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ORIGINAL ARTICLE

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Direct electrochemical characterization of hyperthermophilic *Thermococcus celer* metalloenzymes involved in hydrogen production from pyruvate

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Abstract The reduction potentials of the metalloproteins pyruvate ferredoxin oxidoreductase (POR), ferredoxin, and hydrogenase isolated from hyperthermophilic *Thermococcus celer* (T_{opt}=88 °C) were determined as a function of temperature from 10 to 85 °C. Square-wave voltammetry experiments were carried out on 15 μL samples directly at an unmodified "edge-polished" pyrolytic graphite electrode using $MgCl_2$ as an electrode promoter. POR exhibited two voltammetric waves with peaks at –280 and –403 mV at room temperature, indicating multiple redox centers, and a single wave at –420 mV at 85 °C. These waves displayed different temperature-dependent peak positions and peak heights, indicating that these redox centers have different thermodynamic and kinetic properties. Ferredoxin displayed a single linear temperature-dependent voltammetric wave at –280 mV at room temperature and –327 mV at 85 °C. Hydrogenase displayed a single biphasic temperature-dependent voltammetric wave at –197 mV at room temperature and –211 mV at 85 °C. Thermodynamic parameters associated with electron transfer, namely standard enthalpies and entropies for the redox centers in the various proteins, are reported.

Keywords *Thermococcus celer* · Hyperthermophiles · Reduction potentials · Square-wave voltammetry

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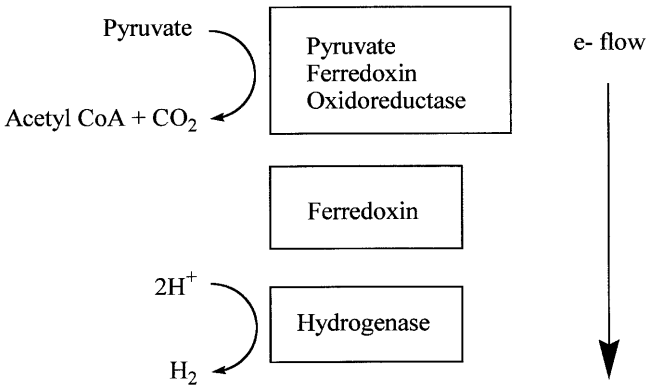
Introduction

Hyperthermophiles, which were first isolated more than three decades ago, are unusual microorganisms that are capable of growing near or above the boiling point of water [1]. Most of these organisms have been isolated near deep sea or shallow marine hydrothermal vents, where temperatures approach or exceed 100 °C and pressures may exceed 400 atmospheres. For example, the archaea *Thermococcus celer* was isolated from a marine water hole near Italy in 1983, and it has an optimal growth temperature of 88 °C [2]. The biochemistry of a few hyperthermophilic organisms have been investigated to some extent, but many questions concerning their incredible thermostability and ability to carry out biochemical reactions under such environmental extremes are not well established [3].

Relatively few hyperthermophilic enzymes involved in anaerobic carbohydrate metabolism have been isolated and characterized (see, e.g. [3, 4, 5]). In hyperthermophilic microorganisms, as well as in other microorganisms that grow at more moderate temperatures, pyruvate is considered an intermediate of carbohydrate fermentation; this intermediate serves as a major source of reductant for hydrogen production [6, 7]. It has been proposed that pyruvate generated from fermentation is typically oxidatively decarboxylated by pyruvate ferredoxin oxidoreductase (POR) to yield acetyl CoA, CO_2, and reduced ferredoxin [8]. The reduced ferredoxin, in turn, transfers electrons to hydrogenase, resulting in the evolution of H_2. This overall pathway, as shown in Scheme 1, is thought to exist in hyperthermophilic microorganisms [6, 7], although this pathway remains to be clearly demonstrated by in vitro assays.

In this investigation, the enzymes from hyperthermophilic *T. celer* thought to be involved in the oxidation of pyruvate through the reduction of protons to



Scheme 1 Schematic diagram illustrating electron flow from pyruvate ferredoxin oxidoreductase, ferredoxin, and hydrogenase

hydrogen (see Scheme 1), pyruvate ferredoxin oxidoreductase, ferredoxin, and hydrogenase, have been electrochemically characterized. The equilibrium reduction potentials ($E^{\circ\prime}$) of these hyperthermophilic enzymes were determined by direct electrochemistry as a function of temperature. As previously noted [9], it is important to measure their equilibrium reduction potentials under more relevant physiological conditions since these enzymes operate at elevated temperatures.

