A Variable-Temperature Direct Electrochemical Study of Metalloproteins from Hyperthermophilic Microorganisms Involved in Hydrogen Production from Pyruvate†

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ABSTRACT: The hyperthermophilic bacterium *Thermotoga maritima* and the hyperthermophilic archaeon *Pyrococcus furiosus* grow optimally at 80 and 100 °C, respectively, by the fermentation of carbohydrates to organic acids, CO_2, and H_2. Pyruvate is a major source of reductant for H_2 production during fermentation, and pyruvate ferredoxin oxidoreductase (POR), a 4Fe-type ferredoxin, and hydrogenase have been previously purified from both species. *P. furiosus* utilizes a copper−iron-containing POR and a nickel−iron-containing hydrogenase, whereas the POR of *T. maritima* lacks copper and its hydrogenase lacks nickel. For all four enzymes and for the two ferredoxins, we have determined their reduction potentials ($E^{\circ\prime}$) and, where possible, thermodynamic parameters associated with electron transfer (ΔS° and ΔH°), using differential pulse voltammetry at temperatures ranging from 25 to 95 °C. At ambient temperature, the $E^{\circ\prime}$ values for all six proteins were comparable and spanned less than 50 mV, but their temperature dependence varied dramatically, even between analogous proteins, such that in the physiological-relevant temperature range the $E^{\circ\prime}$ values became widely separated. In most cases, transition points were observed in $E^{\circ\prime}$/temperature profiles, and these generally corresponded with significant increases in catalytic activity, but occurred at lower temperatures in *T. maritima* than in *P. furiosus*. The two ferredoxins (and also *P. furiosus* rubredoxin) had much more negative entropy terms than were calculated for POR and hydrogenase, and these values were also more negative than those previously reported for mesophilic redox proteins. The reduction potentials measured at high temperatures and likely efficiencies of electron transfer between the various proteins were consistent with in vitro activity measurements. The results show that the electron transport pathways between POR and hydrogenase are very different in these two hyperthermophilic organisms. It is concluded that reduction potentials measured at ambient temperature appear to be of little value in rationalizing electron transfer processes in hyperthermophilic proteins and in fact may be quite misleading.

Hyperthermophiles are a recently discovered group of microorganisms that have the remarkable property of growing at temperatures of 90 °C and above (Stetter *et al.*, 1990; Adams & Kelly, 1992; Adams, 1993, 1994). They have been found in geothermally-heated environments which include shallow and deep sea hydrothermal vents. All but 2 of the 20 or so hyperthermophilic genera isolated have been classified within the domain of Archaea (formerly archaebacteria) (Woese *et al.*, 1990). The exceptions are *Thermotoga* (Huber *et al.*, 1986) and *Aquifex* (Huber *et al.*, 1992), which are classified as Bacteria (formerly eubacteria). The majority of the hyperthermophiles are strictly anaerobic heterotrophs, and most are obligately dependent upon the reduction of elemental sulfur (S^0) for growth. These unusual microorganisms have obviously adapted their biochemistry to function at temperatures close to and even above 100 °C, but the mechanisms by which this is achieved are largely unknown. A number of enzymes have been purified from hyperthermophiles in the last few years, and, as might be anticipated, most are extremely stable upon prolonged incubation at high temperatures, and virtually all have an optimum temperature for catalysis above 95 °C [for review, see Adams (1993)].

Elucidating the mechanisms by which proteins can be stabilized at extreme temperatures and how this is related to the catalytic activity of thermostable enzymes is an extremely challenging and as yet unresolved problem, and one that intimately depends upon a much better understanding of protein folding in general (e.g., Alonso & Dill, 1991; Kotik & Zuber, 1993). The stability of only one hyperthermophilic enzyme has been investigated in any detail, that of glyceraldehyde-3-phosphate dehydrogenase from *Thermotoga maritima* (Wrba *et al.*, 1990; Rehaber & Jaenicke, 1992, 1993). This homotetrameric enzyme exhibits a thermal transition temperature at 110 °C, and several temperature-dependent intermediate folding states have been identified in renaturation studies. However, studies with this (Schultes *et al.*, 1990) and other hyperthermophilic proteins (Zwickl *et al.*, 1990; Blake *et al.*, 1991; Sanangelantoni *et al.*, 1992; Ostendorp *et al.*, 1993) have shown that detailed comparisons of amino acid sequences from homologous hyperthermophilic

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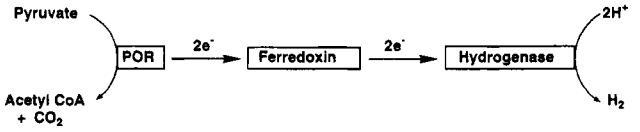
Scheme 1: Electron Transfer Pathway for the Production of Acetyl-CoA, CO_2, and H_2 from Pyruvate in Fermentative Mesophilic Organisms



and mesophilic proteins yield little if any insight into potential stabilizing mechanisms, and there are no apparent general rules for "hyperthermophily". So far, the three-dimensional structure of only one hyperthermophilic protein has been reported, that of the small redox protein rubredoxin (M_r 5500) from *P. furiosus* (Blake *et al.*, 1992a,b; Day *et al.*, 1992; Wampler et al., 1993; Bradley *et al.*, 1993). Comparisons with the crystal structures of mesophilic rubredoxins showed that hyperthermostability appeared to be conferred by rather minor structural changes and mainly in surface residues.

In contrast to protein stability and enzyme catalysis, electron transfer reactions at high temperatures have received virtually no attention. In mesophilic proteins, conformational differences between the oxidized and reduced states have been demonstrated, and these can directly affect the activation energy for electron transfer reactions (Marcus & Sutton, 1985). However, the electrochemical properties of proteins are typically examined only at or near ambient temperature, and the few mesophilic proteins for which thermodynamic terms associated with electron transfer ($\Delta H°$ and $\Delta S°$) have been calculated (Taniguchi *et al.*, 1980; Koller & Hawkridge, 1988; Link *et al.*, 1992) were obtained over limited ranges of temperature. Hence, the effects of temperatures near 100 °C on biological equilibrium electron transfer potentials ($E°'$) remain unexplored. Clearly, formal reduction potentials of redox centers must be obtained at the temperatures at which the proteins normally function. In addition, associated thermodynamic parameters could provide information on how hyperthermophilic redox enzymes are able to optimize catalytic activity at physiologically-relevant temperatures.

