Characterization of an Ancestral Type of Pyruvate Ferredoxin Oxidoreductase from the Hyperthermophilic Bacterium, *Thermotoga maritima*†

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ABSTRACT: The hyperthermophilic bacterium, *Thermotoga maritima*, is a strict anaerobe that grows up to 90 °C by carbohydrate fermentation. We report here on its pyruvate ferredoxin oxidoreductase (POR), the enzyme that catalyzes the oxidation of pyruvate to acetyl-CoA, the terminal oxidation step in the conversion of glucose to acetate. *T. maritima* POR was purified to electrophoretic homogeneity under strictly anaerobic conditions. It has a molecular weight of 113 000 and comprises four dissimilar subunits with M_r values of approximately 43 000, 34 000, 23 000, and 13 000. It contains thiamine pyrophosphate (TPP) and at least two ferredoxin-type [4Fe–4S] clusters per molecule, as determined by iron analysis and EPR spectroscopy. CoASH was absolutely required for pyruvate oxidation activity, while the addition of TPP was stimulatory. The apparent K_m values at 80 °C for pyruvate, CoASH, and TPP were 14.5, 0.34, and 0.043 mM, respectively, and the corresponding apparent V_m values ranged from 154 to 170 μmol of pyruvate oxidized/min/mg (units/mg). The apparent K_m and V_m values for *T. maritima* ferredoxin, the proposed physiological electron carrier for POR, were 26 μM and 280 units/mg, respectively. POR did not use 2-oxoglutarate, phenyl pyruvate, or indolyl pyruvate as substrates. The enzyme was extremely thermostable: the temperature optimum for pyruvate oxidation was above 90 °C, and the time for a 50% loss of activity ($t_{50\%}$) at 80 °C (under anaerobic conditions) was 15 h. The enzyme was also very sensitive to inactivation by oxygen, with a $t_{50\%}$ in air at 25 °C of 70 min. Sodium nitrite was a weak inhibitor of POR activity (K_i = 54 mM), while carbon monoxide (320 μM), sodium cyanide (20 mM), sodium fluoride (5 mM), and or sodium azide (2.5 mM) had no inhibitory effect. This is the first POR to be purified from a hyperthermophilic bacterium. Interestingly, its molecular properties are more similar to those of the POR from a hyperthermophilic archaeon than to those of PORs from mesophilic bacteria. The evolutionary significance of this is discussed.

Hyperthermophiles are a recently discovered group of microorganisms that have the remarkable property of growing at temperatures of 90 °C and above (Stetter, 1986; Kelly & Deming, 1988; Stetter *et al.*, 1990; Adams, 1990, 1992, 1993). They have been found in geothermally heated environments, which include shallow and deep sea hydrothermal vents. Virtually all of them are strictly anaerobic heterotrophs, and most are obligately dependent upon elemental sulfur (S^0) reduction for growth. All but two of the 20 or so hyperthermophilic genera isolated so far fall 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). *Thermotoga maritima* grows by the fermentation of simple and complex carbohydrates. The only identified products of its metabolism are lactate, acetate, CO_2, and H_2 (Huber *et al.*, 1986). This organism is not dependent upon S^0 for growth, but if S^0 is added to the growth medium, it is reduced to H_2S. This appears to be a "H_2 detoxification mechanism", as the H_2 generated during fermentation inhibits cell growth (Janssen & Morgan, 1992).

The discovery of hyperthermophilic organisms has important consequences which span microbial physiology and metabolism, evolution, and biotechnology. For example, they are considered to be the earliest known ancestors of all extant life on this planet, having evolved when the earth was much hotter than it is at present (Woese *et al.*, 1990; Woese, 1987). Clearly, an essential step in elucidating the novel biochemistry required to sustain life at and above 100 °C is the characterization of their primary metabolic pathways and the enzymes involved. With this aim in mind, we have focused on the metabolism of H_2 by *T. maritima* and recently reported on the properties of its H_2-evolving hydrogenase (Juszczak *et al.*, 1991), the enzyme responsible for catalyzing H_2 production. In addition to its high thermostability, the enzyme has several unusual properties compared with its mesophilic relatives. *T. maritima* is thought to couple H_2 production and glucose oxidation via a conventional Embden–Meyerhof–Parnas pathway. Some key enzyme activities have recently been measured (Janssen & Morgan, 1992), but only one enzyme of the pathway, glyceraldehyde-3-phosphate dehydrogenase, has been purified (Wrba *et al.*, 1990).

In fermentative H_2-producing organisms such as *T. maritima*, the terminal oxidative step in the conversion of glucose to acetate and CO_2 is typically catalyzed by pyruvate ferredoxin oxidoreductase (POR[1]). This enzyme generates acetyl-CoA and CO_2 from pyruvate while the electrons are

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[1] Abbreviations: FPLC, fast protein liquid chromatography; DTT, dithiothreitol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); TPP, thiamine pyrophosphate; CoASH, coenzyme A; POR, pyruvate ferredoxin oxidoreductase; SDS, sodium dodecyl sulfate; EPR, electron paramagnetic resonance; EPPS, *N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid; MES, 2-morpholinoethanesulfonic acid; MOPS, 3-morpholinopropanesulfonic acid; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid.

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transferred to ferredoxin for ultimate H_2 production via hydrogenase. POR has been purified from several mesophilic anaerobes (Wahl & Orme-Johnson, 1987; Kerscher & Oesterhelt, 1981; Meinecke *et al.*, 1989) and recently from the hyperthermophilic archaeon, *Pyrococcus furiosus* (Blamey & Adams, 1993), an organism that grows optimally at 100 °C (Fiala & Stetter, 1986). The hyperthermophilic enzyme was found to differ considerably from the mesophilic PORs in its molecular and catalytic properties, differences which have been suggested to have some evolutionary significance (Blamey & Adams, 1993). These differences prompted the current study to characterize the POR from *T. maritima*, which is one of the two known hyperthermophilic bacteria. We show here that the molecular properties of *T. maritima* POR are similar to those of the hyperthermophilic POR from *P. furiosus*. In an accompanying paper (Smith *et al.*, 1994), spectroscopic data are presented to demonstrate that these two hyperthermophilic enzymes have distinctly different mechanisms for catalyzing pyruvate oxidation.