Equilibrium reduction potentials, which determine equilibrium concentrations of oxidized and reduced species, influence the direction of electron flow in electron transfer pathways. Based only on reduction potentials, it would be expected that in order to have electrons flow from POR to hydrogenase, $E^{\circ\prime}_{POR}<E^{\circ\prime}_{ferredoxin}<E^{\circ\prime}_{hydrogenase}$. Previous studies [9] of POR, ferredoxin, and hydrogenase isolated from other hyperthermophiles, *Pyrococcus furiosus* and *Thermotoga maritima*, have shown that their reduction potentials do not follow this favorable thermodynamic trend. It was also demonstrated that all the proteins isolated from these two organisms have different temperature-dependent reduction potentials [9] and catalytic mechanisms [10]. In order to further our understanding of this important biological pathway and compare the pathways of these unusual microorganisms, we have electrochemically characterized the same enzymes from an additional hyperthermophilic microorganism, *T. celer*.

Materials and methods

T. celer cells were grown, harvested, and all proteins were isolated under anaerobic conditions as previously described ([6] [11], Blamey JM, Chiong M, Smith ET, submitted). A 0.5-mL solution containing 0.5 mM protein and 25 mM TRIS (pH 8.0) in a stoppered 8 mL vial was made anaerobic by cycling between a vacuum and nitrogen gas. A Hamilton gas-tight syringe was used to transfer 10–20 µL samples into an electrochemical cell as described below.

Reduction potentials at ambient temperature were measured using a microscale cell as previously described [12]. This cell consisted of an "edge-polished" pyrolytic graphite working electrode, a saturated Ag/AgCl reference electrode ($E^{\circ\prime}$=199 mV), and a platinum wire counter electrode. The pyrolytic graphite electrode was polished with 30 µm aluminum oxide sandpaper prior to each experiment. The cell was flushed with nitrogen passed over a heated RC 11 catalyst (Chemical Dynamics, South Plainfield, NJ).

The isothermal temperature-controlled experiments on 15 µL samples were carried out in an electrochemical cell fabricated from latex tubing (approximately 10 mm in length and 4 mm ID). The reference electrode (4 mm OD diameter), which was also used in the ambient temperature studies, was partially inserted into one end of the latex tubing and placed in an anaerobic chamber (Coy Laboratory Products, Detroit, Mich.). Within the chamber, the latex tubing was filled with approximately 50 µL of 1 M $MgCl_2$. The working electrode was then inserted into the other end of this latex tubing, leaving a <2 mm gap between the two electrodes. Care was taken not to introduce any gas bubbles. The working/reference electrode assembly was transferred out of the glove box. A Hamilton gas-tight syringe filled with 15 µL of an anaerobic protein sample was injected through the tubing into the gap between the reference and working electrodes; this procedure displaced most of the $MgCl_2$. A stainless steel pin was placed into the hole made by the syringe and this was used as the counter electrode. The working and counter electrodes were arranged so that there was no physical contact between them. The assembled three-electrode cell was then connected to the potentiostat, the cell was carefully placed inside a polypropylene bag, and then the bag was immersed in a water bath.

Square-wave voltammograms were obtained using a CV 50W potentiostat (BAS, Lafayette, Ind.) at a frequency of 5 Hz, a step potential of 2 mV, and a pulse potential of 50 mV. All reduction potentials are reported versus SHE. Electrochemical measurements recorded at ambient temperature with the temperature-controlled cell were consistent with the measurements made with the other microscale electrochemical cell. Multiple scans were obtained at each temperature, and the reduction potentials that were obtained were found to be within a few millivolts of each other at each temperature. The saturated Ag/AgCl reference electrode was calibrated against a saturated calomel electrode as a function of temperature. The reaction entropy, ΔS°, was determined from the slope of $E^{\circ\prime}/T$, and the reaction enthalpy was determined from the slope of $E^{\circ\prime}/T$ versus $1/T$ as previously described [9].

Results and discussion

The reduction potentials of the three hyperthermophilic enzymes from *T. celer* determined at room temperature in this study are listed in Table 1, as well as values for hyperthermophilic enzymes determined in a previous study [9]. The room temperature reduction potential for the various *T. celer* proteins is significantly more positive than values previously reported for hyperthermophilic enzymes [9], as well as most mesophilic proteins involved in this pathway [8, 13]. All of the proteins in earlier studies have reduction potentials closer to –400 mV, which is characteristic of low-potential 4FeS centers.