The hyperthermophilic proteins we chose to use in this study were from the bacterium, *Thermotoga maritima* (Huber *et al.*, 1986) and from the archaeon *Pyrococcus furiosus* (Fiala & Stetter, 1986), which grow at temperatures up to 105 and 90 °C, respectively. Both organisms obtain energy for growth by the fermentation of carbohydrates to organic acids, CO_2, and H_2, although they differ in the pathways that are utilized to convert glucose to pyruvate (Adams, 1994; Wrba *et al.*, 1990; Schafer *et al.*, 1994; Kengen *et al.*, 1994; Schroder *et al.*, 1994). In mesophilic organisms, pyruvate generated by fermentation is typically oxidatively decarboxylated by pyruvate ferredoxin oxidoreductase (POR) to yield acetyl-CoA, CO_2, and reduced ferredoxin (e.g., Thauer *et al.*, 1977). As shown in Scheme 1, reduced ferredoxin then couples to hydrogenase to evolve H_2. POR, ferredoxin, and hydrogenase have been purified from both *T. maritima* and *P. furiosus*. However, the POR of *T. maritima* is an iron–sulfur protein (M_r = 120 000: Blamey & Adams, 1994) whereas that of *P. furiosus* although of similar size also contains copper (Blamey & Adams, 1993; Smith *et al.*, 1994). In addition, while the ferredoxins of *T. maritima* (Blamey *et al.*, 1994) and *P. furiosus* (Busse *et al.*, 1992) are of similar size ($M_r \sim 7000$) and contain a single [4Fe-4S] cluster, their hydrogenases are very different. That of *T. maritima* is a homotetramer (M_r = 280 000) and contains only iron–sulfur clusters (Juszczak *et al.*, 1991) whereas *P. furiosus* hydrogenase is a heterotetramer (M_r = 160 000) and also contains nickel (Bryant & Adams, 1989). Hence, the PORs, ferredoxins, and hydrogenases from these two hyperthermophiles provide ideal model systems with which to investigate electron transfer pathways at high temperatures, particularly in view of the distinct molecular properties of their enzymes but not of their ferredoxins. Moreover, there is a significant difference in the optimum growth temperatures of these two hyperthermophiles (80 °C *versus* 100 °C), and they represent two distinct domains of life (archaeal *versus* bacterial).

We report here the results from a direct electrochemical study on the high-temperature redox properties by these three types of hyperthermophilic metalloprotein involved in the production of H_2 from pyruvate. The technique entails the potentiometric equilibration between a redox protein and an electrode in the absence of electron mediators, and is a very attractive approach as it is rapid and does not require large volumes or high sample concentrations. Until recently, the use of direct electrochemistry was restricted to a few small redox proteins (e.g., Armstrong *et al.*, 1987; Frew & Hill, 1988) as it was generally assumed that large highly charged enzymes with inaccessible redox centers would not interact well at an electrode surface. However, we show here for the first time that direct electrochemistry can be observed with hyperthermophilic enzymes.

METHODS

Protein Sources. Thermotoga maritima (DSM 3109) and *Pyrococcus furiosus* (DSM 3638) were each grown in a 600 L fermentor as previously described (Bryant & Adams, 1989; Juszczak *et al.*, 1991). *P. furiosus* rubredoxin (Blake *et al.*, 1991) and the ferredoxins (Aono *et al.*, 1989; Blamey *et al.*, 1994), PORs (Blamey & Adams, 1993, 1994), and hydrogenases (Bryant & Adams, 1989; Juszczak *et al.*, 1991) of *P. furiosus* and *T. maritima* were purified under anaerobic conditions as previously described. The 3Fe form of *P. furiosus* ferredoxin was prepared by ferricyanide treatment (Conover *et al.*, 1990). Solutions of the various proteins (typically 0.2 mM) were prepared anaerobically in 50 mM Tris/HCl, pH 8.0, containing 0.1 M NaCl, and these were used directly unless stated otherwise. For direct electrochemical experiments involving the 3Fe form of *P. furiosus* ferredoxin, 10 μL of neomycin (10 mM), a promoter of pyrolytic graphite electrodes (Butt *et al.*, 1991), was added anaerobically to a 25 μL aliquot of the protein solution to give a final neomycin concentration of approximately 1 mM. The conversion of the 3Fe form of *P. furiosus* ferredoxin to the 4Fe form (Conover *et al.*, 1990) was accomplished by the anaerobic addition of 2 μL of $FeCl_3$ (5 mM) to 25 μL of a solution of the 3Fe-ferredoxin (0.2 mM), and the sample was equilibrated at an ambient potential < -500 mV. Excess iron was not removed from the sample.

Direct Electrochemistry. Electrochemical experiments were performed utilizing both a temperature-controlled cell with a sample volume of 250 μL (Smith & Adams, 1992) and a room temperature micro-cell utilizing sample volumes of 25 μL (Smith *et al.*, 1991). The pyrolytic graphite electrode was polished in an alumina slurry as previously described (Smith & Adams, 1992). No additional pretreat-

ments were necessary. The addition of neomycin (Butt *et al.*, 1991) did not enhance the electrochemical data obtained with any of the proteins except for the 3Fe form of *P. furiosus* ferredoxin. Temperature-controlled electrochemical methods have been previously described (Smith & Adams, 1992). Electrochemical data were recorded with a computer-interfaced PARC 263A Polarographic Analyzer. Reduction potentials are reported versus the standard hydrogen electrode and were determined at the potential at which the peak current occurred. The peak width at half-height for a reversible electron transfer process is 98 mV/n where n = the number of electrons transferred per redox site. Reduction potentials corresponding to the net peak current were determined to be independent of scan direction at 2 mV/s scan rate, and this scan rate was used throughout. The pulse height was 50 mV for all experiments. Peak current and peak position *versus* scan rate profiles did not correlate to theoretical expectations for reversible, "quasireversible", or irreversible systems. We assume that nonfaradiac current, which is generated not by an electron transfer process (faradaic current) but by the movement of ions in response to a change in applied potential, contributed to the overall net current and this prevented a quantitative analysis of the data [see Smith *et al.* (1991)]. In all the cases we examined, the peak current position was not significantly influenced by scan rates up to 50 mV/s. We conclude, therefore, that practical reversibility was observed under our experimental conditions. Reduction potentials were measured under isothermal conditions, and the Ag/AgCl reference electrode was routinely calibrated against a saturated calomel electrode as previously described (Smith & Adams, 1992).

