

Pyrococcus furiosus glyceraldehyde 3-phosphate oxidoreductase has comparable $W^{6+/5+}$ and $W^{5+/4+}$ reduction potentials and unusual [4Fe-4S] EPR properties

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Abstract *Pyrococcus furiosus* glyceraldehyde 3-phosphate oxidoreductase has been characterized using EPR-monitored redox titrations. Two different W signals were found. W_1^{5+} is an intermediate species in the catalytic cycle, with the midpoint potentials $E_m(W^{6+/5+}) = -507$ mV and $E_m(W^{5+/4+}) = -491$ mV. W_2^{5+} represents an inactivated species with $E_m(W^{6+/5+}) = -329$ mV. The cubane cluster exhibits both $S = 3/2$ and $S = 1/2$ signals with the same midpoint potential: $E_m([4Fe-4S]^{2+/1+}) = -335$ mV. The $S = 1/2$ EPR signal is unusual with all g values below 2.0. The titration results combined with catalytic voltammetry data are consistent with electron transfer from glyceraldehyde 3-phosphate first to the tungsten center, then to the cubane cluster and finally to the ferredoxin.

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Key words: Glyceraldehyde 3-phosphate oxidoreductase; Tungsten; Reduction potential; Electron paramagnetic resonance; *Pyrococcus furiosus*

1. Introduction

Pyrococcus furiosus is a hyperthermophilic archaeon growing optimally at 100°C. It has three tungsten-containing oxotransferases: aldehyde oxidoreductase (AOR), formaldehyde oxidoreductase (FOR) and glyceraldehyde 3-phosphate oxidoreductase (GAPOR). All three enzymes presumably have ferredoxin as a natural electron carrier. Recently the crystal structures of both AOR and FOR have been published [1,2] showing the tungsten coordinated by a bis-pterin cofactor and with a [4Fe-4S] cluster close by. AOR has been shown to exhibit several different W^{5+} EPR signals at different potentials [3]. Only one signal, the 'low-potential signal', has been attributed to a catalytically competent species undergoing two subsequent one-electron reductions from W^{6+} to W^{4+} . *P. furiosus* GAPOR is to date the only enzyme of the three with a known biological function [4]. It is highly specific for the substrate glyceraldehyde 3-phosphate. Contrarily, FOR and AOR have a broader in vitro specificity for short chain and longer chain aldehydes [5,6]. The sequence homology with AOR (15% identity) [7] and FOR (23% identity) [5] is relatively low compared to the homology between AOR and FOR (40% identity) [5]. GAPOR has not been characterized in detail spectroscopically. Only a W^{5+} EPR signal with g values near 1.96, 1.89 and 1.83 has been reported for the reduced enzyme [7,8]. We characterized GAPOR using EPR-monitored redox titrations. The results are compared with what

is known about the other two tungsten-containing oxotransferases from *P. furiosus*.

2. Materials and methods

2.1. Cultivation and protein purification

P. furiosus (DSM 3638) was cultivated as previously described [9]. Cells were broken by osmotic shock, diluting with 5 volumes 30 mM Tris-HCl, pH 8.0 (anaerobic) containing 1 mM dithiothreitol, 1 mM cysteine, 5 mM $MgCl_2$, 0.1 mg/l DNase I, 0.1 mg/l RNase. Cysteine was used as a mild reductant instead of dithionite, which is an inhibitor for GAPOR [4]. A cell-free extract was obtained as the supernatant after 1 h centrifugation at $26000 \times g$. GAPOR was purified anaerobically as described previously [7]. GAPOR activity was measured as described [7]. SDS-polyacrylamide gel electrophoresis was performed on a Phast System (Pharmacia) holding a PhastGel SDS 8–25%. The purified GAPOR had a specific activity of 30 U/mg at 50°C which compares well with the 25 U/mg previously described with the same purification procedure [7]. The purified protein was shown to be pure by SDS-PAGE (not shown).

