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Molecular and biochemical characterization of bifunctional pyruvate decarboxylases and pyruvate ferredoxin oxidoreductases from *Thermotoga maritima* and *Thermotoga hypogea*

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Hyperthermophilic bacteria *Thermotoga maritima* and *Thermotoga hypogea* produce ethanol as a metabolic end product, which is resulted from acetaldehyde reduction catalysed by an alcohol dehydrogenase (ADH). However, the enzyme that is involved in the production of acetaldehyde from pyruvate is not well characterized. An oxygen sensitive and coenzyme A-dependent pyruvate decarboxylase (PDC) activity was found to be present in cell free extracts of *T. maritima* and *T. hypogea*. Both enzymes were purified and found to have pyruvate ferredoxin oxidoreductase (POR) activity, indicating their bifunctionality. Both PDC and POR activities from each of the purified enzymes were characterized in regards to their optimal assay conditions including pH dependency, oxygen sensitivity, thermal stability, temperature dependency and kinetic parameters. The close relatedness of the PORs that was shown by sequence analysis could be an indication of the presence of such bifunctionality in other hyperthermophilic bacteria. This is the first report of a bifunctional PDC/POR enzyme in hyperthermophilic bacteria. The PDC and the previously reported ADHs are most likely the key enzymes catalysing the production of ethanol from pyruvate in bacterial hyperthermophiles.

Keywords: hyperthermophiles/pyruvate decarboxylase/pyruvate ferredoxin oxidoreductase/*Thermotoga hypogea*/*Thermotoga maritime*.

Abbreviations: AcDH, acetaldehyde dehydrogenase; ADH, alcohol dehydrogenase; CFEs, cell-free extracts; CoA, coenzyme A; DCM, dichloromethane; DNPH, dinitrophenylhydrazine DTT, dithiotheritol; PDC, pyruvate decarboxylase; POR, pyruvate ferredoxin oxidoreductase; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; SDT, sodium dithionite; TPP, thiamine pyrophosphate.

The genus *Thermotoga* is comprised of a group of Gram-negative heterotrophic bacteria belonging to the order *Thermotogales*. Members of *Thermotogales* are generally characterized by the presence of an outer sheath-like structure surrounding the cells called 'toga' (*1*). Many of them are hyperthermophiles that can grow optimally at $\geq$80°C (*2*) or capable of growing at 90°C (*3*). They use the conventional Embden-Meyerhof and Entner-Doudoroff pathways to produce pyruvate as a metabolic intermediate and acetate, lactate, alanine, CO_2 and H_2 as major metabolic end products (*4*, *5*). It has been shown that some heterotrophic extreme thermophiles and hyperthermophiles including some members of the order *Thermotogales*, namely *Thermotoga hypogea* (*6*), *Thermotoga lettingae* (*7*), *Thermotoga neapolitana* (*8*), *Kosmotoga olearia* (*9*) and *Thermosipho affectus* (*10*) are able to produce ethanol. However, the pathways and enzymes responsible are not fully understood. Several NADPH-dependent alcohol dehydrogenases (ADH, EC 1.1.1.1) have been purified and characterized from different hyperthermophiles including *T. hypogea* with kinetics data suggesting that they catalyse aldehyde reduction and alcohol production *in vivo* (*11–14*).

Acetaldehyde production is commonly known to be catalysed by either pyruvate decarboxylase (PDC, EC 4.1.1.1) which converts pyruvate directly to acetaldehyde via a non-oxidative decarboxylation reaction or CoA-dependent acetaldehyde dehydrogenase (AcDH, EC 1.2.1.10) that reduces acetyl-CoA to acetaldehyde (*15*). The presence of either PDC or AcDH is essential for any biological ethanol production as ADH reduces the aldehydes to the corresponding alcohols. No PDC or AcDH has been detected from any hyperthermophilic bacterium (*15*). In accordance with the missing activities, no *pdc* or *acdh* gene homologue could be found in the fully sequenced genomes of any hyperthermophilic organism (*15*, *16*). The question rises about how acetaldehyde is produced in these organisms.

There are reports of a bifunctional PDC/pyruvate ferredoxin oxidoreductase (POR) of hyperthermophilic archaea *Pyrococcus furiosus* (*17*) and *Thermococcus guaymasensis* (*16*). POR (EC 1.2.7.1) is a metalloenzyme that catalyses the ferredoxin-dependent oxidative decarboxylation of pyruvate to acetyl-CoA in a TPP- and CoA-dependent manner. The electrons released during the reaction are transferred to low potential single electron carrier, ferredoxin or flavodoxin (*18–20*).

