Hyperthermophilic redox chemistry: a re-evaluation

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Abstract The redox chemistry of *Pyrococcus furiosus* rubredoxin and ferredoxin has been studied as a function of temperature in direct voltammetry and in EPR monitored bulk titrations. The E_ms of both proteins, measured with direct voltammetry, have a normal (linear) temperature dependence and show no pH dependence. EPR monitoring is not a reliable method to determine the temperature dependence of the E_m: upon rapid freezing the proteins take their conformation corresponding to the freezing point of the solution.

Key words: Rubredoxin; Ferredoxin; Reduction potential; Hyperthermophile; *Pyrococcus furiosus*

1. Introduction

Hyperthermophiles grow optimally at temperatures above 80°C. Their biochemistry is expected to have characteristics that may differ fundamentally from that of mesophiles. Several claims have been made on unusual redox chemistry in *Pyrococcus furiosus*. The temperature dependent E_m of ferredoxin has been reported to be biphasic and extrapolates to approximately −600 mV at 100°C, as measured by EPR monitored redox titrations [1]. However, a recent report of direct electrochemistry on heterologously expressed wild type ferredoxin shows a normal linear temperature dependence of the E_m [2]. The E_m of rubredoxin has been reported to be pH dependent [3], although no significant pH dependence is expected based on the structure of the cluster and its environment. Furthermore, the E_m of rubredoxin has a non-linear temperature dependence [3]. The observations described above have several implications which have been discussed in the literature [4]. The non-linear and biphasic temperature dependences of the E_m of rubredoxin and ferredoxin respectively, measured by redox titrations, have been interpreted in terms of a protein dielectric constant that changes non-linearly with temperature [5]. Furthermore, molecular dynamics simulations have been used to determine temperature dependent changes in the physical properties of the protein [6].

The differences in the observations of direct electrochemistry and EPR monitored redox titrations on ferredoxin raise the question with which method and under which conditions redox potentials can best be obtained. Furthermore, the strange redox behavior reported for rubredoxin has only been measured in redox titrations. We have studied the temperature and pH dependence of the E_m of ferredoxin and rubredoxin with cyclic voltammetry, and we have compared these with the results obtained with EPR monitored redox titrations.

2. Materials and methods

2.1. Cultivation and protein purification

P. furiosus (DSM 3638) was cultivated as previously described [7]. Cells were broken by osmotic shock, diluting with 5 volumes 50 mM Tris pH 8.0 (anaerobic) containing 2 mM sodium dithionite, 5 mM $MgCl_2$, 0.1 mg/l DNase I, 0.1 mg/l RNase. A cell-free extract was obtained as the supernatant after 1 h centrifugation at 3200×*g*.

Rubredoxin was purified as described previously [8].

Ferredoxin was not purified as described previously [9], but with a new method. Ammonium sulfate was added to cell-free extract to 60% saturation. After centrifugation (15 min, 3200×*g*, 4°C) the supernatant was collected and diluted to 40% ammonium sulfate saturation. The supernatant was passed through a phenyl-Sepharose column (Pharmacia), equilibrated with 40% ammonium sulfate in 50 mM Tris pH 8.0 (anaerobic), yielding pure ferredoxin. As isolated ferredoxin was reconstituted by incubation with 10-fold excess of $FeSO_4$ and 2 mM sodium dithionite under anaerobic conditions. Excess iron was removed on a Bio-Gel P-6DG desalting column (Bio-Rad).

Horse heart cytochrome *c* was from Boehringer Mannheim.

2.2. Electrochemistry and EPR monitored titrations

Cyclic voltammograms were recorded with a BAS CV27 potentiostat (Bioanalytical systems) connected to a Kipp and Zonen x-y-t recorder. The electrochemical experiments were performed with a three electrode microcell using the method described preciously [10]. The working electrode was a nitric acid activated glassy carbon disc (Le Carbon Loraine). As the counter electrode a micro platinum electrode was used. And the potential was measured with reference to an Ag/AgCl reference electrode (Radiometer). All reported potentials have been recalculated with respect to the normal hydrogen electrode (NHE). During the experiments the electrochemical cell was immersed in a thermostatted waterbath. A typical experiment was performed on a 20 μl droplet of 0.15 mM rubredoxin with 0.10 mM $SmCl_3$ (promoter) or 0.14 mM ferredoxin with 6.7 mM neomycin (promoter) in 35 mM buffer. The buffers used were either MES (pH 5.6), Bis-Tris (pH 6.5), MOPS (pH 7.2), TAPS (pH 8.4), CHES (pH 9.2), or CAPS (pH 10.4). To prevent evaporation 15 μl nujol oil (Perkin Elmer) was added on top of the droplet. At high pH values (above pH 8) 24 mM $MgSO_4$ was used instead of $SmCl_3$.