2.2. EPR spectroscopy and EPR-monitored redox titrations

GAPOR was investigated with a dye-mediated reductive titration as described previously [10] at 50°C in 30 mM Tris-HCl, pH 8.0, using 47 μ M GAPOR. Sodium dithionite was used as reductant to reach low potentials. However, sodium dithionite is an inhibitor of GAPOR activity. Therefore another titration without mediators was performed using the substrate/product couple glyceraldehyde 3-phosphate/3-phosphoglycerate (GAP/3PG) to poise the potentials. Potentials were calculated using the literature value for the GAP/3PG couple, which is -614 mV vs. NHE at 50°C and pH 8.0 [11]. GAPOR was incubated with different ratios of GAP/3PG for 10 min at 50°C. This temperature was chosen to obtain a rapid equilibrium, but at the same time to prevent thermal degradation of GAP. The GAPOR concentration ranged from 65 to 80 μ M. The GAP and 3PG concentrations ranged from 0.1 to 480 mM. Light-reduced GAPOR was prepared by irradiating 35 μ M GAPOR, 25 μ M deazaflavin, 2 mM EDTA in 30 mM Tris-HCl, pH 8.0 with light from a 150 W tungsten lamp for 60 min at ambient temperature. Samples were rapidly frozen in cold isopentane. EPR spectra were recorded on a Bruker ER-200D spectrometer with peripheral equipment and data handling as has been described previously [12]. W^{5+} EPR signals were simulated as previously described [9].

2.3. In vitro reconstitution of electron transfer chain

Voltammograms of *P. furiosus* ferredoxin were recorded as previously described [13]. The 20 μ l droplet contained 0.15 mM Fd in 25 mM MOPS pH 7.25 plus 6.7 mM neomycin as promoter. GAPOR was added to a final concentration of 3 μ M and GAP was added to a final concentration of 8 mM. Catalytic waves were recorded with a scan rate of 10 mV/s. The pseudo-first order rate constant was calculated from the ratio of catalytic and diffusion-controlled current using a numerical method adapted from Nicholson and Shain [14].

3. Results

3.1. EPR spectroscopy

Based on the amino acid sequence and the structures of

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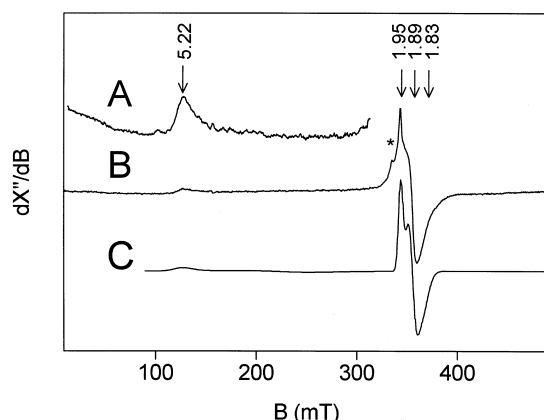


Fig. 1. $[4\text{Fe-4S}]^{1+}$ EPR signals of *P. furiosus* GAPOR poised at -664 mV in the GAP/3PG titration. Trace A, $S=3/2$ signal. EPR conditions: microwave frequency, 9.41 GHz; modulation frequency, 100 kHz; modulation amplitude, 1.0 mT; microwave power, 200 mW; temperature, 7.5 K. Trace B, $[4\text{Fe-4S}]^{1+}$ $S=3/2$ and $S=1/2$ signal. EPR conditions: same as for trace A except: gain, 10 times lower and temperature, 11.8 K. Trace C, simulation of $S=1/2$ and $S=3/2$ signals. Simulation parameters $S=1/2$: $g_{x,y,z}=1.830, 1.885, 1.952$; line width $_{x,y,z}=0.032, 0.019, 0.013$ (in g value units). Simulation parameters $S=3/2$: $g_{x,y,z}=1.398, 1.920, 5.218$; line width $_{x,y,z}=0.450, 0.450, 0.450$. The minor feature indicated with an asterisk is not part of $[4\text{Fe-4S}]^{1+}$ EPR signals because it has different power saturation behavior and the intensity is independent of potential.