POR has been isolated and characterized from the hyperthermophilic fermentative archaeon *P. furiosus* (21), the sulphate-reducing hyperthermophilic archaeon *Archaeoglobus fulgidus* (22), the hyperthermophilic bacterium *Thermotoga maritima* (23), the extremely thermophilic bacterium *Hydrogenobacter thermophilus* (24) and more recently from the hyperthermophilic archaeon *Thermoc. guaymasensis* (16). The PDC/POR bifunctionality has only been reported in enzymes from archaeal hyperthermophiles (16, 17). It was unclear if such bifunctionality was only a property of archaeal enzymes or if it would be also present in bacterial hyperthermophiles. In this study, the bifunctional PDC/PORs were purified from two hyperthermophilic members of the genus *Thermotoga*, *T. maritima* and *T. hypogea*.

Materials and Methods

Reagents and chemicals

Desulfo-CoA was prepared by mixing 100 mg CoA with 1 g of activated Raney nickel catalyst in 5 ml H_2O and stirred at room temperature (25). The reaction was run to completion, determined spectrophotometrically by analysing 10 µl aliquots with 5,5′-thiobis(2-nitrobenzoic acid) (26). Upon completion, the liquid phase was filtered through a 0.2 µm syringe filter and loaded onto a mono Q 5/50 GL (5 × 50 mm) column. The desulfo-CoA eluded by the addition of LiCl using a linear gradient (0–0.2 M LiCl) of solution A (3 mM HCl) and B (3 mM HCl and 0.2 M LiCl). Fractions containing desulfo-CoA were pooled and dialysed overnight in H_2O at 4°C (Float-A-Lyzer G2, MWCO 100–500 Da). The final product was freeze-dried and characterized by mass spectroscopy (ESI-MS) and NMR (JEOL 300 MHz FT-NMR).

Microorganisms and growth conditions

Thermotoga maritima (DSMZ 3109) and *T. hypogea* (DSM 11164) were obtained from DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany) and grown routinely under anaerobic conditions in 20-l glass carboys. The small-scale starter cultures were grown in the media supplemented with vitamin solutions, but no vitamin mixture was used for large-scale (15 l) growth. *Thermotoga maritima* was grown anaerobically on glucose and yeast extract at 80°C as described by Huber *et al.* (27) with modifications as described previously (28). *Thermotoga hypogea* was grown under anaerobic conditions at 70°C as described by Fardeau *et al.* (6) with modifications as described elsewhere (29). The growth was monitored by direct cell enumeration using Petroff-Hausser counting chamber (1/400 mm^2, 0.02 mm deep; Hausser Scientific, Horsham, PA) and a Nikon Eclipse E600 phase-contrast light microscope (Nikon Canada, ON, Canada). When the growth was at the late log-phase, the cultures were cooled down in ice slurry and centrifuged at $13,000 \times g$ using a Sharples continuous centrifugation system (Sharples equipment division, PA) at 150–200 ml/min. The cell pellets were snap-frozen in liquid nitrogen and stored at −80°C until use.

Preparation of cell-free extracts

To purify the bifunctional PDC/POR from *T. maritima* and *T. hypogea*, the cell-free extracts (CFEs) were prepared from the cell pellets. All operations were performed under anaerobic conditions and on ice unless otherwise specified. The cell pellets (50 and 60 g wet weight of *T. hypogea* and *T. maritima*, respectively) were re-suspended in the anaerobic lysis buffer [50 mM Tris-HCl, 5% glycerol, 2 mM dithiotheritol (DTT) and 2 mM sodium dithionite (SDT), 0.1 mg/ml lysozyme, and 0.01 mg/ml DNase I, pH 7.8] in a degassed flask. The ratio of the cell pellets to the lysis buffer was 1:5 (w/v) for *T. hypogea* and 1:4 (w/v) for *T. maritima*. The cell suspensions were incubated with stirring at 37°C for 2 h and subsequently were centrifuged at $10,000 \times g$ for 30 min at 4°C. The supernatants, designated as CFEs, were transferred to anaerobic serum bottles using a syringe pre-rinsed with anaerobic buffer A (50 mM Tris-HCl, 5% glycerol, 2 mM DTT and 2 mM SDT, pH 7.8).