MATERIALS AND METHODS

Methods

Thermotoga maritima (DSM 3109) was grown in a 400-L fermentor using glucose as a carbon source, as previously described (Juszczak *et al.*, 1991). POR activity was routinely determined spectrophotometrically using serum-stoppered cuvettes under argon at 80 °C by the pyruvate-dependent reduction of methyl viologen (Blamey & Adams, 1993). The standard assay mixture contained sodium pyruvate (5 mM), $MgCl_2$ (1 mM), coenzyme A (CoASH, 0.1 mM), and methyl viologen (1 mM) in 50 mM EPPS buffer (pH 8.4). Enzyme activity is expressed as units/milligram of protein, where 1 unit equals the oxidation of 1 μmol of pyruvate/min. Protein concentrations were routinely estimated by the Lowry method using bovine serum albumin as the standard (Lowry *et al.*, 1951). The protein content of samples of pure POR was also determined by the quantitative recovery of amino acids from compositional analyses (see below). The amounts of protein in all samples were 107 ± 7% of those measured by the colorimetric protein assay (from three separate determinations). All analytical values for the pure protein that were based on the Lowry method have therefore been corrected by a factor of 1.07. Molecular weights were estimated by gel filtration on columns (1.6 × 50 cm) of Superdex 200 and Superose 12 (both from Pharmacia LKB), using 50 mM Tris-HCl (pH 8.0) containing 0.2 M NaCl as the equilibration buffer. The standard proteins used (and their molecular weights) were as follows: ferritin (440 000), β-amylase (200 000), alcohol dehydrogenase (150 000), phosphorylase *b* (96 000), and bovine serum albumin (68 000). Electrophoresis in the presence and absence of SDS was carried out using 12% and 20% (w/v) acrylamide (Weber *et al.*, 1972). The standard proteins used (and their molecular weights) were the following: bovine serum albumin (68 000), ovalbumin (45 000), glyceraldehyde-3-phosphate dehydrogenase (36 000), carbonic anhydrase (29 000), trypsinogen (24 000), soybean trypsin inhibitor (20 100), and α-lactalbumin (14 200). All molecular weight standards were obtained from Sigma Chemical Co.

Amino acid analyses were performed on an Applied Biosystems Model 4240A analyzer after the hydrolysis of POR samples under Ar at 165 °C for 1 h in the presence of 6 M HCl, phenol (1%, w/v), and thioglycolic acid (8%, w/v). Serine and threonine were corrected for destruction. The N-terminal sequences of the subunits of *T. maritima* POR and *P. furiosus* POR were determined using an Applied Biosystems Model 477 sequencer. The subunits of the two enzymes were separated by SDS gel electrophoresis and electroblotted onto PVDF protein-sequencing membranes using a Bio-Rad electroblotting system. Electroblotting was carried out in 10 mM CAPS buffer (pH 11.0) containing methanol (10%, v/v) for 1 h at 50 V (Matsudaira, 1987). POR apoprotein was prepared and reduced with DTT using the methods described for *P. furiosus* ferredoxin (Aono *et al.*, 1989). Tryptophan was determined from the A_{280} value of the apoprotein after correction for tyrosine (Edelhoch, 1967). The cysteine content of the reduced apoprotein was estimated by the reaction with 5,5′-dithiobis(2-nitrobenzoic acid) (Riddles *et al.*, 1983). The amount of TPP in pure POR was determined by fluorescence spectroscopy (Penttinen, 1979), and the iron and acid-labile sulfide contents were measured using *o*-phenanthroline (Lovenberg *et al.*, 1963) and by methylene blue formation (Chen & Mortenson, 1977), respectively, using the 8Fe-ferredoxin from *Clostridium pasteurianum* (Mortenson, 1964) as a reference protein. A complete metal analysis (40 elements) was carried out by plasma emission spectroscopy using a Jarrel Ash Plasma Comp 750 instrument at the Department of Ecology of the University of Georgia. EPR spectra were recorded on an IBM-Bruker ER 300D spectrometer interfaced to an ESP 3220 data system and equipped with an Oxford Instruments ITC-4 flow cryostat. Spin quantitations were determined by double integration of the first derivative spectra recorded at 8 K using 20 μW of microwave power (Aasa & Vänngård, 1975). These were compared with spectra of Cu (1 mM) in EDTA (100 mM) recorded under identical conditions. The purification of *T. maritima* ferredoxin will be described elsewhere.[2] *P. furiosus* POR was purified as previously described (Blamey & Adams, 1993).

Purification of Pyruvate Ferredoxin Oxidoreductase

POR was routinely purified from 200 g of cells (wet weight). All procedures were carried out at 23 °C under strictly anaerobic conditions. The buffer used throughout the purification was 50 mM Tris-HCl containing 10% glycerol, 2 mM sodium dithionite, and 2 mM DTT at the indicated pH. The purification procedure was the same as that for *T. maritima* hydrogenase (Juszczak *et al.*, 1991) up to and including the first Q-Sepharose column. The POR activity eluted from this column as 250 mM NaCl at pH 8.2 was applied, just prior to the hydrogenase.

DEAE Sephacel Chromatography. Fractions (100 mL) from the Q-Sepharose column (Pharmacia LKB) with POR activity above 6 units/mg were diluted with 3 vol of buffer (pH 7.6) and applied to a column (2.5 × 40 cm) of DEAE Sephacel (Pharmacia LKB) equilibrated at 3 mL/min with the same buffer. POR activity eluted as 235–290 mM NaCl was applied to the column using sequential gradients of 0–100 mM (80 mL) and 100–500 mM NaCl (1400 mL).

Second Q-Sepharose Chromatography. Fractions (100 mL) from the previous column containing POR activity above 6 units/mg were applied directly to a column (2.5 × 30 cm) of hydroxyapatite (high resolution, Behring Diagnostics) equilibrated with buffer at pH 8.0. The absorbed proteins were eluted at 2.5 mL/min with a gradient (1150 mL) from 0 to 300 mM NaCl in the same buffer. POR activity eluted as 250–285 mM NaCl was applied to the column.

[2] J. M. Blamey and M. W. W. Adams, manuscript in preparation.