Both rubredoxin and ferredoxin were redox titrated at 20°C and 80°C in presence of a mixture of 13 dye mediators as described previously [11]. A typical titration was done on 18.5 μM rubredoxin in 25 mM Bis-Tris pH 6.5 or 0.88 mM ferredoxin in 25 mM CHES pH 9.3 with equimolar amounts of mediators. Nujol mineral oil (Perkin Elmer) was added to prevent evaporation at 80°C. The potential was measured at a platinum wire versus an Ag/AgCl reference electrode (Radiometer). Substoichiometric amounts of sodium dithionite were added for a stepwise reduction of the protein. Samples were injected in pre-heated Ar, flushed EPR tubes and subsequently rapidly frozen in liquid N_2/isopentane. The freezing time was 0.5 s [12].

3. Results

3.1. New purification method for ferredoxin

The new purification yields 260 mg of pure ferredoxin from 400 g of cells (wet weight). The protein proved to be pure

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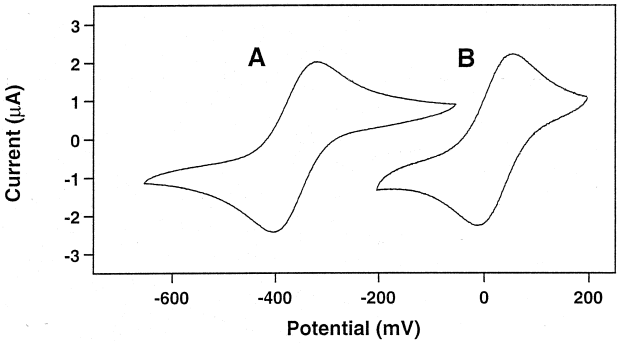

Fig. 1. Cyclic voltammograms of *P. furiosus* ferredoxin (A) and rubredoxin (B) at ambient temperature. Trace A, voltammogram of 0.14 mM ferredoxin in 25 mM MOPS pH 7.2 with 6.7 mM neomycin. Experimental conditions: scan rate 10 mV/s, temperature 24.9°C. Trace B, voltammogram of 0.32 mM rubredoxin in 25 mM Bis-Tris pH 6.5 with 0.1 mM $SmCl_3$. Experimental conditions: scan rate 10 mV/s, temperature 24.5°C.

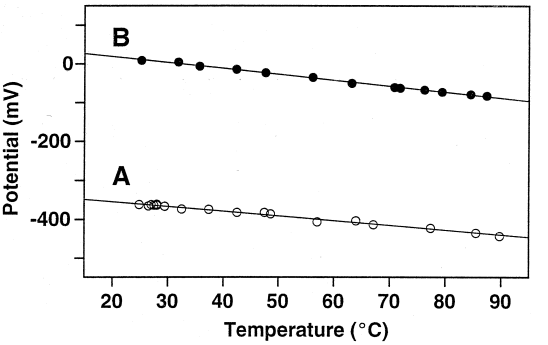

Fig. 2. Temperature dependence of the midpoint potential as determined by cyclic voltammetry of *P. furiosus* ferredoxin (A) and rubredoxin (B). Trace A, 0.14 mM ferredoxin (○) in 25 mM MOPS pH 7.2 with 6.7 mM neomycin, scan rate 10 mV/s. Trace B, 0.15 mM rubredoxin (●) in 25 mM Bis-Tris pH 6.5 with 0.1 mM $SmCl_3$, scan rate 10 mV/s.

according to analytical gel filtration, electronic absorption spectroscopy and EPR. The yield is similar to the five step purification method described previously [9]. However, the two step purification significantly reduces time and effort needed to obtain the pure protein.