Representative electrochemical data and temperature profiles of *T. celer* proteins are shown in Figs. 1, 2, 3. Thermodynamic data for electron transfer, namely standard entropy and enthalpy, obtained in

this work and those previously obtained for similar proteins isolated from other hyperthermophiles are listed in Table 1. The positive enthalpy values observed for *T. celer* ferredoxin and hydrogenase, in contrast to POR, indicated that the electron transfer reactions are endothermic and that the reduced protein is more stable at higher temperatures. The entropy values for all three proteins were negative (except for hydrogenase below 45 °C), which indicates that the oxidized form of the protein is favored at higher temperatures.

Pyruvate ferredoxin oxidoreductase

Representative square-wave voltammograms and the reduction potential of *T. celer* POR as a function of temperature are both shown in Fig. 1. The multiple waves observed for this protein suggest multiple redox centers with different reduction potentials. This result is consistent with the fact that POR isolated from other hyperthermophiles are known to contain multiple 2Fe and 4Fe centers [6, 7]. The resolution of multiple waves via direct electrochemistry, however, has not been reported for any hyperthermophilic POR. It was noted, though, that there was a shoulder in the square-wave voltammogram of *P. furiosus* POR [9]. Separate multiple waves would only be observed for enzymes that contain redox centers with significantly different reduction potentials (>100 mV). Unfortunately, direct electrochemistry yields no information as to the identity of redox centers, and therefore additional spectroscopic studies would be necessary in order to identify the reduction potentials of individual centers within *T. celer* POR.

Although the identities of a redox center cannot be determined from voltammetry, the voltammetric wave

Table 1 Comparison of the reduction potentials of hyperthermophilic iron-sulfur proteins determined at 25 °C by direct electrochemistry

Enzyme	$E°'$ (mV)	$\Delta S°$ (cal K^{-1} mol^{-1})	$\Delta H°$ (kcal mol^{-1})
T. celer			
POR	–280, –403	–45	–26
Ferredoxin	–280	–16	2
Hydrogenase	–197	+5[b], –10[c]	4
P. furiosus[a]			
POR	–408	–19	3
Ferredoxin	–370	–50	–5
Hydrogenase	–390	0	nd[d]
T. maritima[a]			
POR	–421	27	18
Ferredoxin	–388	–28	1
Hydrogenase	–411	–20	3

[a]Extracted from [9]
[b]Below 45 °C
[c]Above 45 °C
[d]Not determined

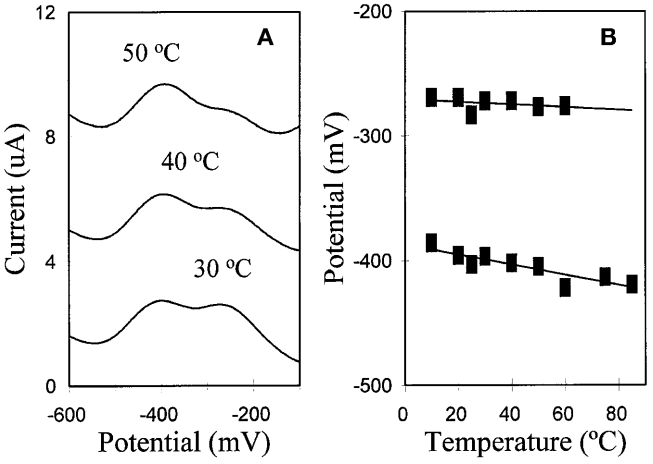


Fig. 1 **A** Square-wave voltammograms of 40 μM *T. celer* POR at three different temperatures. **B** Reduction potentials of *T. celer* POR (indicated by separated traces for each peak as illustrated in **A**) as a function of temperature

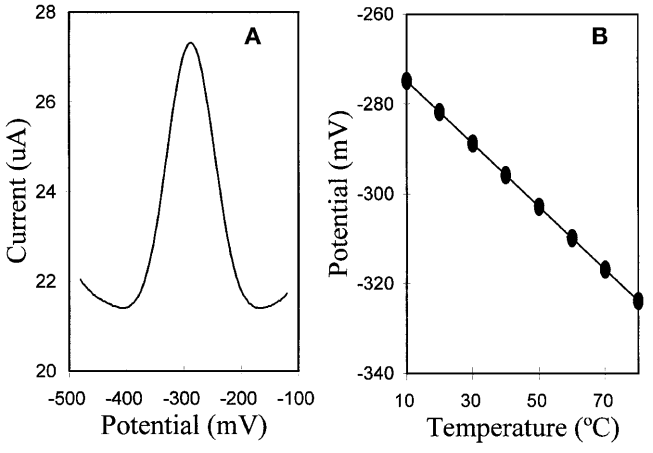


Fig. 2 **A** Square-wave voltammogram of 65 μM *T. celer* ferredoxin at 25 °C. **B** Reduction potential of *T. celer* ferredoxin versus temperature