Table 1: Purification of *T. maritima* Pyruvate Ferredoxin Oxidoreductase

step	activity (units)	protein (mg)	specific activity (units/mg)	recovery (%)	purification (-fold)
cell-free extract	32000	4900	6.5	100	1.0
Q-Sepharose	10800	970	11.2	33	1.7
DEAE Sephacel	13400	748	17.9	42	2.7
Q-Sepharose	9300	380	24.5	29	3.8
DEAE Fast Flow	7300	273	30.8	23	4.7
Sephacryl S-200	5700	65	87.4	18	13.4

DEAE Fast Flow Chromatography. Fractions (25 mL) from the previous column containing POR activity above 12 units/mg were diluted with 3 vol of buffer (pH 7.8) and applied to a column (2.5 × 40 cm) of DEAE Fast Flow (Pharmacia LKB) equilibrated at 3 mL/min with the same buffer. POR activity eluted as 230–280 mM NaCl was added when a linear gradient of 0–280 mM NaCl (1200 mL) was applied. Fractions (20 mL) with POR activity above 18 units/mg were concentrated to approximately 5 mL by ultrafiltration (Amicon PM-30).

Sephacryl S-200 Chromatography. The concentrated sample was applied to a column (2.5 × 100 cm) of Sephacryl S-200 (Pharmacia LKB) equilibrated with buffer (pH 8.0) containing 100 mM NaCl at 1.5 mL/min. Fractions (10 mL) with POR activity above 30 units/mg were analyzed by nondenaturing and SDS gel electrophoresis. Those judged pure were concentrated by ultrafiltration (Amicon PM-30) to approximately 20 mg/mL and stored as pellets in liquid N_2.

RESULTS

Purification of POR. Cell-free extracts from more than 10 different batches of *T. maritima* cells contained 3.5–6.5 units/mg of POR activity when measured at 80 °C. No activity was detected in the particulate fraction of cell-free extracts of *T. maritima*, indicating that it is a cytoplasmic enzyme. POR was sensitive to inactivation by O_2: less than 50% of the initial activity remained after a cell-free extract prepared anaerobically using either 50 mM Tris (pH 8.0) or 50 mM phosphate (pH 7.0) was incubated for 75 min at 23 °C in air. No POR activity was lost, even after 48 h, when the extract was prepared anaerobically in the same buffers containing sodium dithionite (2 mM), glycerol (10% v/v), and DTT (2 mM) and was maintained under Ar to protect it against traces of O_2 contamination. However, POR appeared to be unstable to some extent even under anaerobic conditions, as in the absence of glycerol and DTT only about 50% of the initial activity remained after a 48-h incubation. The reasons for this are not known. This dithionite/glycerol/DTT buffer system also stabilized the O_2-sensitive hydrogenase of *T. maritima* (Jusczcak *et al.*, 1991), and since both enzymes were routinely purified from the same batch of cells, the same buffer system could be conveniently used for the purification of POR. On the other hand, attempts to purify POR from cell-free extracts by Q-Sepharose or hydroxyapatite chromatography resulted in a recovery of activity of less than 40%, and this was unaffected by the presence or absence of glycerol and DTT. Because subsequent chromatography steps did not result in similar losses of POR activity (see Table 1), it appeared that the initial loss of activity was due to the separation of some activating factor during the first chromatography step rather than to inactivation of POR. The results of a typical purification procedure are summarized in Table 1. Approximately 65 mg of purified POR was obtained from 200 g (wet weight) of cells.

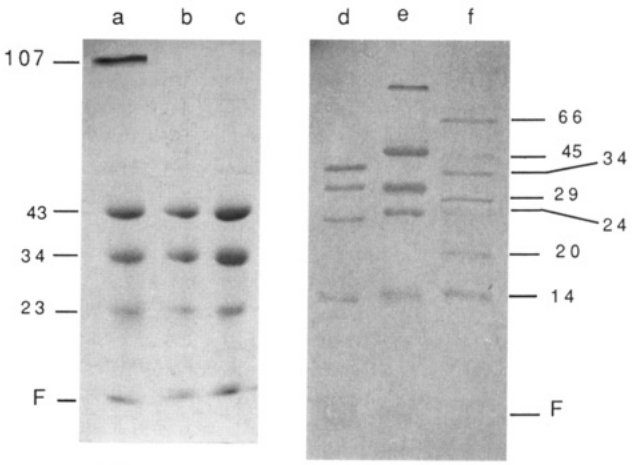

FIGURE 1: SDS–polyacrylamide gel electrophoresis gels of the pyruvate ferredoxin oxidoreductases of *T. maritima* and *P. furiosus*. The pure enzymes (4.0 mg/mL) were incubated for 30 min at the specified temperature with an equal volume of SDS (1%, w/v) and 2-mercaptoethanol (5%, v/v) prior to electrophoresis in the presence of SDS (0.1%, w/v) using 12% (lanes a–c) or 20% (lanes d and e) acrylamide gel. The samples were as follows: (a) *T. maritima* POR, 80 °C; (b) *T. maritima* POR, 90 °C; (c) *T. maritima* POR, 100 °C; (d) *T. maritima* POR, 100 °C; and (e) *P. furiosus* POR, 80 °C. Lane f contained protein markers for the 20% gel with the indicated molecular weights. F indicates the position of the dye fronts.