Enzyme assays

The PDC activity was determined by measuring the rate of acetaldehyde production. In principle, the acetaldehyde produced during the enzymatic reaction is derivatized with an acidic solution of 2, 4-dinitrophenylhydrazine (DNPH). The formation of the corresponding hydrazone derivative produces a yellow-reddish coloured complex which is then quantified by reverse-phase high performance liquid chromatography (RP-HPLC). Enzymatic reactions were carried out under anaerobic conditions and at 80°C unless otherwise specified. The standard assay mixture (1 ml final volume) containing N-(2-Hydroxyethyl)-piperazine-N′-3-propanesulfonic acid (EPPS) buffer (100 mM, pH 8.4), 1 mM $MgCl_2$, 0.1 mM TPP, 10 mM sodium pyruvate and 1 mM CoA was pre-warmed by incubation at 80°C water bath for 4 min. The reaction was started by adding the enzyme. The reaction was stopped after 20 min by transferring the assay vials on the ice and adding 2 ml of freshly prepared saturated DNPH solution in 2 N HCl (stirred at room temperature and dark for 1 h). The vials were then incubated overnight at room temperature with shaking (150–200 rpm). The resulting hydrazone (acetaldehyde-DNPH) derivative was then extracted twice each with 1 ml of dichloromethane (DCM) by vigorous shaking at room temperature for 15 min. The organic (lower) phase was transferred to a new clean vial covered with a piece of Parafilm M membrane with few holes. The assay vials were placed in a vacuum desiccator to evaporate the DCM and the resulting yellowish-red powder was dissolved in 4 ml of pure (HPLC grade) acetonitrile by incubation at 4°C overnight.

An aliquot of the assay product was filtered through a 0.2 µm nylon syringe filter (National Scientific, Rockwood, TN), and analysed using a Perkin-Elmer LC Series4 HPLC system (Norwalk, CT) fitted with a reversed-phase Allure C18 column (150 × 4.6 mm, 5 µm, 60 Å). Isocratic elution conditions with a mobile phase of acetonitrile/water (80:20 v/v) were used at a flow rate of 1 ml/min. A micrometrics model 788 dual variable wavelength detector (Norcross, GA) was used and operated at 365 nm. The sample was applied using a Rheodyne Model 7125 injection valve (Rheodyne Inc., CA) with a 20 µl sample loop. The HPLC system was operated at room temperature. The final concentration of acetaldehyde and isobutyraldehyde were determined using a calibration curve prepared by linear regression plotting of known concentrations of each product which was processed under the same assay conditions.

POR activity was measured using the pyruvate-dependent reduction of methyl viologen as described previously (21). The assays were carried out at 80°C in an assay mixture (2 ml) containing 100 mM EPPS, pH 8.4, 1 mM $MgCl_2$, 5 mM sodium pyruvate, 0.4 mM TPP, 100 µM CoA, 1 mM of methyl viologen and enzyme in stoppered optical glass cuvettes with 1 cm light path (Starna cells, Inc., Atascadero, CA).

The anaerobic assay buffer was transferred to a pre-degassed cuvette and then was incubated in a water-jacketed cuvette holder on a Genesys 10 UV-Vis spectrophotometer (Thermo Scientific, MA) for 4 min. After pre-warming the cuvette was taken out and the assay components were added using a pre-rinsed Hamilton gas-tight syringe (Hamilton Company, Reno, NV) in a rapid succession, and the SDT was added at a very small amount until a light blue colour appeared in the cuvette. Subsequently, the cuvette was placed back in the holder for another 30 s. The reaction was started by adding the enzyme and the absorbance change at 578 nm was monitored. The extinction coefficient of methyl viologen is $\varepsilon_{578} = 9.8\ mM^{-1}cm^{-1}$ (24). The activity was determined based on the linear part of the enzymatic reaction progress curve and one unit of enzyme activity was defined as the oxidation of 1 µmol of the substrate or the reduction of 2 µmol MV per minute. Tests were done to establish the linear correlation between the activity and the amount of protein in the assay mixture. To determine the ability of the enzymes to catalyse the oxidative and non-oxidative decarboxylation of 2-ketoisovalerate, it replaced pyruvate in the PDC and POR assays (21, 30) for measuring the activities of 2-ketoisovalerate decarboxylase (KDC) and 2-ketoisovalerate ferredoxin oxidoreductase (VOR), respectively.

Enzyme purification

Protein purification was carried out using multistep chromatography at ambient temperature and under anaerobic conditions. The columns were run using a fast performance liquid chromatography (FPLC) system. The purification steps were monitored by measuring the POR activity, as the assay is simpler and faster. The purity of the fractions at each step was analysed using sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). In the case of *T. maritima* the co-elution of both PDC and POR activities were followed throughout FPLC purification steps by assaying both POR and PDC activities.