3.2. Cyclic voltammetry of rubredoxin

Reproducible voltammograms of rubredoxin were obtained in the temperature range from 20 to 90°C. At a scan rate of 10 mV/s the peak separation was 51 mV where 57 mV is expected for a diffusion controlled response. The slightly lower value may indicate that a minor fraction of the rubredoxin adsorbs onto the electrode. However, anodic and cathodic peak currents were similar, and the peak current increased linearly with the square root of the scan rate. Both observations indicate a (quasi) reversible system. A voltammogram of rubredoxin at ambient temperature is given in Fig. 1. The temperature dependence of the $E_{m,6.5}$ of rubredoxin is linear, contrary to previous observations [5], as can be seen in Fig. 2. A temperature dependence of −1.53 mV/°C is found for the $E_{m,6.5}$. Thermodynamic parameters calculated from this temperature dependence are given in Table 1. Furthermore, the E_m is not dependent of the pH in the range 5.6–10.4 (Fig. 3). This contradicts the observation of a non-linear temperature and pH dependence of the E_m reported previously [3].

3.3. Cyclic voltammetry of ferredoxin

Well defined, reversible and reproducible voltammograms of the ferredoxin were recorded in the temperature range from 20 to 90°C. A voltammogram of ferredoxin at ambient temperature is given in Fig. 1. At a scan rate of 2 mV the peak separation was 59 mV as expected for a fully reversible electron exchange at 25°C. However, a scan rate of 10 mV/s was used to make measurements at higher temperatures feasible. The peak separations of the voltammograms used to determine the $E_m(T)$ were between 60 and 80 mV. The temperature dependence of the E_m of ferredoxin is linear, as can be seen in Fig. 2, and not biphasic as reported previously [1]. A temperature dependence of −1.23 mV/°C is found for the $E_{m,7.2}$. Thermodynamic parameters calculated from this temperature dependence are given in Table 1. The E_m of the ferredoxin is independent of the pH in the range 5.6–9.2.

3.4. EPR monitored redox titrations

As a standard laboratory routine in redox titrations we use a set of 13 mediators [11]. Two of these, phenazine ethosulfate and neutral red, are known to be unstable at higher temperatures. The 11 remaining dyes have been studied in cyclic voltammetry to determine reduction potential as a function of temperature. All experiments were done in 50 mM EPPS, pH 8.4 at 22°C, in view of the minimal temperature dependence of this buffer (−0.011 pH units per degree [13]). For indigo carmine no cathodic wave was identifiable; the asymmetric shape of the voltammogram was virtually independent of temperature (not shown). The 10 remaining dyes afforded reasonably well defined cyclic voltammograms in the temperature range 25–80°C. The cathodic-to-anodic peak separation was usually greater than the theoretical values of 29 or 59 mV for two or one electron transfer, respectively, indicating quasi reversibility, i.e. relatively slow heterogeneous electron transfer. The apparent reduction potential was determined as the average of the potentials of cathodic and anodic peak currents.

The results are presented in Fig. 4. All dyes have apparent E_ms that are a linear function of temperature. Thus, unlike findings with, e.g. the redox protein cytochrome *c* [14], the dyes do not exhibit significant structural changes in the tested temperature range. Although the curves differ in slope, the magnitude of these slopes is relatively small. As a result the subsequent E_ms at 80°C are still reasonably spaced, therefore, this set of dyes is a good redox buffering system over the whole temperature range. The only restriction is on the oxi-

Table 1
Thermodynamic parameters of *P. furiosus* ferredoxin and rubredoxin as determined by cyclic voltammetry

Protein	$\Delta G^{\circ\prime}$ (kJ/mol)	$\Delta S^{\circ\prime}$ (J/mol/K)	$\Delta H^{\circ\prime}$ (kJ/mol)
Rubredoxin	−0.8	−213	−62.6
Ferredoxin	34.9	−184	−19.9

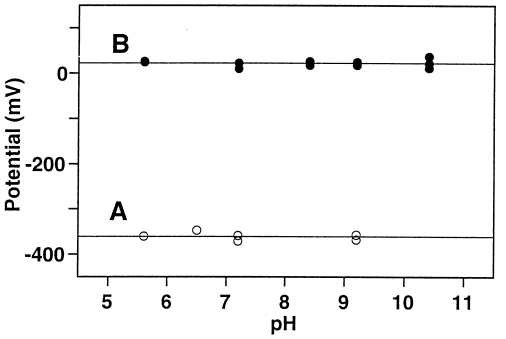

Fig. 3. pH dependence of the midpoint potential as determined by cyclic voltammetry of *P. furiosus* ferredoxin (A) and rubredoxin (B). Trace A, 0.14 mM ferredoxin (○) in 25 mM buffer with 6.7 mM neomycin, scan rate 10 mV/s. Trace B, 0.15 mM rubredoxin (●) in 25 mM buffer with 0.1 mM $SmCl_3$, scan rate 10 mV/s. The buffers used were MES (pH 5.6), Bis-Tris (pH 6.5), MOPS (pH 7.2), TAPS (pH 8.4), CHES (pH 9.2), and CAPS (pH 10.4).