Thermodynamic Parameters. $\Delta S°$ was determined from the slope of the plot of reduction potential *versus* temperature (eq 1):

$$\Delta S° = nF(\mathrm{d}\Delta E°/\mathrm{d}T)_p \quad (1)$$

Since $\Delta H°$ cannot be assumed to be independent of temperature, it was determined using the Gibbs–Helmholtz equation (eq 2) from the slope of the plot of reduction potential/temperature *versus* 1/temperature:

$$\Delta H° = -nF[\mathrm{d}(\Delta E°/T)/\mathrm{d}(1/T)]_p \quad (2)$$

Reduction potentials obtained from data recorded between 25 and 90 °C were used to calculate the thermodynamic parameters.

Break points in temperature/reduction potential profiles were determined by direct inspection. All reduction potentials were determined from duplicate measurements, typically before and after raising the temperature. Due to the limited number of data points, all reduction potentials were included in the calculation of thermodynamic parameters associated with electron transfer, and, therefore, these values represent average thermodynamic parameters. No attempt was made to deconvolute current/potential data to obtain separate reduction potentials for the various redox centers within a single protein.

RESULTS

We recently described the construction and operation of a temperature-controlled, anaerobic cell for direct electrochemical studies and examined the redox properties of some

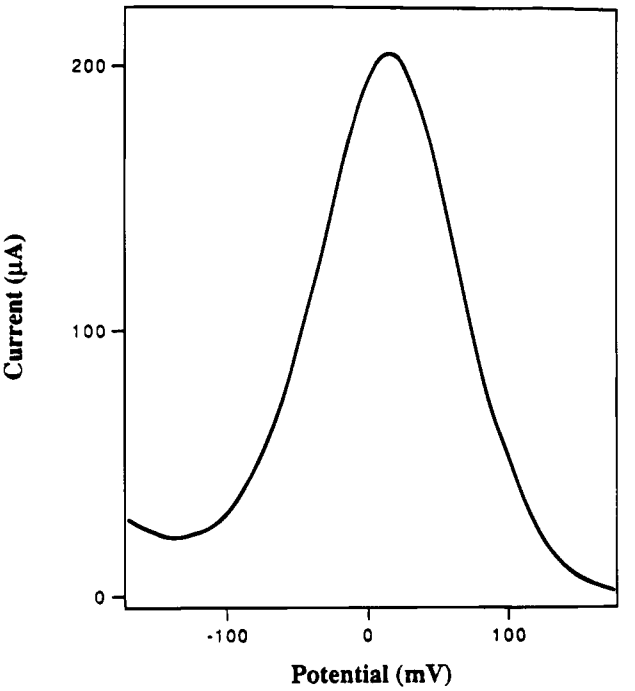


FIGURE 1: Differential pulse voltammogram of *P. furiosus* rubredoxin. The sample contained 100 μM protein in 0.1 M NaCl, 50 mM Tris/HCl, pH 8.0. The experimental conditions were 50 mV pulse height and 2 mV/s scan rate, and the temperature was 25 °C.

Table 1: Electrochemical Properties of Hyperthermophilic Iron–Sulfur Proteins

organism	protein	$E°'_{25\,°C}$ (mV)	$E°'_T$[a] (mV)	T[b] (°C)	$\Delta S°$ (eu)	$\Delta H°$ (kcal)
P. furiosus	rubredoxin[c]	0	−160	nd	−60	−16
T. maritima	ferredoxin	−388	−453	50	−28	1
P. furiosus	ferredoxin[c]	−365	−600	80	−50	−5
T. maritima	POR	−421	−370	40	27	18
P. furiosus	POR	−408	−470	60	−19	3
T. maritima	hydrogenase	−411	−438	56	−20	3
P. furiosus	hydrogenase[c]	−390	−390	nd	0	nd

[a] T = 80 and 100 °C for *T. maritima* and *P. furiosus*, respectively. [b] Transition temperature. [c] Determined by EPR spectroscopy (Adams, 1992). [d] nd = not determined.

organic redox dyes at temperatures between 30 and 90 °C (Smith & Adams, 1992). To investigate the utility of this system for proteins, we initially chose the rubredoxin from *P. furiosus*, a small redox protein (M_r = 5500) containing a single iron site whose redox properties have been characterized by bulk EPR spectroelectrochemical titrations (Adams, 1992). A differential pulse voltammogram of *P. furiosus* rubredoxin is shown in Figure 1. The reduction potential at room temperature ($E°'_{25\,°C}$) was determined to be 12 mV, which is comparable to the value of 0 mV obtained by bulk electrochemical/EPR techniques (Adams, 1992). Thermodynamic parameters associated with electron transfer of the rubredoxin are listed in Table 1.

Ferredoxin. The 4Fe-ferredoxins from *T. maritima* and *P. furiosus* gave rise to near-symmetric voltammograms at 25 °C similar to those obtained for the rubredoxin. For the *P. furiosus* protein, the value from direct electrochemistry (−370 mV) was in very good agreement with that obtained by bulk electrochemical/EPR redox titrations (−365 mV: Park *et al.*, 1991). The effect of temperature on the measured reduction potentials of the two ferredoxins is shown in Figure 2, and the calculated thermodynamic parameters are listed

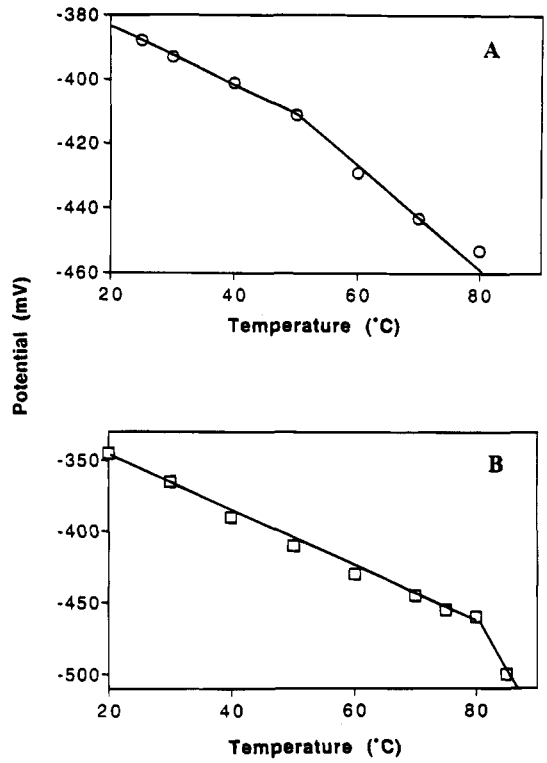


FIGURE 2: Temperature dependence of the reduction potentials of *T. maritima* ferredoxin (A) and *P. furiosus* ferredoxin (B). The experimental conditions for the *T. maritima* protein are as in the legend to Figure 1. The data for *P. furiosus* ferredoxin were taken from Adams (1992).