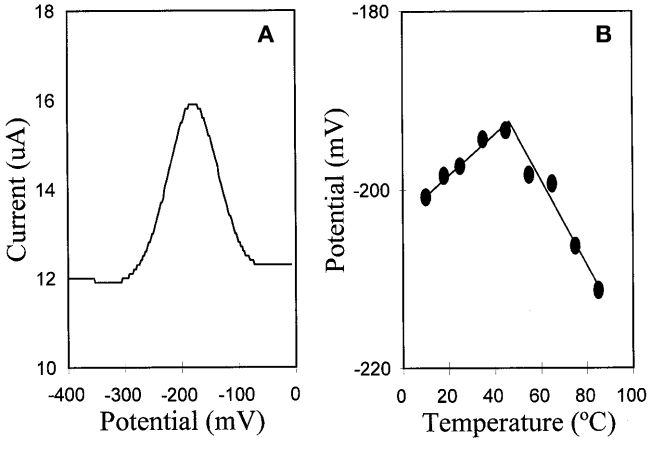


Fig. 3 **A** Square-wave voltammogram of 25 μM *T. celer* hydrogenase at 25 °C. **B** Reduction potential of *T. celer* hydrogenase versus temperature

observed at –403 mV at room temperature is at a reduction potential characteristic of a 4FeS center; the voltammetric wave at –280 mV is more characteristic of a 2FeS center. The peak current for the peak potential at –280 mV is temperature dependent (see Fig. 1), indicating that the electron transfer rate varies between the two different centers. Notably, hyperthermophilic proteins are inactive at room temperature, and show no significant activity below 70 °C. However, our electrochemical experiments indicate that electron transfer appears to be rapid at room temperature for both types of redox centers, and then the rate of electron transfer diminishes for the high potential center as the temperature increases. This observation may be responsible for the change in catalytic activity as a function of temperature. As the temperature increases, catalytic activity increases, the reduction potential of POR decreases, and it becomes a better electron donor to its electron acceptor ferredoxin.

Ferredoxin

A representative square-wave voltammogram and the reduction potential of *T. celer* ferredoxin as a function of temperature are both shown in Fig. 2. Unlike other hyperthermophilic ferredoxins [9], the reduction potential profile of *T. celer* ferredoxin is linear over the temperature ranges examined in this study. In addition, its reduction potential is significantly less temperature dependent than other hyperthermophilic ferredoxins.

Hydrogenase

A representative square-wave voltammogram of *T. celer* hydrogenase and the reduction potential as a function of temperature are both shown in Fig. 3. The reduction potential of this protein was significantly more positive than the reduction potential of other hyperthermophilic hydrogenases, but the value was consistent with other values previously reported for nonhyperthermophilic nickel-containing hydrogenases [14, 15, 16, 17, 18]. The voltammetric wave observed in Fig. 3 is probably due to its nickel center, based on previously reported values for this center. Moreover, this unusually positive reduction potential is consistent with the fact that in vitro assays demonstrate that *T. celer* hydrogenase is more efficient at hydrogen oxidation, and 100-fold more efficient at catalyzing hydrogen oxidation than *T. maritima* or *P. furiosus* hydrogenases (Blamey JM, Chiong M, Smith ET, submitted).

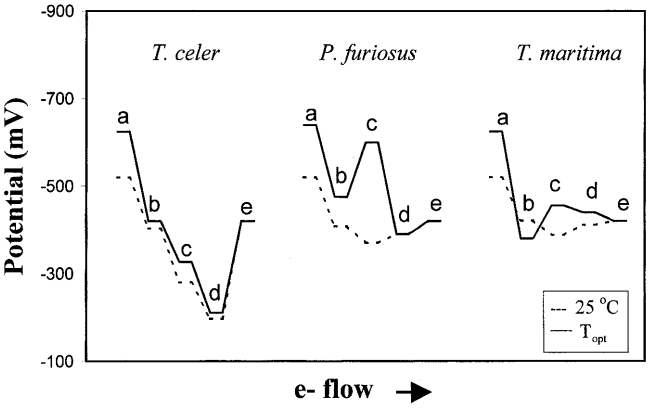
Hydrogenase was the only enzyme in this study that exhibited a positive change in reduction potential as the temperature increased, but only up to 45 °C. This type of behavior was also observed for *T. maritima* POR [9]. In addition, *T. celer* hydrogenase was the only enzyme in this study to exhibit a break point in the temperature-reduction potential profile. Break points have been previously observed in the temperature-reduction potential profiles of most hyperthermophilic enzymes [9], although the reason is not understood. These break points have been ascribed to changes in ionization states of the protein [19]; however, this has not been demonstrated experimentally.