Molecular Composition and Stability of POR. The purified enzyme gave rise to a single protein band after electrophoresis on nondenaturing gels. Electrophoretic analysis using 12% SDS gels after the sample was treated at 80 °C for 10 min showed one major protein band corresponding to a M_r value of approximately 107 000, together with three minor protein bands corresponding to M_r values of 43 000, 34 000, and 23 000. The intensity of the latter three protein bands increased, while that of the M_r = 107 000 band decreased (but did not disappear) when purified POR was treated for 30 min at 80 or 90 °C (Figure 1, lanes a and b). After treatment at 100 °C for 30 min, the M_r = 107 000 protein band was barely visible (Figure 1, lane c). Therefore, it appeared that the POR holoprotein was a stable trimeric species that was not completely dissociated upon SDS and heat treatment. However, analysis of *T. maritima* POR on 20% SDS gels revealed an additional subunit of M_r 13 000 that was not evident on 12% acrylamide gels (Figure 1, lane d). A reexamination of *P. furiosus* POR, which was reported to contain three subunits (Blamey & Adams, 1993), showed that it behaved similarly. That is, using 12% SDS gels, it also gave rise to a protein band of M_r 107 000, which upon prolonged heating dissociated into three subunits with M_r values of 47 000, 31 000, and 24 000 (Blamey & Adams, 1993). As shown in Figure 1 (lane e), a fourth subunit was observed using 20% SDS gels, and this also corresponded to an M_r value of 13 000. Both the *T. maritima* and *P. furiosus* enzymes eluted from Superose 12 and from Superdex 200 gel filtration columns as single protein peaks with apparent M_r values of 155 000 ± 30 000. Increasing the salt concentration of the equilibration buffer from 0.2 to 1.0 M NaCl had no effect on their elution behavior. Note that the molecular weight previously reported for *P. furiosus* POR was underestimated (Blamey & Adams, 1993). Together with the electrophoresis results, therefore, these data suggest that each POR comprises four nonidentical subunits ($\alpha\beta\gamma\delta$), with the M_r values indicated above, and has a minimum molecular weight of approximately 115 000, although in light of the gel filtration results it cannot be ruled out that the holoenzymes contain other combinations of these four subunits.

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Tm α:  M E R V V E - R V A V T G A E A V A N A M R Q I E P D V V A A Y P I T P Q T P I V E Y F
Pf α:  - - - - - - - - K V M - K G N E A A A W
Hh α:  T D D E L I W R I A G - G S G D G I D S T S Q N F A K A L M R S G L D V F T H R H Y P S

Tm β:  P V N X K Q L A Q D E F D K K E I T Q G H R L X P G X G A P I T V K F V M M I A
Pf β:  A V R K P P I T T R E - - - - Y W A P G H A A X A G - G
Hh β:  S K A F S A I D E R E V D R D A F T P G V E P Q P T W C - P G G G D F G V L K A

Tm γ:  X I R K V M K A N E - - A A A W A A K - L A K P K V I A A F P X X P
Pf γ:  M I E V A F H G R G G Q K A V T A A N I L A E - - - - A A F L G

Tm δ:  S L K S W K E I P I G G V I D K P G T A
Pf δ:  - - - - - A E S P F K A D I E R A Q K E L S
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FIGURE 2: N-terminal amino acid sequences of pyruvate ferredoxin oxidoreductases. The abbreviations are as follows: Tm, *Thermotoga maritima*; Pf, *Pyrococcus furiosus*; Hh, *Halobacterium halobium* [data taken from Plaga *et al.* 1992)]. α, β, γ, and δ represent different subunits. Note that the Hh enzyme has only two subunits (α and β). Gaps have been inserted to maximize homology with the Tm enzyme, and indentical residues are underlined.

Table 2: Amino Acid Compositions of Pyruvate Ferredoxin Oxidoreductases from *T. maritima* (Tm) and *P. furiosus* (Pf)

	residues/mole	
amino acid	Tm	Pf[a]
aspartic acid[b]	85	93
threonine	46	46
serine	42	39
glutamic acid[b]	92	137
proline	56	59
glycine	63	87
alanine	80	100
cysteine	13	8
valine	76	73
methionine	52	26
isoleucine	55	57
leucine	70	85
tyrosine	40	40
histidine	16	19
lysine	103	26
arginine	53	61
tryptophan	9	19
phenylalanine	38	45
no. of residues	993	1020
calcd M_r[c]	113412	114963

[a] Data from Blamey and Adams (1993). [b] Glutamic acid and aspartic acid represent both the free acid and the amide forms. [c] Includes iron and inorganic sulfide.

The N-terminal sequences of the four subunits of *T. maritima* POR are given in Figure 2. For comparison, the same analysis was carried out with the tetrameric POR from *P. furiosus*, and the results are also given in Figure 2. The complete amino acid composition of *T. maritima* POR is given in Table 2. It contained 13.1 ± 1.9 cysteine residues per molecule, as determined by reaction with DTNB. Measurements of the iron content of the enzyme using the colorimetric assay yielded values of 9.9 ± 2.5 mol/113 000 g of protein (values from at least three separate determinations), and similar iron values were obtained from analysis by plasma emission spectroscopy. The results from this technique also showed that other metals, including W, Zn, and Mn and also Se, were present in the sample in only trace amounts (<0.05 mol/mol). The acid-labile sulfide content of POR was 14.6 ± 2.7 mol/mol (from six different preparations of the enzyme). Fluorescence spectroscopy showed that the enzyme also contained 0.5 ± 0.1 TPP/mol (from three determinations of the same enzyme preparation).

The sensitivity of pure POR to inactivation by O_2 was similar to that in the cell-free extracts. For example, less than 50% of the original activity remained when the protein (8 mg/mL in 50 mM Tris-HCl buffer, pH 8.0, containing 10% glycerol, 2 mM sodium dithionite, and 2 mM DTT) was shaken briefly in air to oxidize the dithionite and then left exposed to air at 23 °C for 75 min. No activity was recovered when the same sample was degassed and flushed with Ar and rereduced by the addition of sodium dithionite (to 4 mM). No loss of activity was detected after the purified enzyme had been stored for 1 year in liquid N_2 (if thawed anaerobically). The pure enzyme was also very thermostable. The times required for a 50% loss of activity ($t_{50\%}$) after the enzyme was incubated (8.0 mg/mL in the buffer described above, or in 50 mM EPPS buffer, pH 8.4) anaerobically at 80 and at 90 °C were approximately 15 and 11 h, respectively. These results were obtained by the direct transfer of the enzyme from incubation vials to assay cuvettes, which were themselves maintained at either 80 or 90 °C.