in Table 1. In both cases, the reduction potential of the protein became more negative with increasing temperature. The reduction potential of *T. maritima* ferredoxin decreased fairly linearly with increasing temperature with a possible transition near 50 °C (Figure 2A). Bulk electrochemical/EPR redox titrations have previously established a transition near 80 °C for the 4Fe form of *P. furiosus* ferredoxin (Figure 2B: Park *et al*, 1991). Direct electrochemical experiments were also attempted with the [3Fe-4S] form of the *P. furiosus* protein, and with the reconstituted [4Fe-4S] protein generated from the same sample (Figure 3). For unknown reasons, the voltammetric wave centered at a potential of −190 mV and assigned to the $[3Fe\text{-}4S]^{1+/0}$ couple was only observed after the addition of neomycin. Under these conditions, an additional wave was observed at −700 mV from the 3Fe form (Figure 3). A similar result was reported with the 3Fe cluster in the 7Fe-ferredoxin of *Desulfovibrio africanus*, and this was postulated to arise from a $[3Fe\text{-}4S]^{0,2-}$ couple (Butt *et al.*, 1991). In the case of *P. furiosus* ferredoxin, however, the low-potential wave more likely originates from a $[3Fe\text{-}4S]^{0,1-}$ couple based on the observed peak width at half-height. *P. furiosus* ferredoxin contains only a single cluster, thus, the conversion of the 3Fe form to the 4Fe form was readily visualized by voltammetry (Figure 3). The reduction potentials at 25 °C ($E^{\circ\prime}_{25\ ^\circ C}$) for the 3Fe form and for the reconstituted 4Fe-protein were similar to those obtained by EPR methods (Table 2).

Pyruvate Ferredoxin Oxidoreductase (POR). Near-symmetric voltammograms were also observed from the more complex iron−sulfur enzyme POR. The measured reduction potentials as a function of temperature for both the *T. maritima* and *P. furiosus* proteins are shown in Figure 4. Inflection points were observed in both cases, and these are

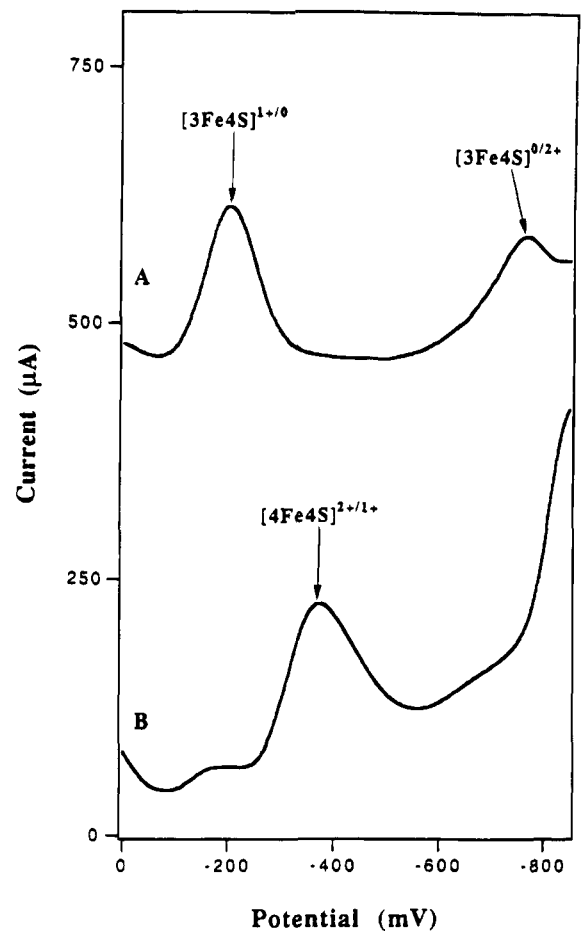


FIGURE 3: Differential pulse voltammograms of the [3Fe-4S] and electrochemically reconstituted [4Fe-4S] forms of *P. furiosus* ferredoxin. The samples were (A) 250 μM *P. furiosus* [3Fe-4S] ferredoxin and (B) electrochemically reconstituted [4Fe-4S] ferredoxin in 1 mM neomycin, 0.1 M NaCl, and 50 mM Tris, pH 8.0. The experimental conditions were as described in the legend to Figure 1.

Table 2: Comparison of the Reduction Potentials of Hyperthermophilic Iron−Sulfur Proteins Determined at 25 °C by Direct Electrochemistry and EPR Spectroelectrochemistry

organism	protein	M_r	redox center(s)	$E^{\circ\prime}$ [a] (mV)	$E^{\circ\prime}$ [b] (mV)
P. furiosus	rubredoxin	6800	[2Fe]	12	0
T. maritima	ferredoxin	7500	[4Fe]	−388	nd[c]
P. furiosus	ferredoxin	7500	[4Fe]	−370	−365
			[3Fe]	−190	−160
T. maritima	POR	113000	[4Fe]	−421	nd
P. furiosus	POR	115000	[4Fe]	−408	nd
T. maritima	hydrogenase	280000	[2Fe]	−411	−440
			[4Fe]	−411	−390
P. furiosus	hydrogenase	185000	[2Fe]	−390	−410
			[4Fe]	−320	−210
			[Ni]	nd	−300

[a] Determined by direct electrochemistry at a pyrolytic graphite electrode. [b] Determined by bulk electrochemical titrations monitored by rapid-freeze EPR spectroscopy. Data taken from Aono *et al.* (1989), Blake *et al.* (1991), Juszczak *et al.* (1991), and Adams (1992). [c] nd = not determined.

listed in Table 1. As was observed with the ferredoxins, the potential of the copper−iron−sulfur-containing POR from *P. furiosus* also decreased with increasing temperature. In contrast, the reduction potential of the iron−sulfur-containing POR from *T. maritima* became more positive with increasing temperature, and particularly above the transition