The CFEs were diluted two times before loading on a diethylaminoethyl (DEAE)-Sepharose column (5.0 × 10 cm) pre-equilibrated with anaerobic buffer A. The column was washed with 300 ml buffer A and then a gradient (0–0.5 M NaCl) of buffer B [50 mM Tris-HCl, 5% glycerol, 1 M sodium chloride, 2 mM DTT and 2 mM SDT, pH 7.8] was applied to the column at a flow rate of 3.0 ml/min. The fractions containing enzyme activities (170–220 mM NaCl for *T. hypogea* PDC/POR and 180–240 mM NaCl for *T. maritima* PDC/POR) were pooled and loaded onto a hydroxyapatite column (HAP, 2.6 × 15 cm) equilibrated with buffer A. The column was washed with 100 ml of anaerobic buffer A and then eluted with a gradient (0–0.5 M potassium phosphate) of buffer C [50 mM Tris-HCl, 5% glycerol, 0.32 M K_2HPO_4, 0.18 M KH_2PO_4, 2 mM DTT and 2 mM SDT pH 7.8] at a flow rate of 2.0 ml/min. The fractions containing enzyme activities (125–150 mM and 115–135 mM phosphate for *T. hypogea* and *T. maritima*, respectively) were pooled and loaded onto a Phenyl Sepharose column (PS, 2.6 × 10 cm) pre-equilibrated with 0.8 M ammonium sulphate in buffer A. The column was washed with 50 ml of 0.8 M ammonium sulphate in buffer A, which was prepared using buffer A and buffer D [50 mM Tris-HCl, 5% glycerol, 2 M ammonium sulphate, 2 mM DTT and 2 mM SDT pH 7.8], and followed by a linear (decreasing) gradient (0.82–0 M ammonium sulphate) using both buffer A and buffer D at a flow rate of 2.0 ml/min. The active enzymes from both *T. hypogea* and *T. maritima* were eluted from the column when 0.3–0.1 M ammonium sulphate was applied. The purified enzymes (as judged by SDS–PAGE) were desalted and concentrated using an ultrafiltration device (Advantech MFS, Inc., CA) with a polyethersulfone membrane having a nominal molecular weight limit of 50,000 (Millipore, MA) under anaerobic conditions and pressure of nitrogen (~30 psi). The concentrated purified enzyme was subsequently stored in liquid nitrogen until use.

Biochemical and biophysical characterization

The optimal pH values were determined for the purified PDCs/PORs from both *T. maritima* and *T. hypogea*, respectively. The activities were assayed at different pH values ranging from 6.0 to 11 using different buffers at a concentration of 100 mM. The pH values expressed here were all measured at room temperature. The sodium phosphate buffer ($\Delta pK_a/^{\circ}C = -0.0028$) was used for pH values of 6.0, 7.0 and 7.5. HEPES buffer ($\Delta pK_a/^{\circ}C = -0.015$) covered pH values of 7.5, 8.0, 8.5 and 9.0 and glycine ($\Delta pK_a/^{\circ}C = -0.0025$) was used for the pH values of 9.0, 9.5, 10.0 and 10.5. For the pH values between 10 and 11.0, CAPS buffer ($\Delta pK_a/^{\circ}C = -0.009$) was used.

Kinetic characterizations of the purified enzymes were carried out using standard assay procedures for each activity (PDC and POR) at optimal pH and 80°C. The kinetic parameters were determined for pyruvate (0–15 mM) and CoA (0–2 mM) by applying various concentrations of each component and other assay components at saturation. In the case of ThPOR the apparent kinetic parameters were also determined for the artificial electron acceptor, methyl viologen (0.04–1.5 mM). The apparent kinetic parameters were calculated from the best fit of data to the Michaelis–Menten equation by non-linear regression using the SigmaPlot software (SYSTAT Software Inc., CA).

The oxygen sensitivity of PDC and POR activities were determined by exposing aliquots of each purified enzyme to ambient atmosphere at 4°C with gentle stirring. The sample preparations for determining oxygen sensitivity contained no extra SDT and DTT, other than the initial 2 mM SDT and DTT supplemented into the buffer used for enzyme dilution. This resulted in final concentrations of SDT and DTT to be ~0.5–1.0 mM. The enzyme activities were determined at different time intervals and compared with the control sample that was kept at 4°C and under anaerobic conditions.

The temperature dependencies of both PDC and POR activities were determined by assaying the enzyme activities at temperature ranges from 30°C to 95°C. The assay mixture was incubated at each temperature for four minutes before starting the reaction. To determine the half-life of the activities at high temperatures, the enzyme was incubated at the selected temperatures and the residual activities at different time points were determined and compared with unheated control stored at 4°C.