Catalytic Properties of POR. The procedure summarized in Table 1 routinely yielded pure POR with a specific activity of 85 ± 4 units/mg in the standard assay at 80 °C. The activity of the enzyme was obligately dependent upon CoASH: no activity was detected if this cofactor was omitted. A double-reciprocal plot of pyruvate oxidation activity *versus* pyruvate concentration (2.5–40 mM, using 0.5 mM CoA) was linear, and the calculated apparent K_m and V_m values were 14.5 mM and 167 units/mg, respectively. Similarly, from a double-reciprocal plot for CoASH, where the concentration was varied between 0.05 and 0.2 mM (using 5 mM pyruvate), the apparent K_m and V_{max} values were 0.34 mM and 125 units/mg, respectively. A solution of CoASH (1 mM in 50 mM EPPS buffer, pH 8.4) lost approximately 50% of its cofactor activity after a 90-min incubation at 80 °C, so that the thermal instability of CoASH appeared not to be a significant factor in determining POR activity. The presence of TPP (0.05 mM) in the routine assay was found to result in an approximate 3-fold increase in POR activity when measured under standard assay conditions using either cell-free extracts or the pure enzyme. When the concentration of TPP was varied (0.01–0.08 mM using 5 mM pyruvate and 0.2 mM CoASH) in the assay of activity of the pure enzyme, a linear double-reciprocal plot of pyruvate oxidation activity *versus* TPP concentration was obtained. The calculated apparent K_m and V_{max} values were 0.043 mM and 154 units/mg, respectively.

Purified POR was unable to oxidize 2-oxoglutarate, phenyl pyruvate, or indolyl pyruvate, either in the presence or absence of CoASH. That is, there was no reduction of methyl viologen at 80 °C when any of these reagents (5 mM) replaced pyruvate in the standard assay mixture. On the other hand, *T. maritima*

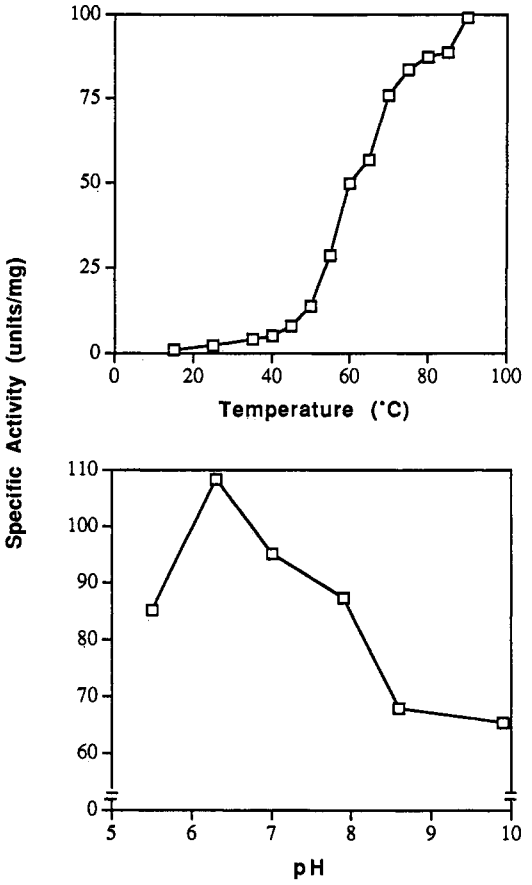

FIGURE 3: Effect of temperature and pH on the catalytic activity of *T. maritima* pyruvate ferredoxin oxidoreductase. The activity of POR was determined as described in the Materials and Methods section, except that the temperature (upper) or pH (lower) was varied as indicated. The buffers used for the pH effects were MES (pH 5.6), MOPS (pH 6.4), phosphate (7.0), EPPS (pH 8.0), glycine (pH 8.6), and CAPS (pH 9.9).

ferredoxin functioned as an efficient electron acceptor for pyruvate oxidation in place of methyl viologen. Using pyruvate (5 mM) and CoASH (0.2 mM), a double-reciprocal plot of pyruvate oxidation activity *versus* ferredoxin concentration was linear over the range 0.06–0.3 mM, and the apparent K_m and V_{max} values were approximately 26.0 μM and 280 μmol of pyruvate oxidized/min/mg, respectively. Figure 3 shows the effect of pH on POR activity at 80 °C under standard assay conditions: there was a substantial decrease below pH 8.0, and maximal activity was observed at pH 6.3. Figure 3 also shows that POR was virtually inactive at ambient temperature with a dramatic increase in activity above 50 °C and an optimum ≥90 °C. The corresponding Arrhenius plot shows a transition point near 60 °C (Figure 4A). The calculated E_{act} values were 11.1 kcal/mol over the range 25–60 °C and 13.5 kcal/mol over the range 60–85 °C. Results from analogous experiments with POR from *P. furiosus* are also shown in Figure 4. In this case, a transition was observed near 70 °C, and the calculated E_{act} values were 19.4 kcal/mol over the range 23–70 °C and 20.8 kcal/mol over the range 70–90 °C.

The inhibition properties of POR were determined both by incubating the enzyme for 5 min at 25 °C with a potential inhibitor and then assaying at 80 °C in its absence and by assaying the untreated enzyme in the presence of the reagent at 80 °C. No loss of activity was observed when CO (1 atm, equivalent to 320 μM), sodium cyanide (20 mM), sodium fluoride (5 mM), or sodium azide (2.5 mM) was used in both assay systems at the indicated concentrations. The enzyme was inhibited by sodium nitrite, albeit at relatively high concentrations, when this was included in the assay mixture. A linear reciprocal plot of the inhibition was obtained using 0–120 mM sodium nitrite in the assay mixture. The apparent K_i was calculated to be 54 mM. *P. furiosus* POR was reported to be inhibited by CO and nitrite (Blamey & Adams, 1993), but other reagents were not tested. *P. furiosus* POR was therefore treated with either sodium cyanide (20 mM), sodium fluoride (5 mM), or sodium azide (2.5 mM) under the conditions described above for *T. maritima* POR. No inhibition of the *P. furiosus* enzyme was observed.