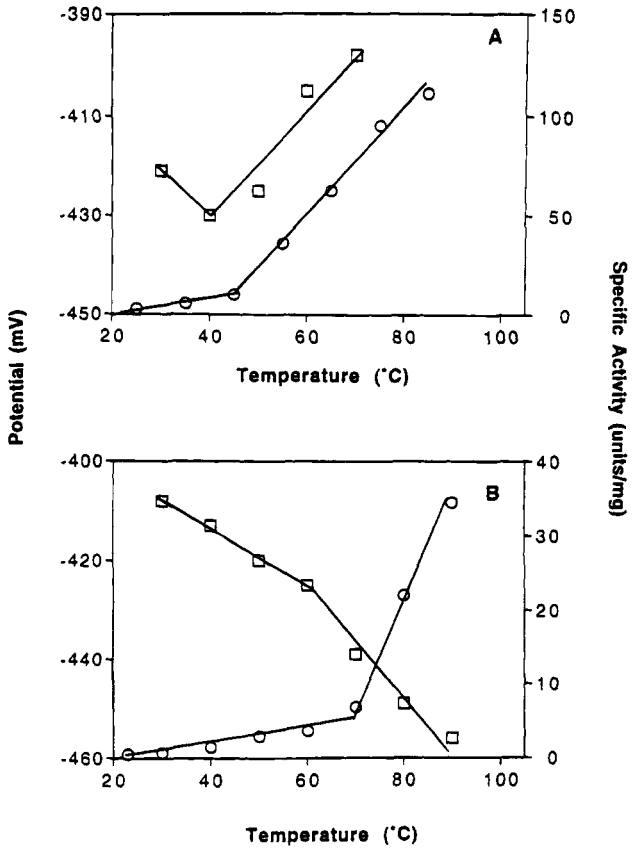


FIGURE 4: Temperature dependence of the reduction potential and catalytic activity of pyruvate ferredoxin oxidoreductases from *T. maritima* (A) and *P. furiosus* (B). Reduction potentials (squares) were determined by direct electrochemistry, and activities (circles) were measured by the pyruvate oxidation as described under Methods. The protein concentration used for the two enzymes was approximately 200 μM, and 1 unit of activity is the oxidation of 1 μmol of pyruvate/min.

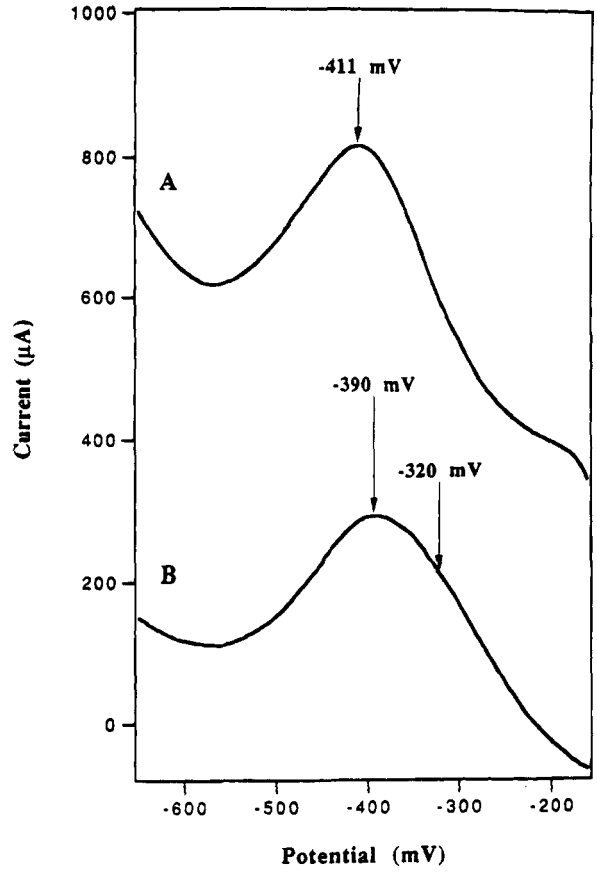


FIGURE 5: Differential pulse voltammograms for the hydrogenases of *T. maritima* (A) and *P. furiosus* (B). Each sample contained 250 μM protein in 0.1 M NaCl, 50 mM Tris/HCl, pH 8.0. The experimental conditions were 50 mV pulse height and 2 mV/s scan rate, and the temperature was 25 °C.

point near 70 °C. The thermodynamic parameters associated with the electron transfer processes for the two PORs are listed in Table 1. The peak current for both enzymes decreased slightly with temperature, which may be indicative of some protein adsorption to the electrode surface. With both enzymes, peak position and peak current were unaffected by the addition of pyruvate, CoASH, or pyruvate plus CoASH at either 25 or 80 °C. Thus, although samples withdrawn from the electrochemical cell showed no significant loss of catalytic activity when assayed under standard conditions, no catalytic current was observed for either enzyme in the presence of substrates. Since catalytic current is manifested in the forward and reverse currents before it is apparent in the net current for differential voltammetric methods (O'Dea *et al.*, 1981), it is possible that catalytic current was generated but was not detected. Alternative approaches, such as rapid quench spectroelectrochemistry, will be required to confirm this. Figure 4 also shows the effect of temperature on the activity of the two enzymes, as determined by the pyruvate- and CoASH-dependent reduction of methyl viologen (Blamey & Adams, 1993, 1994). Temperature-dependent break points were observed for both enzymes, although these are somewhat higher than those seen for the reduction potentials.

Hydrogenase. Differential pulse voltammograms of the iron-only hydrogenase of *T. maritima* and the nickel–iron–sulfur hydrogenase of *P. furiosus* are shown in Figure 5. The voltammetric wave of *P. furiosus* hydrogenase was measurably broader than that of *T. maritima* hydrogenase; however, this broadening was unaffected by scan rate (up to 10 mV/s) and therefore was not a kinetic effect. The broadening most probably arises from differences in the reduction potentials of the various iron–sulfur centers in the *P. furiosus* protein, differences previously shown by bulk electrochemical/EPR techniques (Adams, 1992). Specifically, the broad voltammetric wave could be assigned to two superimposed waves with reduction potentials indicated in Figure 5, and these are also listed in Table 1. They are assigned to the 2Fe and 4Fe centers found in this protein based on the similarity in the values with those obtained by the EPR method. In agreement with the bulk electrochemical technique (Adams, 1992), the reduction potentials of the redox centers in *P. furiosus* hydrogenase were found to be independent of temperature over the range 30–80 °C, and consequently no inflection points were detected. In contrast, the $E^{\circ\prime}$ value of *T. maritima* hydrogenase was temperature-dependent as measured by direct electrochemistry. The data are presented in Figure 6 together with the effect of temperature on the activity of the H_2 production activity of the *T. maritima* enzyme using reduced methyl viologen as the electron donor (Juszczak *et al.*, 1991). Inflection points near 60 °C were observed in both temperature profiles, and these are given in Table 1, together with the corresponding thermodynamic parameters.