AOR and FOR, one $[4\text{Fe-4S}]$ cluster and one tungsten center are expected for GAPOR. The $[4\text{Fe-4S}]^{2+}$ can undergo a one-electron reduction at low potentials to $[4\text{Fe-4S}]^{1+}$, which is paramagnetic. The $[4\text{Fe-4S}]^{1+}$ is expected to show $S=3/2$ or $S=1/2$, or both EPR signals. The tungsten center is expected to undergo two subsequent one-electron reductions from W^{6+} to W^{4+} . The intermediate W^{5+} is paramagnetic and shows a typical $S=1/2$ signal with all g values usually below 2.0, which is still observable at temperatures as high as 100 K. The redox titrations of GAPOR produced $S=3/2$ and $S=1/2$ signals that can be attributed to the $[4\text{Fe-4S}]^{1+}$ cluster (Figs. 1 and 2). The

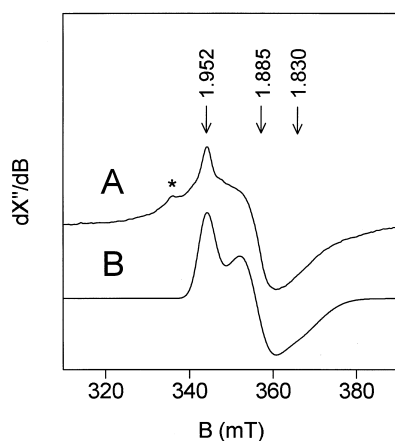


Fig. 2. $[4\text{Fe-4S}]^{1+}$ $S=1/2$ EPR signal of GAPOR poised at -664 mV in the GAP/3PG titration. Trace A, experimental spectrum. EPR conditions: microwave frequency, 9.41 GHz; modulation frequency, 100 kHz; modulation amplitude, 1.0 mT; microwave power, 200 mW; temperature, 11.8 K. Trace B, simulation of A. Simulation parameters given in the legend to Fig. 1. The signal (*) is not part of $[4\text{Fe-4S}]^{1+}$ EPR signal (cf. legend to Fig. 1).

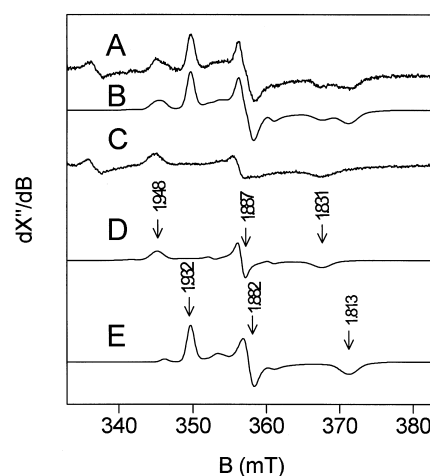


Fig. 3. EPR spectra of *P. furiosus* GAPOR W^{5+} . Trace A, experimental spectrum with potential poised at -510 mV in the GAP/3PG titration. Trace B, sum of simulations D and E. Trace C, experimental spectrum with potential poised at -674 mV. Trace D, simulation of C. Trace E, simulation of A–D. EPR conditions: microwave frequency, 9.42 GHz; modulation frequency, 100 kHz; modulation amplitude, 0.5 mT; microwave power, 3.2 mW; temperature, 34 K.