Electron Paramagnetic Resonance Properties. T. maritima POR as purified in the presence of sodium dithionite (2 mM) gave rise to a $g = 1.94$ type EPR spectrum at low temperatures typical of reduced $[4Fe–4S]^+$ clusters (Figure 5). The general line shape of the spectrum remained unchanged both when the temperature was varied between 8 and 30 K (using 20 mW of power) and when the microwave power was varied between 0.02 and 20 mW (at 8 K). The signal was not observed above 30 K, and no additional resonances were observed at

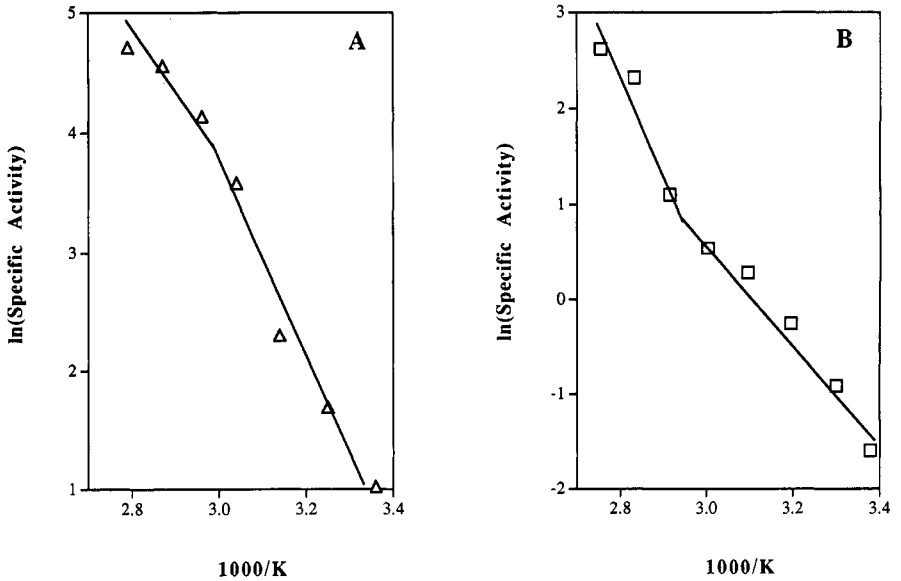

FIGURE 4: Arrhenius plots for the pyruvate ferredoxin oxidoreductases from *T. maritima* (A) and *P. furiosus* (B). The data were taken from Figure 3 and from Blamey and Adams (1993), respectively.

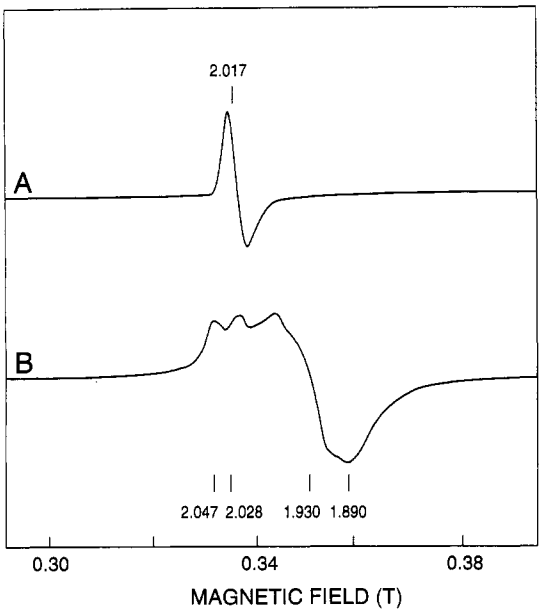

FIGURE 5: EPR spectra of *T. maritima* pyruvate ferredoxin oxidoreductase. The samples contained the enzyme at a concentration of 33.0 mg/mL in 50 mM Tris-HCl (pH 8.0) containing 10% glycerol. They were as follows: (A) oxidized with 2 mM thionine and (B) reduced with 2 mM dithionite and 2 mM DTT. The conditions of measurement were as follows: microwave power, 20 mW; modulation amplitude, 5 G; microwave frequency, 9.45 GHz; gain , 3.2×10^4; temperature, 8 K.

low field (8 K, 20 mW). The complete spectrum represented 1.4 spins/mol. These data suggest that the reduced enzyme contains two nonidentical $[4Fe–4S]^+$ clusters. The EPR spectrum of the enzyme oxidized by thionine (E_m = +60 mV) is also shown in Figure 5. The sample was prepared by removing sodium dithionite (and DTT) followed anaerobically by the addition of excess thionine. The line shape, g values, and temperature dependence of the signal (observable up to 30 K) suggest that it arises from an oxidized $[3Fe–4S]^+$ cluster. As the signal represented less than 0.1 spin/mol, it probably arose by partial oxidative degradation of the 4Fe clusters during sample preparation. However, this is either a minor or a reversible effect, since the reduced enzyme did not lose activity upon thionine oxidation. These results also suggest that the less than integer spin content of the EPR signal from the $[4Fe–4S]^+$ clusters in the dithionite-reduced enzyme is probably due to their incomplete reduction by sodium dithionite, rather than to degradation of the clusters during enzyme purification.

DISCUSSION

The hyperthermophilic bacterium, *T. maritima*, contains significant amounts of POR activity located in its cytoplasm. The specific acitivity of cell-free extracts (3.5–6.5 units/mg at 80 °C) was much higher than that found in mesophilic organisms (typically much less than 1.0 unit/mg at 30 °C; Kerscher & Oesterheldt, 1981; Wahl & Orme-Johnson, 1987; Williams *et al.*, 1987; Brostedt & Nordlund, 1991) and about twice that found with the hyperthermophilic archaeon, *P. furiosus* (1.5–3.0 units/mg at 80 °C; Blamey & Adams, 1993). The ferredoxin from *T. maritima* functioned as an efficient electron carrier for its POR, consistent with it being the physiological electron acceptor for the enzyme. However, *in vitro* studies have shown that this ferredoxin does not appear to be the physiological electron donor to the H_2-evolving hydrogenase of *T. maritima* (Juszczak *et al.*, 1991), suggesting that an intermediate electron carrier is involved in addition to the ferredoxin. *T. maritima* POR appeared to be specific for pyruvate as it did not oxidize 2-oxoglutarate, phenyl pyruvate, or indolyl pyruvate, compounds that might be generated by transamination reactions of amino acids.