Potential/Current Response. In the direct electrochemical studies of all the proteins described above, the voltammagram

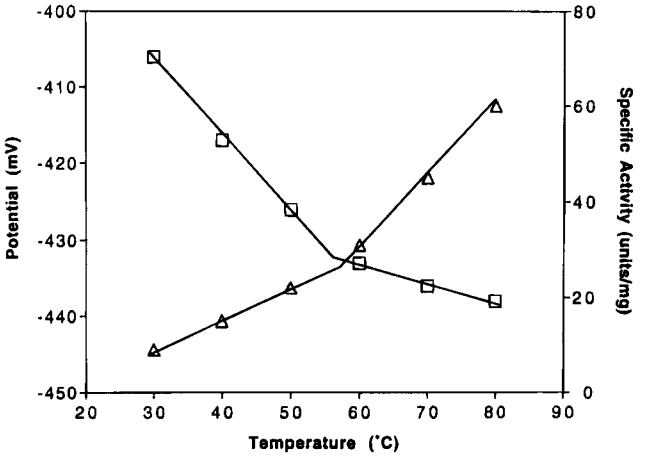


FIGURE 6: Temperature dependence of the reduction potential and catalytic activity of *T. maritima* hydrogenase. The reduction potentials were determined by direct electrochemistry as described under Methods and the legend to Figure 1 using approximately 200 μM hydrogenase. The specific activity data were taken from Juszczak *et al.* (1991).

peak widths at half-height generally remained constant with temperature. Theoretically, the peak width at half-height should be 98 mV/*n* at 25 °C for differential pulse voltammetry, and the width at half-height should decrease with increasing temperature according to the Nernst equation. Except in the case of the PORs at high temperatures, no significant loss in peak current or peak broadening was observed for the proteins under study, suggesting that adsorption processes and/or protein denaturation did not occur to any significant extent under the experimental conditions. Neomycin did not appear to promote or enhance electrode interactions of any protein except for 3Fe form of *P. furiosus* ferredoxin. As noted above, we did not analyze peak current/scan rate profiles because of the significant contribution of charging or nonfaradaic current.

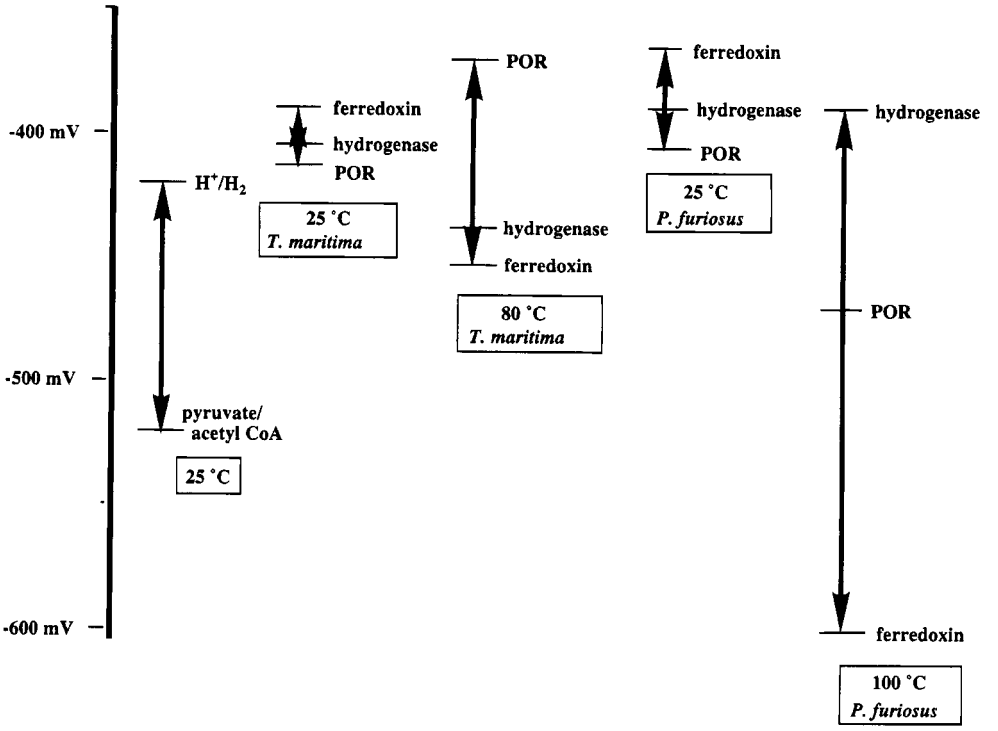
DISCUSSION

We have compared the electrochemical properties of analogous redox proteins obtained from the hyperthermophiles *T. maritima* (T_{opt} = 80 °C) and *P. furiosus* (T_{opt} = 100 °C) involved in the conversion of pyruvate to acetyl-CoA, H_2, and CO_2 (Scheme 1). Predicted or measured reduction potentials for these proteins at ambient temperature and at the optimal growth temperatures of the organism from which they were obtained are shown diagramatically in Scheme 2. It should be noted that the reduction potentials for the substrates and products of the overall reaction at high temperatures are not known. As indicated, the temperature dependence of the potentials of these proteins varied dramatically, even between proteins of similar function. However, it is clear from Scheme 2 that at ambient temperature the redox potentials of the three protein types are comparable and span less than 50 mV, yet they diverge with increasing temperature such that in the physiological-relevant range they are widely separated, particularly in the case of *P. furiosus*. Clearly, ambient temperature reduction potentials are of little value in rationalizing the function of hyperthermophilic redox proteins and in fact might be quite misleading. For example, the reduction potentials of *T. maritima* and *P. furiosus* ferredoxin at their respective growth temperatures are more negative than their respective hydrogenases and PORs, which is in contrast to the situation with their ambient temperature reduction potentials. Moreover, *T. maritima* POR has the most positive reduction potential of the three *T. maritima* proteins at 80 °C, but it is the most negative at 25 °C. Before considering the consequences of these changes in the overall flow of electrons from pyruvate to H_2 in the two organisms, the properties of the analogous proteins will be discussed.