dative side of the potential scale where *N*,*N*,*N*′,*N*′-tetramethyl *p*-phenylene diamine exhibits a voltammogram at 80°C that rapidly decreases in amplitude during continuous cycling.

The results of the EPR monitored redox titrations of *P. furiosus* rubredoxin and ferredoxin at ambient and high temperatures are given in Table 2. The EPR tubes were either directly frozen in liquid nitrogen (dead time, $\tau \approx 5$ s) or in cold isopentane ($\tau = 0.5$ s). The midpoint potentials obtained are virtually independent of the freezing time or the temperature at which the titrations were performed.

4. Discussion

4.1. The redox chemistry of P. furiosus rubredoxin is regular

Proteins of hyperthermophilic origin clearly differ from mesophilic and psychrophilic counterparts in their intrinsic thermostability. The molecular nature of this added stability is beginning to emerge (cf. [15]), however, this matter was not addressed in our present research. We sought to answer the question whether hot redox biochemistry has molecular characteristics sufficiently unusual for a meaningful distinction, in terms of structure-function relationships, from regular redox biochemistry. Several claims in the literature regarding hyperthermophilic redox proteins with quite unexpected properties [1,3,16], followed up by several claims of explanation by theoretical modelling [5,6], have incited us to initiate a systematic research effort into this matter.

The mononuclear, high-spin Fe(III/II) site in rubredoxins is tetrahedrally coordinated by four cysteinate ligands. The reduction potential of this center is expected to be essentially independent of pH, because protonation of the thiolate(s) would lead to demetallation, and Cys has no protonatable side groups. Consistent with this prediction we have previously found, in a direct electrochemical study, the redox potential of *Megasphaera elsdenii* rubredoxin to be virtually independent of pH in the pH range 5.5–9.5 [17]. In contrast to this observation Adams reported the E_m of *P. furiosus* rubredoxin to be significantly dependent on the pH, with $\Delta E \approx -25$ mV/pH at 20°C, on the basis of EPR monitored redox titrations. In addition, the E_m was reported [3] to be non-linearly (approximately quadratically, cf. [5]) dependent on the temperature, while, to our knowledge, every $E_m(T)$ plot reported thus far for any redox protein is linear in the temperature. Direct voltammetry, however only at room temperature, on a synthetic rubredoxin identical to the *P. furiosus* rubredoxin has been reported giving the same E_m value as EPR monitored redox titration on the native protein [18].

Table 2
Reduction potentials determined in EPR monitored bulk titrations of *P. furiosus* ferredoxin and rubredoxin

Protein	Potential (mV)	Conditions
Rubredoxin	+80	20°C to liquid N_2
	+76	80°C to liquid N_2
	+79	80°C to isopentane
Ferredoxin	−363	20°C to liquid N_2
	−359	90°C to isopentane

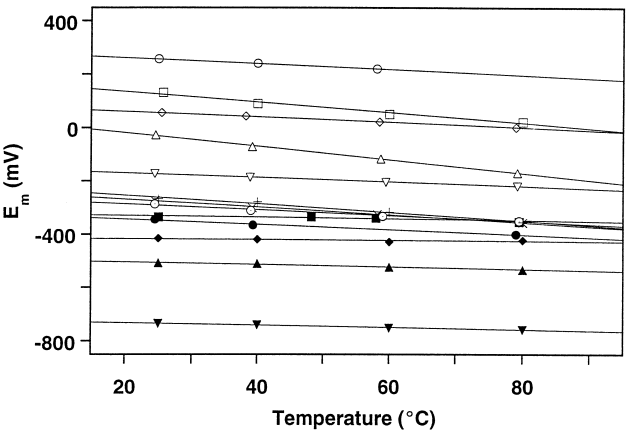

Fig. 4. Apparent midpoint potentials (of indigo carmine only the anodic peak potential) of redox mediators as a function of temperature. Mediators: *N*,*N*,*N*′,*N*′-tetramethyl *p*-phenylene diamine (○), 2,6-dichlorophenol indophenol (□), methylene blue (◇), indigo carmine (△), resorufin (▽), benzyl viologen 1 (+), anthraquinone 2-sulfonate (×), 2-hydroxy-1,4-naphthoquinone (⊙), 3,7-diamino-2,8-dimethyl-5-phenylphenazinium chloride (■), phenosafranin (●), methyl viologen 1 (◆), benzyl viologen 2 (▲), methyl viologen 2 (▼).