We show here that *T. maritima* POR has an M_r value of approximately 113 000 and comprises four dissimilar subunits. As shown in Table 3, this in stark contrast to the PORs that have been well-characterized from mesophilic organisms, which include several anaerobic bacteria (Uyeda & Rabinowitz, 1971; Wahl & Orme-Johnson, 1987; Meinecke *et al.*, 1989), a phototroph (Brostedt & Nordlund, 1991), and an anaerobic protozoan (Williams *et al.*, 1987). These enzymes have M_r values of approximately 240 000 and contain two identical subunits. POR from the aerobic archaeon, *H. halobium* (Kerscher & Oesterhelt, 1981), has a similarly high molecular weight, but it is a tetramer of two different types of subunit. On the other hand, like the *T. maritima* enzyme, we show here that the POR from the hyperthermophilic archaeon, *P. furiosus*, is also a heterotetrameric protein with an M_r value of approximately 115 000 (Blamey & Adams, 1993). Thus, the PORs from the two hyperthermophiles appear to be about one-half the size of the corresponding mesophilic enzymes and equivalent to their $M_r \sim 120\,000$ subunit. In addition to molecular weight, the compositions of the two hyperthermophilic enzymes are virtually identical

Table 3: Properties of Pyruvate Ferredoxin Oxidoreductases

source	molecular weight	subunits (kDa)	Fe/mol	pyruvate K_m (mM)	CoASH K_m (μM)	specific activity[a]	reference
Thermotoga maritima	113000	1 × 43 1 × 34 1 × 23 1 × 13	10	14.5	340	87 (80 °C)	this work
Pyrococcus furiosus	115000	1 × 47 1 × 31 1 × 24 1 × 13	8	0.46	110	22 (80 °C)	this work Blamey & Adams, 1993
Klebsiella pneumoniae	240000	2 × 120	16	2.0	4.0	6.0	Wahl & Orme-Johnson, 1987
Clostridium thermoaceticum	240000	2 × 120	16	2.0	4.0	6.0	Drake *et al.*, 1981 Wahl & Orme-Johnson, 1987
Clostridium acidiurici	240000	2 × 120	6	2.2	nd[b]	9.0	Uyeda & Rabinowitz, 1971
Clostridium acetobutylicum	246000	2 × 123	8	0.32	3.7	25	Meinecke *et al.*, 1989
Rhodospirillum rubrum	252000	2 × 114	13	0.18	9.0	15	Brostedt & Nordlund, 1991
Trichomonas vaginalis	240000	2 × 123	7	0.14	2.5	18	Williams *et al.*, 1987
Halobacterium halobium	256000	2 × 86 2 × 42	8	0.10	nd	0.36	Kerscher & Oesterhelt, 1981

[a] Expressed as micromoles of pyruvate oxidized/minute/milligram at 25–30 °C unless indicated otherwise. [b] nd, not determined.

for most of the amino acids, the main exceptions being higher amounts of Met and Lys in the *T. maritima* enzyme (Table 2). It is not known whether these residues are related to differences in the thermal stability of the two enzymes (see below).

T. maritima POR was found to contain two [4Fe–4S] clusters per molecule, which is similar to the number found with *P. furiosus* POR (Blamey & Adams, 1993) and equivalent to the Fe content of the PORs from the bacteria *C. thermoaceticum* and *K. pneunomiae* (Wahl & Orme-Johnson, 1987). As indicated in Table 3, this suggests that some of the other PORs purified so far may have lost some Fe during purification. All of these enzymes are O_2-sensitive and most also appear to lose the prosthetic group, TPP, during purification, whereupon some have to be purified in buffers containing this cofactor, for example, see Wahl and Orme-Johnson (1987) and Brostedt and Nordlund (1991). Indeed, all of the purified PORs contain less than stoichiometric amounts of TPP, and all of the bacterial enzymes, including that of *T. maritima*, are activated by its addition. Curiously, only the PORs from the archaea (*H. halobium* and *P. furiosus*) and the eukaryote (*T. vaginalis*) do not recover any activity upon TPP addition. Substantial amounts of POR activity were lost when cytoplasmic extracts of *T. maritima* were initially separated by column chromatography, suggesting that some activating moiety is removed from the enzyme. This factor appeared not to be TPP, as the enzyme in cell-free extracts and in the purified state was stimulated to the same extent by the addition of TPP to the assay medium. A similar loss occurred during the first chromatographic separation (but not in subsequent ones) used in the purification of *P. furiosus* POR (Blamey & Adams, 1993).

T. maritima POR is by far the most thermostable enzyme of this type reported so far as, surprisingly, it was more resistant to heat denaturation ($t_{50\%}$ = 11 h at 90 °C) than the POR from the hyperthermophile, *P. furiosus* ($t_{50\%}$ = 18 min at 90 °C), although both enzymes have a temperature optimum for catalysis above 90 °C. In addition to their molecular compositions and stability, these two hyperthermophilic enzymes have in common very low apparent affinities for CoASH (at 80 °C), which are at least an order of magnitude lower than those of the mesophilic enzymes (Table 3). This is in spite of the fact that CoASH is stable at 80 °C, at least over the time period of the assay. Indeed, *T. maritima* POR (but not the *P. furiosus* enzyme) also shows a very low affinity for pyruvate (Table 3). In addition, the specific pyruvate-oxidizing activity of *T. maritima* POR (at 80 °C) is significantly higher than that of the other PORs (at 30 °C), including the *P. furiosus* enzyme (at 80 °C). This difference might be reflected in the activation energy for pyruvate oxidation. The values for the *T. maritima* enzyme (11.1–13.5 kcal/mol) were about one-half of those determined for *P. furiosus* POR (19.4–20.8 kcal/mol) over the temperature range 23–90 °C. Notably, both hyperthermophilic PORs exhibit transition points close to the minimum growth temperatures of the two organisms from which they were obtained.