same signals were observed in deazaflavin/light-reduced GAPOR (not shown). The $S=1/2$ signal has $g_{z,y,x}=1.95, 1.89, 1.83$. Integration of the $S=1/2$ signal yielded 0.43 spins/molecule. The signal could not be exactly simulated assuming only g -strain broadening (Fig. 2). The experimental spectrum has broad wings at high and low field that are not reproduced in the simulation. Apparently the $[4\text{Fe-4S}]^{1+}$ interacts with another paramagnet close by. Of the $S=3/2$ signal only a low field feature at $g=5.22$ is observed. No additional signals, with higher g values, were observed at temperatures from 4.5 to 9.0 K, where the $S=3/2$ signal is best measured, that could account for the g_z of the higher doublet. Apparently the effective g_z values are almost equal for the two doublets. Under the standard spin Hamiltonian $H=D[S_z^2-S(S+1)]+E(S_x^2-S_y^2)+\beta B \cdot g \cdot S$ this can be explained either with a $D \gg h\nu$ ($=0.31 \text{ cm}^{-1}$), $g \approx 1.92$ and $E/D \approx 1/3$ or with a $D/h\nu \approx 1.30$ (i.e. $D \approx 0.41 \text{ cm}^{-1}$), $g \approx 2.00$ and $E/D \approx 1/3$. The $S=3/2$ signal can approximately be simulated assuming $E/D=1/3$ and $g=1.92$. The ratio ($S=1/2$):($S=3/2$) is estimated to be 1:2 using the simulations for both spectra. If the $S=3/2$ signal represents the sum of both doublets the total quantitation of the $[4\text{Fe-4S}]^{1+}$ signals is 0.86 spins/mol. If only the ground state doublet of the $S=3/2$ species is observed the total quantitation would be 1.3 spins/mol.

A W^{5+} EPR signal is detected at potentials lower than -300 mV (Fig. 3) which resembles the W^{5+} signals that have been reported for GAPOR previously [7,8]. Integration of this W^{5+} signal yields 0.12 spins/molecule. However, another W^{5+} signal is also found with different g values and a quantity up to 0.30 spins/molecule. This signal has not been reported previously. The latter signal will be designated W_1^{5+} , and the other, with the lower quantity, W_2^{5+} . The designations used in the literature for FOR and AOR (i.e. low-, mid- and high-potential W^{5+}) are not functional for GAPOR, since both W^{5+} signals found are low potentials. The simulation parameters for both tungsten EPR signals are given in Table 1. The saturation behavior at 19 K of the tungsten signals and

Table 1
EPR parameters of *P. furiosus* GAPOR W^{5+} species

Species	g_z	g_y	g_x	A_z (mT)	A_y (mT)	A_x (mT)	W_z (mT)	W_y (mT)	W_x (mT)
W_1	1.923	1.882	1.813	7.0	6.0	6.0	0.09	0.10	0.20
W_2	1.948	1.887	1.831	7.0	8.0	6.0	0.20	0.07	0.15

the $S = 1/2$ signal of the $[4Fe-4S]$ cluster can be seen in a power plot (Fig. 4).

3.2. Dye-mediated redox titration

The results of the dye-mediated titration with sodium dithionite are presented in Fig. 5. The midpoint potentials of the $[4Fe-4S]^{2+/1+}$ and $W_2^{6+/5+}$ couples have been determined and are given in Table 2. The standard deviations for the fits were between 5 and 15 mV for all the potentials given in Table 2. The actual uncertainty may be larger depending on the number of samples in a titration curve. No reduction to W_2^{5+} was found. W_1^{5+} was not found in the dye-mediated redox titration. The $S = 3/2$ and $S = 1/2$ signals attributed to $[4Fe-4S]^{1+}$ have the same midpoint potential.

3.3. Substrate/product redox titration

In the GAP/3PG titration both W^{5+} signals were found. No redox changes for the W_2 species was found (Fig. 5). The E_m values of the W_1^{5+} species can be seen in Table 2 and are consistent with two subsequent one-electron reductions from W^{6+} to W^{4+} . The maximum quantity of W_1^{5+} is 0.30 spins/molecule while the quantity of the W_2^{5+} species is unchanged during the titration and amounts to only 0.09 spins/mol (cf. Fig. 5). W_2 is likely due to inactivated enzyme. The $S = 1/2$ and $S = 3/2$ signals of the cubane cluster are also observed. The GAP/3PG titration gives a much lower apparent E_m value for the $[4Fe-4S]^{2+/1+}$ couple because electrons must flow via the tungsten in this titration (Fig. 5). Therefore, the tungsten center has to be reduced before the cubane cluster can be reduced. This is of course not the case with the dye-mediated titration. The results of the dye-mediated and GAP/3PG titrations are complementary.