Sequencing of POR genes of T. hypogea

Full-genome sequence of *T. hypogea* was not available at the time of the study. The genes encoding the POR and parts of the neighbouring genes were sequenced using a primer walking and inverse PCR strategies. The primers were designed based on highly conserved regions within *por* operon of the *Thermotoga* species which were available at the time of the study, including *T. maritima*, *Thermotoga petrophila* and *T. lettingae*. The overlapping primers were designed within the confirmed sequenced fragment to be used for amplification and sequencing of the neighbouring sequence. The overlapping sequenced segments were subsequently assembled and the resulting nucleotide sequence was deposited in the EMBL/GenBank/DDBJ nucleotide sequence databases under the Accession No. KC686826.

Genomes in the NCBI microbial genome database (http://www.ncbi.nlm.nih.gov/genomes/MICROBES/microbial_taxtree.html) were used to retrieve the amino acid and nucleotide sequences of PORs from various hyper/thermophiles. The deduced amino acid sequences were compared to protein sequences retrieved from NCBI, Swiss Prot and EMBL. The search in these databases was carried out using the programs FASTA, BLAST and PROSITE. ClustalW version 2.0 was used for DNA and/or protein sequence alignments and comparisons.

Results and Discussion

Unlike the ADHs that are widely distributed (*31*, *32*), only a few PDCs are found to be present in prokaryotes. The commonly known PDCs (*i.e.* homolog of *Zymomonas mobilis* and *Saccharomyces cerevisiae* PDCs) are rare in bacteria and completely absent from hyper/thermophiles (*15*, *16*). The AcDHs which are present in mesophilic and some thermophilic bacteria like *Thermoanaerobacter* (*33–35*) and *Geobacillus* species (*36*), are not present in any hyperthermophilic bacterium or archaeon (*15*). PDC activities of 80 and 120 mU/mg were determined in CFEs of *T. hypogea* and *T. maritima*, respectively. During the purification of the corresponding enzymes, *T. maritima* PDC and POR activities were co-eluted throughout the purification and the PDC to POR ratios were constant after each chromatography step (Supplementary Fig. S1).

The purification of PDC/POR of *T. hypogea* and *T. maritima* was achieved using three chromatography steps (Supplementary Tables SI and SII), and their purity was confirmed using SDS–PAGE where four bands were revealed (Fig. 1A and B). The estimated apparent molecular masses of the subunits from *T. hypogea* were 45, 35, 22 and 14 kDa. The subunits of the enzyme from *T. maritima* showed molecular masses of 46, 35, 23 and 13 kDa which are similar to the POR that has previously been characterized and reported (*23*). This further confirms that the TmPDC/POR characterized here is indeed the same POR characterized by Blamey and Adams (*23*). Each purified enzyme eluted as a single peak from the gel filtration column verifying the purity of the enzymes. The apparent native molecular weights of the bifunctional PDC/POR were estimated to be 157,000 ± 6000 Da for *T. hypogea* and 156,000 ± 4500 Da for *T. maritima*, suggesting that both enzymes are heterotetramers (αβγδ). The purified enzymes from both microorganisms exhibited a brownish colour,

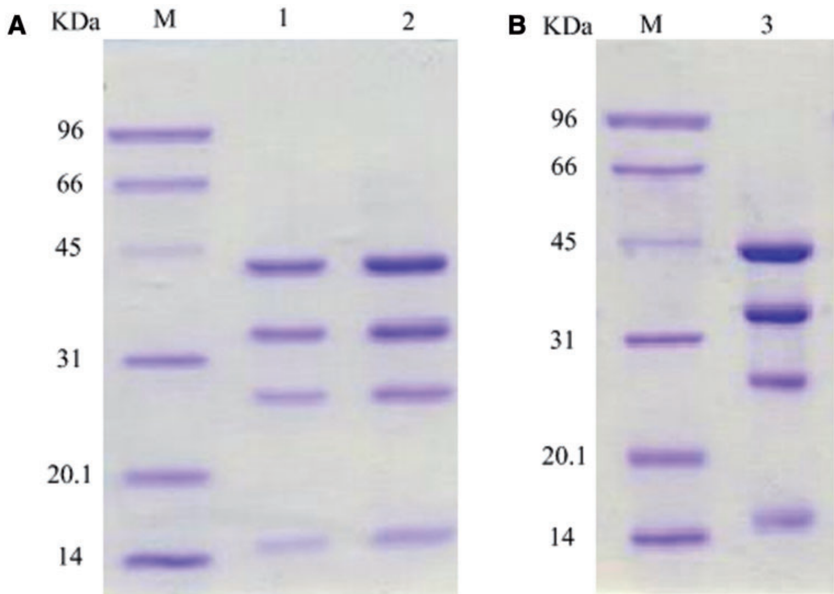

Fig. 1 Analysis of purified PDC/POR from *T. maritima* (**A**) and *T. hypogea* (**B**) using SDS–PAGE (12.5%). Lane 1, 2 μg of purified TmPDC/POR; lane 2, 4 μg purified TmPDC/POR; lane 3, 6 μg purified ThPDC/POR; lanes M, molecular weight standard markers (Bio-Rad Laboratories, ON, Canada).

characteristic of iron–sulphur containing proteins. This is the first report that bifunctional PDC/POR enzymes were purified from bacterial hyper/thermophiles.