Electrochemical response of proteins

Significant background currents were observed for both hydrogenase and ferredoxin (note the baseline current as shown in Figs. 2 and 3). This additional current was attributed to charging or nonfaradaic current, and no attempt was made to correct for it. We have observed that highly charged proteins often contribute to the charging current, and therefore subtracting out current due to the buffer alone is not completely adequate as a method for baseline correction. At the frequency used in this study, the positions of voltammetric waves were independent of scan direction, and peak widths at half-height for ferredoxin and hydrogenase were slightly less than predicted by theory (126 mV/n). Smaller peak widths suggest that some protein was adsorbed to the electrode surface. No attempt was made to deconvolute the multiple waves observed for POR to determine peak widths at half-height.

Electron transport chain

Scheme 2 illustrates the reduction potentials of all three *T. celer* enzymes at room temperature and near the organism's optimal growth temperature. It is apparent that the change in reduction potential of *T.*



Scheme 2 Schematic diagram illustrating reduction potentials of the substrates (pyruvate/acetyl CoA (a) and protons/molecluar hydrogen (e)), pyruvate ferredoxin oxidoreductase (b), ferredoxin (c), and hydrogenase (d) (isolated from *T. celer, T. maritima,* and *P. furiosus)*, at both room temperature (*dashed lines*) and near their optimal growth temperature (*solid lines*)

celer enzymes with temperature is relatively small in comparison to other hyperthermophilic enzymes [9]. Therefore, since there is no predictable trend in reduction potentials as a function of temperature, it is very important to obtain reduction potentials at the temperature where an enzyme exhibits optimal activity.

As stated earlier, in order for electrons to flow from POR to hydrogenase as illustrated in Scheme 1, it is logical that $E^{\circ\prime}_{POR} < E^{\circ\prime}_{ferredoxin} < E^{\circ\prime}_{hydrogenase}$. This is exactly what was observed experimentally for *T. celer*, but contrary to what has been previously reported for other hyperthermophiles [9]. It appears that the reduction potentials of the *T. celer* enzymes are thermodynamically optimized for electron flow from POR to hydrogenase using ferredoxin as an electron carrier.

The reduction potentials of the half-reactions involved in Scheme 1 are also shown in Scheme 2 at room temperature and near the organism's optimal growth temperature. The reduction potential of the hydrogen electrode is defined as 0 V at all temperatures, and it was calculated to be –420 mV at pH 7. While the reduction potential of pyruvate/acetyl CoA is not known at elevated temperatures, a rough estimate of its reduction potential was made based on certain assumptions. It was assumed that the equilibrium constant for this half-reaction does not change significantly over the temperature range used in this study. This was a reasonable assumption, based on previous equilibrium studies as a function of temperature for related half-reactions [20]. Thus, based on the relationship between Gibb's standard free energy and the equilibrium constant ($\Delta G^{\circ} = -RT\ln K$), it was calculated that the reduction potential of the pyruvate/acetyl CoA half-reaction was about –620 mV at 85 °C.

The estimated value for the pyruvate/acetyl CoA half-reaction did not take into account the change in the equilibrium constant with temperature. It was reported [20] that equilibrium constants of related substances change about threefold for a 10 °C temperature change. Based on this value and considering the 60 °C temperature range covered in this study, it was approximated that a 20-fold change in the equilibrium constant for the pyruvate/acetyl CoA half-reaction would only result in a less than ±50 mV change in reduction potential (depending on whether the reaction was endothermic or exothermic) from our estimated value.

Thus it appears that the electron transport chain as shown in Scheme 1 is thermodynamically feasible based on the reduction potentials of the *T. celer* enzymes (as shown in Scheme 2) for electron flow from pyruvate to hydrogenase. Based on reduction potentials alone, it is not thermodynamically favorable for electrons to flow from hydrogenase to hydrogen. In vitro assays also indicate that *T. celer* hydrogenase has a greater activity for hydrogen oxidation. If Scheme 1 is correct, it would require hydrogenase to catalyze the reduction of protons in a thermodynamically unfavorable direction based only on reduction potentials. As previously noted [9], however, reduction potentials alone do not explain the direction of electron flow; the concentrations of substrate and product need to be considered as well. For example, this pathway is possible if the concentration of substrate, $[H^+]$, is high and the partial pressure of H_2 is low. We are currently developing an assay to determine if protons can indeed serve as the final electron acceptor for the proposed electron transport chain illustrated in Scheme 1.

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