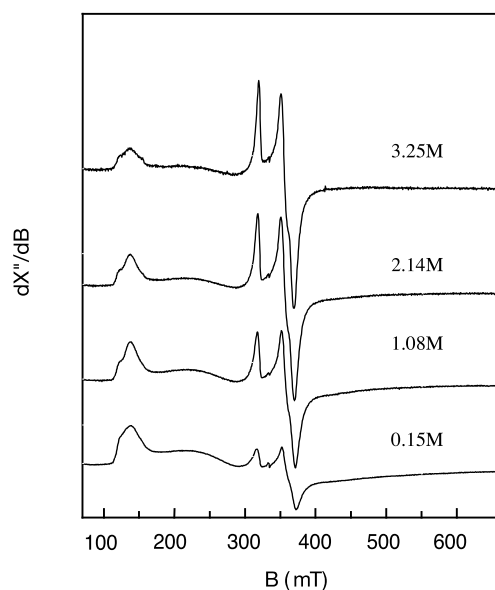


Fig. 4. EPR spectra of anaerobically purified *Pfu* Fd at different ionic strengths. The traces from bottom to top are at 0.15 M, 1.08 M, 2.14 M and 3.25 M NaCl, respectively. The spectral amplitudes have been normalized to that of the bottom trace (7.5 mg/ml protein). EPR conditions: Microwave frequency, 9.42 GHz; microwave power, 8 mW; modulation frequency, 100 kHz; modulation amplitude, 5 mT; temperature, 8 K.

#### 4. Discussion

The 7.5 kDa Fd from *P. furiosus* has been studied intensively for 13 years. The protein has posed significant problems apparently related to conformational inhomogeneities. A 3D structural determination has thus far not been achieved either with X-ray crystallography or with multi-dimensional NMR [8]. Part of these problems may well be related to modification of the Fe-S cluster upon exposure to oxygen and/or to multiple forms of the putative Cys–Cys disulfide bridge (open/closed and *cis/trans*) [8]. However, we would like to put forth here the contention that a major determinant of the *Pfu* Fd inhomogeneity problem can be traced back to the question of its oligomeric state.

We find the early claim of Smith et al. that the MALDI-MS experiment indicated monomeric *Pfu* Fd, unconvincing, where they also noted that “proteins consisting of non-covalently bound subunits break during ion formation and only individual subunits are detected”. Blamey et al. have more recently reported gel electrophoresis and gel filtration experiments on a Fd from *Pyrococcus woesei*, which indicate a monomer/dimer equilibrium for that protein, however with a cross-over concentration between 0.2 and 0.5 M NaCl [23].

On the basis of gel filtration and gel electrophoresis experiments we propose that the *Pfu* Fd is a mixture of monomeric and dimeric protein, in an equilibrium that is far to the dimeric side under physiological ionic strength conditions. The EPR spectroscopic and electrochemical experiments suggest that there is a significant difference in electronic structure and reactivity between the dimeric and monomeric form, namely, the dimer is very predominantly  $S = 3/2$  (at low temperature) and exhibits fast heterogeneous electron transfer, while the monomer is for a significant part  $S = 1/2$  and is impaired in its electron transfer at least with activated-carbon electrodes. Based on the previous we hypothesize that *Pfu* Fd is a functional dimer in vivo, and we note, in addition that all (putative) natural electron-transfer partners of *Pfu* Fd are redox enzymes catalyzing two-electron reactions.

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