The biophysical and biochemical properties of the enzymes were characterized towards both oxidative and non-oxidative decarboxylation of pyruvate. The optimal pH values for both POR and PDC activities of *T. maritima* were determined to be the same at pH 8.4 (Supplementary Fig. S2A and B). The POR activity for this enzyme was previously reported to have an optimal pH value of 6.3 (*23*); however, the optimal pH value of 8.4 reported herein seems to be more consistent with optimal pH values reported for different mesophilic and thermophilic PORs (see Supplementary Table SIII). The purified ThPDC/POR also showed an optimal pH for both PDC and POR activities at 8.4 (Supplementary Fig. S2C and D). These values are different from the archaeal bifunctional PDC/PORs, which have higher pH optima for PDC activities (pH 10.2 for *P. furiosus* and pH 9.5 for *Thermoc. guaymasensis*) compared with their POR activities (*16*, *17*) that have optimal pH values closer to neutral (Supplementary Table SIII). The optimal pH for the archaeal bifunctional PDC/PORs described so far (*16*, *17*) is much higher than most of the commonly known PDCs from different mesophilic bacteria and yeasts, which generally prefer slightly acidic pH values (pH 5.5–6.5) for optimal activity (*37*).

Both POR and PDC activities of the purified enzymes from *T. maritima* and *T. hypogea* were extremely oxygen sensitive. The previously reported time required for a 50% loss of activity ($t_{1/2}$) of TmPOR upon exposure to air at room temperature was ~70 min (*23*). The $t_{1/2}$-value determined in this study was <15 min for both POR and PDC activities of the *T. maritima* enzyme (Supplementary Fig. S3A and B). The disparity is presumably the result of difference in the amounts of reducing agents and protein concentrations used in the experiment. The *T. hypogea* enzyme was also exceedingly sensitive to oxygen with a $t_{1/2}$-value determined for both the POR and PDC activities to be 5 min or less (Supplementary Fig. S3C and D). The oxygen sensitivity is a common property of majority of PORs. The only exceptions are the enzymes characterized from the halophilic archaeon *Halobacterium salinarium* (*38*) and the mesophilic sulphate-reducing bacterium *Desulfovibrio africanus* (*39*).

Both PDC and POR activities of ThPDC/POR were thermostable with $t_{1/2}$-values of ~3 h and 2 h at 80°C, respectively. The time required for a 50% loss of POR activity ($t_{1/2}$) of the purified *T. hypogea* enzyme (1.7 mg/ml in 50 mM Tris-HCl, pH 7.8 containing 0.5–1.0 mM SDT and 0.5–1.0 mM DTT) was ~180 min (~3 h) at 80°C (data not shown). The $t_{1/2}$-value for PDC activity of the same enzyme (0.9 mg/ml in 50 mM Tris-HCl, pH 7.8 containing 0.5–1.0 mM SDT and 0.5–1.0 mM DTT) was ~130 min (data not shown). The POR of TmPDC/POR was stable under anaerobic conditions with a $t_{1/2}$-value of 11 h at 80°C, signifying it as the most thermostable POR characterized so far (*23*). *Pyrococcus furiosus* POR has a $t_{1/2}$-value of only ~18 min at 80°C (*21*). Considering both TmPOR and *P. furiosus* POR are active optimally at above 90°C, the difference between their thermal stabilities is intriguing. It was not unexpected that ThPDC/POR would display less thermal stability than TmPDC/POR because of the lower optimal growth temperatures (T_{opt}) of 70°C and 80°C for *T. hypogea* and *T. maritima*, respectively.