3.4. In vitro reconstitution of electron transfer chain

The cyclic voltammogram of Fd only shows a reversible

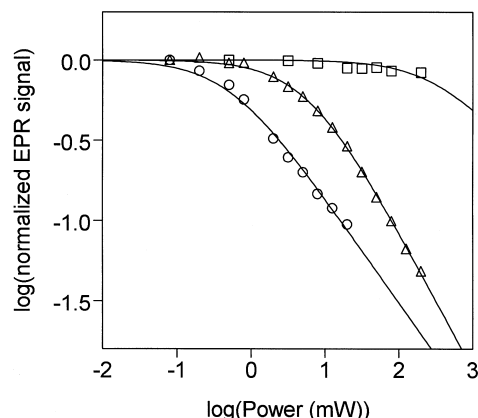


Fig. 4. Saturation plot of *P. furiosus* GAPOR W^{5+} and $[4Fe-4S]^{+} S = 1/2$ signals at 19 K. Trace A, $[4Fe-4S]^{+} S = 1/2$ signal (\square). Trace B, W_1^{5+} signal (Δ). Trace C, W_2^{5+} signal (\circ). Normalized EPR signal \equiv (signal/ $\sqrt{\text{power}}$)/(low-power signal/ $\sqrt{\text{low-power}}$).

electron transfer between the Fd and the electrode (Fig. 6). The peak separation is 60 mV which is close to the expected 63 mV for a fully reversible system at 55°C [14]. Diffusion of Fd from the bulk solution to the electrode and not the electron transfer from Fd to the electrode is rate-limiting. Addition of GAP or GAPOR separately to Fd did not significantly change the voltammogram of Fd. However, if GAP was added to GAPOR a catalytic wave appeared as a large increase of the anodic peak as can be seen in Fig. 6. The catalytic current increased with temperature, but was already detectable at room temperature. Using the ratio of the catalytic and the diffusion-controlled currents the pseudo-first order rate constant of the rate-determining step can be calculated. The rate-determining step can be either the GAP oxidation or the Fd reduction by GAPOR because, as was concluded from

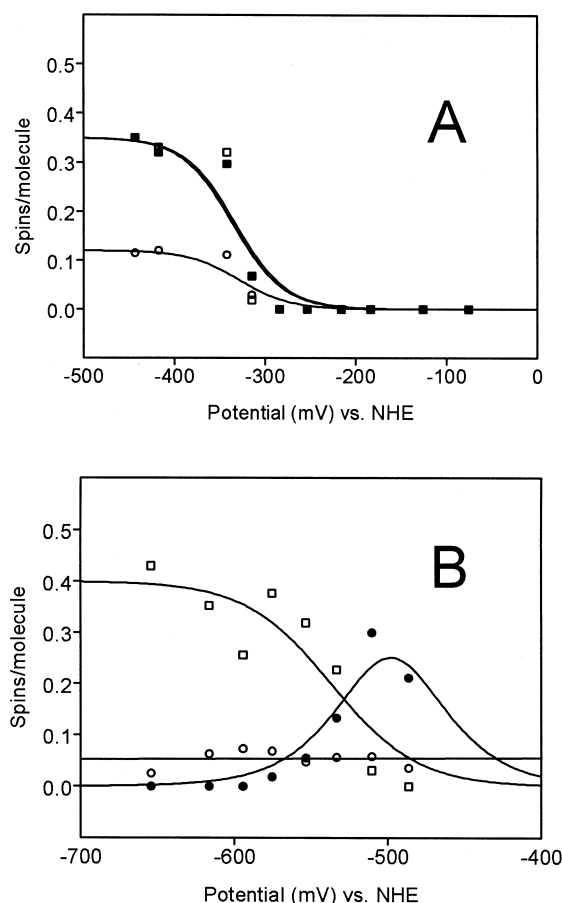


Fig. 5. Redox titrations of *P. furiosus* GAPOR. Dye-mediated redox titration (A) and GAP/3PG redox titration (B). The $[4Fe-4S]^{1+} S = 3/2$ signal (\blacksquare) is monitored at $g = 5.22$ with the EPR conditions as in the legend to Fig. 2. The $[4Fe-4S]^{1+} S = 1/2$ signal (\square) is monitored as the amplitude at $g_y = 1.890$ with the same EPR conditions. The W_1^{5+} signal (\bullet) is monitored at $g_z = 1.923$ and the W_2^{5+} signal (\circ) is monitored at $g_z = 1.948$ at 40 K with EPR conditions as in the legend to Fig. 1. The solid lines are fits for $n = 1$ redox transitions with the midpoint potentials given in Table 2.