On the other hand, there are some distinct differences between the properties of the two hyperthermophilic PORs from *T. maritima* and *P. furiosus*. They show different sensitivities to inhibitors such as nitrite and CO. In particular, CO is a potent inhibitor of the *P. furiosus* enzyme (K_i = 7.2 μM), yet even at saturating concentrations (equivalent to 320 μM) CO had no effect on *T. maritima* POR (the response of mesophilic PORs to CO has not been reported). This suggests mechanistic differences between these two hyperthermophilic enzymes, a difference that is also reflected in their EPR properties. For example, this technique indicated the presence of an organic radical species in *P. furiosus* POR (Blamey & Adams, 1993), but this was not evident with *T. maritima* POR. Moreover, this appears to reflect a general difference between the archaeal and bacterial enzymes, as a radical-type EPR signal was also reported with *H. halobium* POR but not with the PORs from *C. thermoaceticum* or *K. pneumoniae* (Wahl & Orme-Johnson, 1987). Additional evidence for distinct bacterial and archaeal mechanisms for pyruvate oxidation using the hyperthermophilic PORs is presented in the accompanying paper (Smith *et al.*, 1994).

We have previously suggested that, because hyperthermophiles such as *T. maritima* and *P. furiosus* are considered to be some of the most ancient of known organisms (Woese *et al.*, 1990), their PORs may represent some ancestral form of a pyruvate-oxidizing enzyme (Blamey & Adams, 1993). Thus, the four different subunits that these PORs uniquely contain may represent separate catalytic domains: one containing TPP for pyruvate decarboxylation, one or perhaps two for acetyl transfer and binding CoASH, and one containing one or both [4Fe–4S] clusters for interaction with, and for electron transfer to, ferredoxin, the external electron acceptor. These subunit-specific domains may then have evolved into the single subunit of the homodimeric mesophilic PORs (Table 3) and eventually into the E1, E2, and E3 enzymes of the pyruvate dehydrogenase multienzyme complex (Guest *et al.*, 1981), with essential retention of fundamental catalytic function. As shown in Figure 2, there is some homology between the N-terminal sequences of the four subunits of *T. maritima* POR and those of *P. furiosus*, and to some extent with those of *H. halobium* (Plaga *et al.*, 1992), the only ones for which some sequence information is available. Complete sequences of the hyperthermophilic enzymes are obviously required to investigate the proposed evolutionary relationships, and studies to obtain them are currently underway.

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REFERENCES

Aasa, R., & Vänngård, T. (1975) *J. Magn. Reson. 19*, 308–315.
Adams, M. W. W. (1990) *FEMS Microbiol. Rev. 75*, 219–238.
Adams, M. W. W. (1992) *Adv. Inorg. Chem. 38*, 341–396.
Adams, M. W. W. (1993) *Annu. Rev. Microbiol. 47*, 627–658.
Blamey, J. M., & Adams, M. W. W. (1993) *Biochim. Biophys. Acta 1161*, 19–27.
Brostedt, E., & Nordlund, S. (1991) *Biochem. J. 279*, 155–158.
Chen, J.-S., & Mortenson, L. E. (1977) *Anal. Biochem. 79*, 157–165.
Drake, H. L., Shou-Ih, H., & Wood, H. G. (1981) *J. Biol. Chem. 256*, 11137–11144.
Edelhoch, H. (1967) *Biochemistry 6*, 1948–1954.
Fiala, G., & Stetter, K. O. (1986) *Arch. Microbiol. 145*, 56–60.
Guest, J. R., Cole, S. T., & Jeyaseelan, K. (1981) *J. Gen. Microbiol. 127*, 65–79.
Huber, R., Langworthy, T. A., Koning, H., Thomm, M., Woese, C. R., Sleytr, U. B., & Stetter, K. O. (1986) *Arch. Microbiol. 144*, 324–333.
Huber, R., Wilharm, T., Huber, D., Trincone, A., Burggraf, S., König, H., Rachel, R., Rockinger, I., Fricke, H., & Stetter, K. O. (1992) *Arch. Microbiol. 15*, 340–351.
Janssen, P. H., & Morgan, H. W. (1992) *FEMS Microbiol. Lett. 96*, 213–218.

Juszczak, A., Aono, S., & Adams, M. W. W. (1991) *J. Biol. Chem. 266*, 13844–13841.
Kelly, R. M., & Deming, J. W. (1988) *Biotechnol. Prog. 4*, 47–62.
Kerscher, L., & Oesterhelt, D. (1981) *Eur. J. Biochem. 116*, 587–594.
Lovenberg, W., Buchanan, B. B., & Rabinowitz, J. C. (1963) *J. Biol. Chem. 238*, 3899–3913.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Matsudaira, P. (1987) *J. Biol. Chem. 262*, 10035–10038.
Meinecke, B., Bertram, J., & Gottschalk, G. (1989) *Arch. Microbiol. 152*, 244–250.
Mortenson, L. E. (1964) *Biochim. Biophys. Acta 81*, 71–76.
Penttinen, H. K. (1979) *Methods Enzymol. 62*, 58–69.
Plaga, W., Lottspeich, F., & Oesterhelt, D. (1992) *Eur. J. Biochem. 205*, 391–397.
Riddles, P. W., Blackeley, R. L., & Zerner, B. (1983) *Methods Enzymol. 91*, 49–60.
Shah, V. K., Stacey, G., & Brill, W. J. (1983) *J. Biol. Chem. 258*, 12064–12068.
Smith, E. T., Blamey, J. M., & Adams, M. W. W. (1994) *Biochemistry 33*, following article in this issue.
Stetter, K. O. (1986) in *The Thermophiles: General, Molecular and Applied Microbiology* (Brock, T. D., Ed.) pp 39–74, John Wiley, New York.
Stetter, K. O., Fiala, G., Huber, G., Huber, R., & Segerer, G. (1990) *FEMS Microbiol. Rev. 75*, 117–124.
Uyeda, K., & Rabinowitz, J. C. (1971) *J. Biol. Chem. 246*, 3111–3119.
Wahl, R. C., & Orme-Johnson, W. H. (1987) *J. Biol. Chem. 262*, 10489–10496.
Weber, K., Pringle, J. R., & Osborn, M (1972) *Methods Enzymol. 26*, 2–27.
Williams, K., Lowe, P. N., & Leadlay, P. F. (1987) *Biochem. J. 246*, 529–536.
Woese, C. R. (1987) *Microbiol. Rev. 51*, 221–271.
Woese, C. R., Kandler, O., & Wheelis, M. L. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 4576–4579.
Wrba, A., Schweiger, A., Schultes, V., Jaenicke, R., & Zavodszky, P. (1990) *Biochemistry 29*, 7584–7592.