The PDC activity of TmPDC/POR was highly temperature dependent and had a maximal activity above 95°C (Fig. 2A), which was similar to the previous

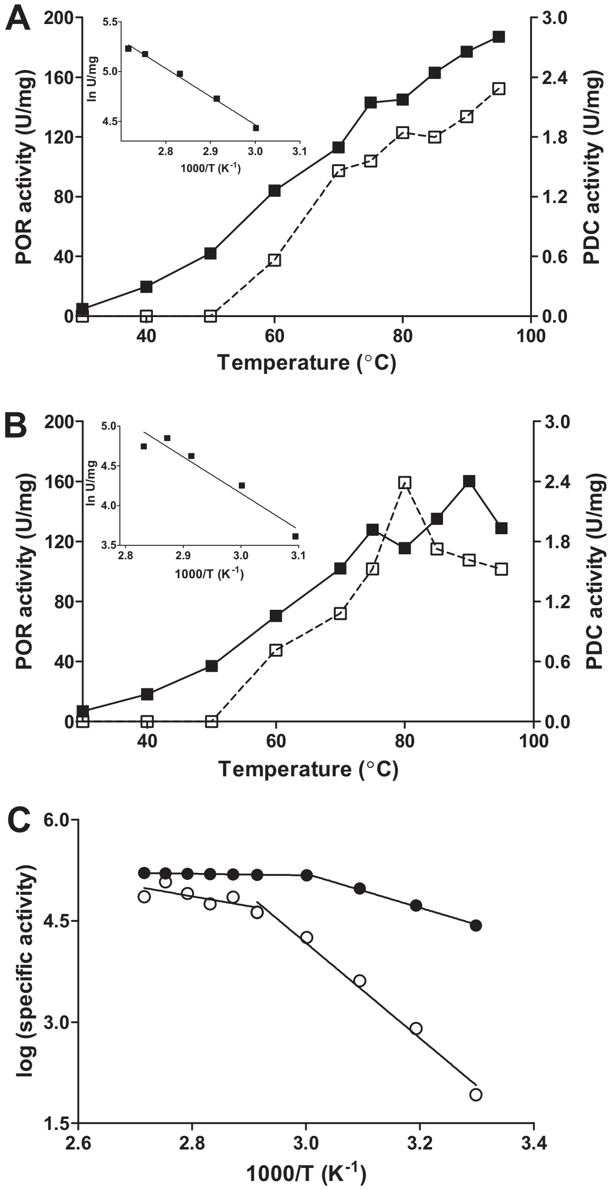

Fig. 2 Effect of temperature on the POR and PDC activities. The temperature dependence was determined for (**A**) TmPDC/POR and (**B**) ThPDC/POR in the temperature range of 30–95°C. The inset shows the Arrhenius plot based on the linear part of the plot A (temperatures 60–95°C) and plot B (temperatures 50–80°C) (**C**) The Arrhenius plot of the POR specific activities. Closed squares (■): POR activity; open squares (□): PDC activity; closed circle (●): TmPOR; open circle (○): ThPOR.

report (*23*). However, PDC and POR activities of ThPDC/POR showed different temperature dependencies as the PDC activity showed an increasing trend starting from 50°C up to 80°C, and decreasing activity at temperatures higher than 80°C, while the POR activity continually increased from 30°C up to 90°C (Fig. 2B). The difference between minimum temperatures determined for both activities might be reflective of the lower sensitivity limit of the PDC assay procedure compared with the POR assay. The thermal lability of the assay components does not appear to be a factor, as all of the assay components are relatively stable at high temperatures (*40*). Besides, the POR assay mixture contained less pyruvate and CoA and still exhibited full activity at temperatures higher than 80°C. Moreover, such temperature differences were not observed for TmPDC/POR.

To more accurately compare the temperature dependency of the enzymes from *T. hypogea* and *T. maritima*, the corresponding Arrhenius plots for the POR activities were depicted for the POR activities of *T. maritima* and *T. hypogea* (Fig. 2C). The TmPOR shows a transition at close to 60°C (Fig. 2C), similar to the previous report of Blamey and Adams (*23*) with calculated activation energy (E_{act}) of 23.6 kJ/mol over the range of 60–95°C (Fig. 2A). The POR from *T. hypogea* shifted at higher temperature, close to 70 °C (Fig. 2C) with a calculated E_{act} of 34.8 kJ/mol over a temperature range 50–80°C (Fig. 2B).

Both purified enzymes catalysed non-oxidative decarboxylation of pyruvate with specific activities of 1.9 ± 0.4 U/mg and 1.4 ± 0.2 U/mg for *T. hypogea* and *T. maritima*, respectively, and their POR activities were 119 ± 16 and 78 ± 9 U/mg, respectively. The specific activity of the purified TmPOR was close to the previously reported value of ~85 U/mg (*23*).