the Fd voltammogram, the Fd oxidation at the electrode is not rate-limiting. At 55°C and pH 7.25 a pseudo-first order rate constant of 36 s^{-1} was found, which compares reasonably well with the value of 94.5 s^{-1} based on the V_{max} reported for GAPOR with Fd as electron acceptor at 70°C and pH 8.4 (90 U/mg) [4]. The rate constant was not determined at 70°C because GAP was unstable at that temperature.

4. Discussion

4.1. W_1^{5+} is an intermediate state of the tungsten during the catalytic cycle

The W_1 undergoes a two-step one-electron transfer, similar to the W in *P. furiosus* AOR and many molybdenum enzymes [3]. Both E_m values are low and close to the E_m value calculated for the GAP/3PG couple [11]. The quantity of the W_1^{5+} is maximally 0.30 spins/mol. This can be explained assuming that the midpoint potential for the $W_1^{6+/5+}$ couple is 15 mV more negative than for the $W_1^{5+/4+}$ couple and that 90% of the tungsten is W_1 (Fig. 5 and Table 2). W_2 is a minority species of only 10% of the tungsten and undergoes only a one-electron reduction from W_2^{6+} to W_2^{5+} in the potential range tested. Although crossing of midpoint potentials of the $M^{6+/5+}$ and $M^{5+/4+}$ couples has been found for several molybdenum-containing oxotransferases [15–17], this is the first example for a tungsten enzyme. GAPOR has only two different W^{5+} species, not as many as have been found for AOR [3] and FOR [8,18]. This confirms that the signals, other than the ‘low-potential’ signals, that do not undergo a two-step one-electron transfer do not represent catalytically relevant species. Even though the titrations were performed at 50°C, the E_m values may refer to a protein conformation at a much lower temperature. *P. furiosus* ferredoxin and rubredoxin have been shown to take their conformation corresponding to the freezing point of the solution upon rapid freezing [13]. However, temperature-dependent redox titrations of *P. furiosus* AOR and FOR showed significant shifts in the midpoint potentials of the tungsten centers and cubane clusters, indicating that for complex enzymes the high temperature conformation may at least partially be maintained in the frozen samples (P.L. Hagedoorn, I. Landa and W.R. Hagen, unpublished results). Investigations on the temperature-dependent redox chemistry of the tungsten enzymes of *P. furiosus* are in progress.

4.2. The $[4\text{Fe-4S}]^+$ exhibits an $S=3/2$ and an unusual $S=1/2$ EPR signal

As is common for many cubane clusters, the $[4\text{Fe-4S}]^+$ cluster of GAPOR exhibits both a $S=3/2$ and a $S=1/2$ EPR signal. The cubane in AOR, however, has been found to be $S=3/2$ only [3]. The *Thermococcus litoralis* FOR cubane

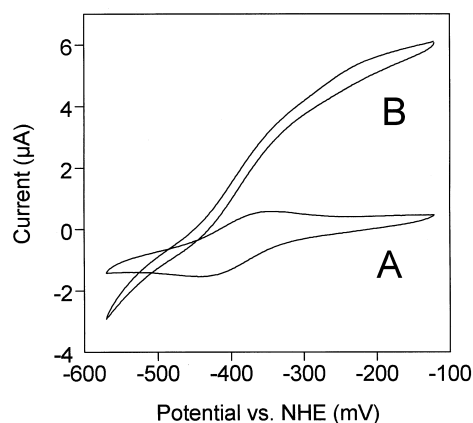


Fig. 6. Cyclic voltammograms of *P. furiosus* ferredoxin without (A) and with GAPOR and GAP (B) at 55°C. Trace A, *P. furiosus* ferredoxin 0.15 mM in 25 mM MOPS pH 7.2 with 6.7 mM neomycin, scan rate 10 mV/s. Trace B, same as in A with 3 µM GAPOR and 8 mM GAP.