Smith and collaborators have proposed to explain this non-linearity on the basis of a protein dielectric constant that varies non-linearly with temperature [5]. Also, Swartz and Ichiye have carried out molecular dynamics simulations to evaluate temperature dependent differences in structure, solvation and energies of *P. furiosus* rubredoxin. These authors claim that a temperature dependent calculated average electrostatic potential at the Fe site, $\Delta\phi$, correlates very well with the experimentally determined temperature dependent E_m [6]. In the present work previously reported experimental $E_m(T)$ results have been found to be erroneous, therefore, the validity of the theoretical studies on rubredoxin [5,6], based on these results, has also been falsified.

4.2. The $E_m(T)$ of small proteins cannot be monitored with EPR

From optically monitored titration studies the E_m of horse heart cytochrome *c* is known to be linearly dependent on the temperature with a break point around 45°C [14]. We have confirmed this in direct cyclic voltammetric experiments (not shown). We have also found the redox potential of rubredoxin and ferredoxin from *P. furiosus* to be linearly dependent on the temperature over the whole range measured when deter-

mined in cyclic voltammetry. When the E_m of these two proteins is determined in bulk, mediated titrations at room temperature and at high temperature, with subsequent monitoring in low temperature EPR spectroscopy, we find apparent E_m values that are independent of the temperature and that correspond approximately to the voltammetrically determined E_ms when extrapolated to a temperature of 0°C or less. The result is independent of the time of freezing the EPR samples, as freezing the filled, thermally equilibrated EPR tubes in liquid nitrogen (dead time, $\tau \approx 5$ s) or in cold isopentane (τ=0.5 s) gives identical results.

To explain the above results we propose that the small electron transferring proteins, such as the ones studied by us, have sufficient flexibility, both in the oxidized and in the reduced state, to allow for rapid (i.e. within 0.5 s) structural adjustment upon temperature change such that the frozen state always approximately corresponds to the equilibrated state near the freezing point of the aqueous solution. A major implication of this proposal is that EPR monitored redox titrations of small proteins can only determine E_ms near 0°C.

Our experimental EPR titration results on rubredoxin and ferredoxin are different from those previously reported by Adams [3]; we are unable to reproduce those results.

4.3. *P. furiosus ferredoxin redox chemistry is regular*

The coordination of clusters in most iron-sulfur proteins is by Cys only, therefore, also here no significant dependence of E_m on the pH is expected. Indeed, only very minor dependences were found in a study of seven different [4Fe-4S] containing proteins [19]. However, the [4Fe-4S] cluster in *P. furiosus* ferredoxin has one aspartate as a presumably monodentate ligand [20] and a significant pH dependence of the E_m is possible. However, our voltammetric study shows the E_m to be virtually independent of pH. A similar conclusion was recently reached for the *P. furiosus* ferredoxin when expressed in *Escherichia coli* [2].

In contrast to the early EPR studies [1,3], our work also indicates the E_m to have a regular linear dependence on temperature without break points up to 90°C, and similar results have now been reported for the heterologously expressed wild type ferredoxin [2]. It should be noted, however, that in the latter study the voltammetrically derived E_m values were reported as plain numbers without presentation of primary data or discussion of uncertainties. This is remarkable because in two earlier studies from the same laboratory rather sluggish responses of ferredoxin were reported as evidenced by considerably broadened differential pulse voltammograms even for very low potential scan rates [4,21]. However, as reported in the present work, well defined and reversible voltammograms can be obtained even at high temperatures.

Smith et al. have also attempted to theoretically explain the break point in the initially reported $E_m(T)$ curve of ferredoxin [5]. Now that these early data have been found to be incorrect, both for the native protein and for the heterologously expressed wild type ferredoxin, the theoretical analysis has also become irrelevant.

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