The two ferredoxins (and also *P. furiosus* rubredoxin) had much more negative entropy terms than were calculated for the larger hyperthermophilic redox enzymes (Table 1). Moreover, the $\Delta S°$ (or $\Delta S°_{rc}$) values for these small hyperthermophilic redox proteins (−28 to −60 eu) were also more negative than those previously reported for seven small mesophilic redox proteins (range of −2 to −16 eu: Taniguchi *et al.*, 1980). In the latter case, the $\Delta S°_{rc}$ values were smaller for proteins with solvent-exposed redox centers, but the entropy terms of all the proteins were significantly more negative than those of small inorganic complexes. Thus, it was proposed (Taniguchi *et al.*, 1980) that a large negative $\Delta S°$ term can be attributed to either a combination of conformation/solvation effects, a more rigid reduced enzyme, or a charge neutralization in the reduced enzyme by more ordered water molecules. The significantly more negative $\Delta S°$ terms we determined for the small hyperthermophilic redox proteins could be explained by any of these effects, but a significantly more rigid reduced enzyme would seem most likely in the case where $\Delta S° < 0$. However, the reduced and oxidized forms of *P. furiosus* rubredoxin show no striking structural differences in crystallographic analyses (to 1.8 Å: Day *et al.*, 1993), and a preliminary calorimetric study has shown that the reduced protein has a significantly lower melting point (T_m) than the oxidized form (Klump *et al.*, 1994). It is also not clear how meaningful the entropy terms are for the larger hyperthermophilic enzymes, especially as there is variation in both magnitude and sign (Table 1).

The two hyperthermophilic hydrogenases used in this study have multiple redox centers. As indicated in Table 2, both contain 2Fe and 4Fe centers that by bulk redox titrations monitored by rapid-freeze EPR can be distinguished in each enzyme by their reduction potentials (Juszczak *et al.*, 1991; Adams, 1992). In *T. maritima* hydrogenase, the values for the two clusters are within 40 mV (Juszczak *et al.*, 1991), and only a single voltammetric peak at the expected potential was observed by direct electrochemistry. Redox centers with reduction potentials separated by more than 100 mV should theoretically have distinct voltammetric waves (Bard & Faulkner, 1980), but this was not observed with *P. furiosus* hydrogenase. For example, the reduction potentials of the two cluster types in this enzyme differ by 200 mV as determined by EPR spectroscopy (Adams, 1992), but only a broad voltammetric wave was seen by direct electrochemistry, although again it was at the appropriate potential (Figure 5). The absence of two distinct waves probably arises from the presence of an additional redox site in this enzyme, namely, the Ni center, which has a potential intermediate between the 2Fe and 4Fe centers (Adams, 1992). Hence, the broad electrochemical wave observed with *P. furiosus* hydrogenase most likely represents three distinct redox sites. However, we made no attempt to deconvolute the voltammograms, and qualitatively assigned reduction potentials based on previous EPR-monitored titrations.

Scheme 2: Reduction Potentials of Substrates and Proteins Involved in H_2 Production from Pyruvate as a Function of Temperature



The reduction potential of the two hyperthermophilic PORs was approximately −400 mV at 25 °C (Table 1), a value characteristic of ferredoxin-type [4Fe-4S] centers, at least two of which are present in each enzyme (Smith *et al.*, 1994). However, that of *T. maritima* POR becomes 100 mV more positive than that of the *P. furiosus* enzyme at physiological temperatures. Since POR catalyzes the oxidation of a substrate, one might expect its reduction potential to be more positive than the coupled half-reaction, which for pyruvate/acetyl-CoA is −520 mV at 25 °C (Thauer *et al.*, 1977). Although this has not been determined at extreme temperatures and is therefore an unknown variable, the higher specific activity for pyruvate oxidation by *T. maritima* POR (87 units/mg: Blamey & Adams, 1994) compared to the *P. furiosus* enzyme (22 units/mg: Blamey & Adams, 1993) is consistent with the fact that the iron−sulfur centers of *T. maritima* POR are easier to reduce at high temperatures. Similarly, the calculated $E_{act.}$ value for *T. maritima* POR (12 kcal/mol over the range 25−85 °C: Blamey & Adams, 1994) is about half of that calculated for the *P. furiosus* enzyme (20 kcal/mol over the range 23−90 °C: Blamey & Adams, 1993). On the other hand, the reduction potentials of the two ferredoxins, the proposed physiological electron acceptors for the two PORs, are more negative than the reduction potentials of their respective PORs, and particularly in the case of *P. furiosus* (Scheme 2). Again, this difference between the proteins of the two organisms is consistent with measurements of catalytic activity. For example, the apparent V_m value of *P. furiosus* POR using ferredoxin (apparent K_m = 93 μM) as an electron acceptor is only 33% of that obtained with methyl viologen ($E^{\circ\prime}$ = −510 mV at 80 °C: Blamey & Adams, 1993) as the electron carrier, whereas for POR and ferredoxin from *T. maritima* (apparent K_m = 26 μM) the analogous value is 322% (Blamey & Adams, 1994). In other words, in spite of the fact that thermodynamic and kinetic data are not strictly comparable, the ferredoxin of *T. maritima* does appear to be a much more efficient electron carrier for POR than is the *P. furiosus* protein for its POR, as suggested by the data represented in Scheme 2.

We now turn to the overall implications of the values of the reduction potentials of the various proteins at the optimum growth temperatures of the particular organism (Scheme 2). From Scheme 1, the following order of reduction potentials can be logically inferred: $E'_{POR} < E'_{Fd} < E'_{hydrogenase}$. However, this is in contrast to our experimental results at the physiologically-relevant temperatures, which show $E'_{Fd} \ll E'_{POR} < E'_{hydrogenase}$ for *P. furiosus*, and $E'_{Fd} < E'_{hydrogenase} < E'_{POR}$ for *T. maritima*. Nevertheless, thermodynamically unfavorable reactions in the pathway can be transduced if they are driven by differences in relative concentrations. For example, although it was determined that $E'_{Fd} \ll E'_{POR}$ in *P. furiosus*, in vitro assays have shown that oxidized ferredoxin at a 2000-fold molar excess compared to POR does serve as an electron acceptor for the enzyme (Blamey & Adams, 1993). Notably, the intracellular concentration of ferredoxin (~70 nmol/g of *P. furiosus* cells, wet weight: Aono *et al.*, 1989) is much higher than the intracellular concentration of POR (~10 nmol/g: Blamey & Adams, 1993). Thus, it can be determined from the Nernst equation (eq 3) that at high concentrations of oxidized

$$E_{eq} = E'_{POR} - E'_{Fd} + 59 \log\left(\frac{[POR_{ox}][Fd_{red}]^2}{[POR_{red}][Fd_{ox}]^2}\right) \quad (3)$$

ferredoxin (Fd_{ox}) a significant amount is reduced (to Fd_{red}) by POR. Accordingly, *P. furiosus* ferredoxin is completely reduced at 80 °C in vitro by a catalytic amount of POR as determined by visible spectroscopy (Ma *et al.*, 1994).