cluster exhibits both $S=3/2$ and $S=1/2$ signals [19]. The $S=3/2$ signal of GAPOR $[4\text{Fe-4S}]^{1+}$ is rhombic with unknown D . A large D of $+4\text{ cm}^{-1}$ has been found for the $[4\text{Fe-4S}]^{1+}$ $S=3/2$ signal of *P. furiosus* AOR [3] and a small D of -0.7 cm^{-1} has been found for the A33Y mutant of *P. furiosus* Fd [20]. The signal also looks similar to the $S=3/2$ signal found for *T. litoralis* FOR, of which only a low field feature at $g=5.39$ was observed [19]. However, no detailed analysis of that signal is currently available.

The $S=1/2$ signal of GAPOR $[4\text{Fe-4S}]^{1+}$ is different from those of most other cubane clusters. All g values are below 2.0, which is unusual. Similar EPR spectra have only been reported for putative $[4\text{Fe-4S}]$ clusters in NADH-reduced glutamate synthase [21] and NADPH-reduced sulfide dehydrogenase (ferredoxin NADPH oxidoreductase) [22]. As can be seen in the saturation plot (Fig. 4), the $S=1/2$ species is relatively fast relaxing, which is normal for these types of clusters [23]. Therefore it is unlikely that it is a W^{5+} signal. The broad $S=1/2$ signal appears to reflect dipolar interaction with another center. The only paramagnet close enough to interact with is W in the same molecule (GAPOR is monomeric). However, the $[4\text{Fe-4S}]^{1+}$ $S=1/2$ signal is already found at potentials where all the W_1 is expected to be W^{6+} , which is diamagnetic (Fig. 5), although the effect that dithionite may have on W_1 is not known.

The previous conclusion may not be valid if the midpoint potentials of W_1 strongly shift towards more negative values as a response to the presence of substrate and/or product. In the dye-mediated titration reduction of the $[4\text{Fe-4S}]$ cluster may then coincide with the reduction of W_1^{6+} causing the anomalous $S=1/2$ signal. We consider this an unlikely possibility because in addition to the presumed interaction signal the signals from non-interaction centers should also be observable unless a strong positive cooperativity occurred.

Interaction of the cubane with W_2 is unlikely because it is a minority species of only about 10% of the total enzyme. The immediate surrounding of the cubane cluster may contribute to the unusual EPR spectrum. Sequence alignment of the AOR family members has shown that between C334 and C338 there is a proline residue which is not present in AOR and FOR [7].

Table 2
Reduction potentials of *P. furiosus* GAPOR W center and $[4\text{Fe-4S}]$ cluster

Couple	Potential vs. NHE (mV)
$W_1^{6+/5+}$	−506
$W_1^{5+/4+}$	−491
$W_2^{6+/5+}$	−329
$[4\text{Fe-4S}]^{2+/1+}$ $S=1/2$	−336
$[4\text{Fe-4S}]^{2+/1+}$ $S=3/2$	−333

Reductive dye-mediated and GAP/3PG titrations were performed at 50°C in 30 mM Tris-HCl, pH 8.0.

4.3. The electron transfer chain from GAP to Fd can be reconstituted *in vitro*

The electrochemical data clearly show that GAPOR can reduce Fd using GAP. The GAP/3PG titrations demonstrated that the tungsten has to be reduced before the cubane cluster can be reduced, even though dithionite reduces the cubane cluster first. Taken together with the values for the midpoint potentials obtained from the bulk titrations, an electron transfer pathway from GAP first to the W then to the [4Fe-4S] cluster and finally to the [4Fe-4S] cluster of Fd can be proposed. This pathway has already been proposed for *P. furiosus* FOR based on the position of the redox centers in the enzyme [2]. No evidence, e.g. pterin radical signals [24], has been found that the pterin cofactor participated in the redox chemistry of GAPOR.

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