On the other hand, the data of Scheme 2 suggest that *P. furiosus* ferredoxin should be an efficient electron carrier

for *P. furiosus* hydrogenase, although only at temperatures above 80 °C, the minimum temperature for significant growth of the organism (Fiala & Stetter, 1986). Indeed, this was thought to be the case as the ferredoxin when chemically reduced by sodium dithionite does support H_2 evolution by the hydrogenase (Bryant & Adams, 1989). However, a very recent study has shown that when ferredoxin is reduced by POR it does not transfer electrons to the hydrogenase for H_2 production, and that the hydrogenase can utilize NADPH as a sole electron donor in vitro (Ma *et al.*, 1994). Moreover, H_2 production from pyruvate was observed at 80 °C from a system containing POR, ferredoxin, NADP, and hydrogenase when NADP:ferredoxin oxidoreductase, which was recently purified from *P. furiosus* (Ma & Adams, 1994), was also added (Ma *et al.*, 1994). Thus, in *P. furiosus*, POR-reduced ferredoxin is apparently unable to function as an electron donor to hydrogenase, even though the results presented herein show that this is a thermodynamically favorable reaction. The reason for this may lie with the metabolism of *P. furiosus*, which is both saccharolytic and proteolytic (Fiala & Stetter, 1986). That is, NADPH, the proposed physiological electron donor to the hydrogenase (Ma *et al.*, 1994), presumably has a reduction potential ($E^{\circ\prime} = -340$ mV at 25 °C) near 100 °C, which is more positive than that of the ferredoxin. Thus, excess reductant from fermentative processes can be disposed of as H_2 via NADP-dependent dehydrogenases that oxidize substrates, e.g., amino acids, whose reduction potentials would not enable the direct reduction of ferredoxin. In addition, *P. furiosus* (but not *T. maritima*, see below) can use pyruvate as the sole carbon source for growth (Schafer *et al.*, 1994; Schroder *et al.*, 1994), and pyruvate oxidation directly generates not only reduced ferredoxin but also NADPH (via NADP:ferredoxin oxidoreductase), which can be utilized either for H_2 production or for biosynthetic purposes.

In contrast to the case with *P. furiosus*, the data presented in Scheme 2 suggest that *T. maritima* ferredoxin may not be a good electron carrier for the hydrogenase of this organism at the optimal growth temperature. Indeed, the chemically-reduced redox protein does not serve as an electron donor for the hydrogenase in an in vitro H_2 production assay at 80 °C, and an additional electron carrier between the two proteins in vivo has been proposed (Juszczak *et al.*, 1991; Blamey *et al.*, 1994). Moreover, the overall flow of electrons between pyruvate and H_2 would appear to be limited by the fact that $E'_{\text{hydrogenase}} < E'_{\text{POR}}$ for *T. maritima*. Similarly, a ferredoxin/POR relative concentration difference cannot be used to explain electron transfer between these proteins as the concentration of ferredoxin in *T. maritima* (~8 nmol/g of cells, wet weight: Blamey *et al.*, 1994) is about an order of magnitude less than that in *P. furiosus* (70 nmol/g). In fact, it is comparable to the intracellular concentration of POR (~10 nmol/g: Blamey & Adams, 1994), which is similar to the concentration of POR in *P. furiosus*. It is also worth noting that the intracellular concentration of *T. maritima* hydrogenase (~4 nmol/g: Juszczak *et al.*, 1991) is much higher than that of *P. furiosus* hydrogenase (~0.3 nmol/g: Bryant & Adams, 1989). Hence, other relative concentration differences (or more specifically, the relative concentrations of particular redox states) as well as other coupled spontaneous reactions very likely influence the energetics of the electron transport pathway of *T. maritima*. Of course, the data presented herein do not take into account the redox properties of other low-potential electron carriers in *T. maritima* which might facilitate electron transfer between its ferredoxin and hydrogenase. In any event, the differences in reduction potentials (Scheme 2) and intracellular concentrations of the analogous proteins in *P. furiosus* and *T. maritima* demonstrate that the mechanisms by which electrons are transduced from pyruvate to H_2 are not the same in these two organisms. Moreover, in order to rationalize the direction of electron flow, it is clear that one must also consider the relative concentrations of oxidized and reduced species of donor/acceptor pairs, which are determined by the effective ambient solution potential of the intracellular space, as well as reduction potentials at physiological temperatures for key substrates and products, e.g., for pyruvate/acetyl-CoA and NADP/NADPH.

It should also be noted that three of the four hyperthermophilic enzymes examined in this study exhibited break points in their reduction potentials with increasing temperature, and these correlated reasonably well with the temperature dependence of their catalytic activities (see Figures 4 and 6). In addition, transition temperatures were also seen with the ferredoxins in their reduction potential profiles. As shown in Table 1, these transition temperatures occurred at lower temperatures with the *T. maritima* proteins than with those from *P. furiosus*. This presumably reflects the 20 °C difference in optimal growth temperatures of these microorganisms, and also suggests that the observed break points are not artifacts. Previous studies have reported a transition point at 40 °C in the reduction potential/temperature profiles of cytochrome *c*, and this was essentially independent of the technique utilized (Koller & Hawkridge, 1988; Tanaguichi *et al.*, 1993). It was concluded that this transition was not due to protein denaturation, was an intrinsic property of the protein, and probably reflected a phase transition due to a change in hydration. Thus, an analogous transition also appears to occur in hyperthermophilic redox proteins and redox enzymes, albeit at much higher temperatures. Although the sources of such inflection points are unclear at present, there does appear to be some relationship between them and the onset of catalytic activity.

Finally, an important issue not at all understood is the tremendous variation observed in the temperature dependence of the reduction potentials of the various hyperthermophilic proteins examined herein, particularly since in most cases the responsible redox entity is a [4Fe-4S] center (Table 2). Obviously, this cluster must be in significantly different environments within the different proteins, and the mechanisms that lead to different responses to temperature may be similar to those that stabilize the same cluster at different redox potentials in different proteins [e.g., see Cammack (1992) and Smith *et al.* (1993)]. Clearly, much remains to be understood about FeS cluster/protein interactions, but the results from the present study do demonstrate that determining the reduction potentials of redox centers at physiological temperatures is essential for any rationalization of electron transfer processes involving hyperthermophilic proteins.

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