The kinetic parameters were calculated by fitting the data to the Michaelis–Menten equation for pyruvate and CoA (Table I). When POR activities of CFEs were assayed in the absence of added TPP, the enzyme of *T. hypogea* and *T. maritima* showed 67% and 80% of their full POR activity levels with the addition of TPP, respectively. However, without the addition of TPP, only negligible activity of PDC or POR could be detected for the purified ThPDC/POR (5% and 12% for POR and PDC activities, respectively). The purified TmPDC/POR displayed ~30% of its full activities (both POR and PDC) when TPP was omitted from its assay mixture. Hence, it seems that most of the enzyme-bound TPP in ThPDC/POR and TmPDC/POR has been dissociated during purification. This is in accordance with previous reports on thermophilic (*23*) and mesophilic PORs (*41*, *42*). Due to highly oxygen sensitive nature of enzymes, the complete removal of the bond TPP by further procedures such as extended on-column washes were not pursued. Consequently, the kinetic parameters resulted from the Michaelis–Menten plot could not be treated as accurate apparent K_m value. However, such value (~40 μM) served only as a reference for determining the requirement of TPP added to the assay mixtures. Therefore, approximately ≥10 times higher TPP concentrations (0.4 mM) were used in the assay mixtures to make certain that the reactions proceed in the presence of excess TPP to prevent it from being a rate-limiting factor.

The activities *T. maritima* and *T. hypogea* PDC were strictly CoA-dependent, which is similar to the previously described archaeal bifunctional PDC/POR enzymes (*16*, *17*). When CoA was substituted by desulfo-CoA (a CoA analog), both purified enzymes lost their POR activity, however the PDC activity was almost unchanged (data not shown), indicating that the thiol group of CoA is not playing a catalytic role in PDC activity and binding of either CoA or desulfo-CoA seems to be essential from rather an

Table I. Kinetic parameters for PDC and POR activities of *T. hypogea* and *T. maritima*.[a]

Source	Activity	Pyruvate[b] K_m^{app} (mM)	Pyruvate[b] V_{max}^{app} (U/mg)	CoA[c] K_m^{app} (μM)	CoA[c] V_{max}^{app} (U/mg)	MV[d] K_m^{app} (mM)	MV[d] V_{max}^{app} (U/mg)	Specific activity[e]
T. maritima	POR[f]	0.4 ± 0.1	81 ± 6	63 ± 6	94 ± 2	ND	ND	78 ± 9
	PDC	0.92 ± 0.3	1.4 ± 0.04	3.1 ± 1.2	1.3 ± 0.03	NA	NA	1.4 ± 0.2
T. hypogea	POR	0.13 ± 0.03	99 ± 3	21 ± 2	73 ± 4	0.14 ± 0.03	81.4 ± 6	119 ± 16
	PDC	1.4 ± 0.4	2.5 ± 0.18	1.4 ± 0.02	1.6 ± 0.13	NA	NA	1.9 ± 0.4

[a]NA, not applicable; ND, not determined. [b]POR measured using 0.1 mM CoA, 1 mM methyl viologen, 0.4 mM TPP and PDC measured using 1 mM CoA, 0.1 mM TPP. [c]POR measured using 5 mM pyruvate, 1 mM methyl viologen, 0.4 mM TPP and PDC measured using 10 mM pyruvate, 0.1 mM TPP. [d]POR measured using 0.1 mM CoA, 0.4 mM TPP and 5 mM pyruvate. [e]Expressed as micromoles of pyruvate oxidized per min per mg of enzyme under standard assay conditions described in the Materials and Methods section. [f]K_m values for pyruvate and CoA reported by Blamey and Adams (*23*) are 14.5 mM and 340 μM, respectively. The assays were conducted using EPPS (50 mM, pH 8.4) and at 80°C.

structural point of view (*17*). A re-reduction cluster mechanism has been proposed for the non-oxidative decarboxylation of pyruvate to acetaldehyde in the presence of CoA catalysed by *Sulfolobus tokodaii* 2-oxoacid:ferredoxin oxidoreductase via steps for the generation of $[4Fe\text{-}4S]^0$ from $[4Fe\text{-}4S]^{2+}$ (*43*). However, it is not clear where the two-electrons come from. It is unlikely that TmPDC/POR and ThPDC/POR would have such a mechanism for catalysing the non-oxidative decarboxylation of pyruvate to acetaldehyde, and further investigation is needed.

ThPDC/POR had lower pyruvate apparent K_m for its POR activity (0.13 mM) than the PDC activity (1.4 mM, Table I) and higher V_{max} for its POR activity (99 U/mg) than the PDC activity (2.5 U/mg, Table I), indicating that POR is probably the dominant catalytic activity under physiological conditions. Previously reported apparent K_m values for pyruvate and CoA of TmPOR (*23*), were unusually high (14.5 mM and 340 μM, respectively), compared with the corresponding K_m of the similar enzyme from the closely related *T. hypogea* POR as well as the other PORs (Supplementary Table SI). Therefore, we set to redetermine these kinetic parameters. As expected, the K_m values were much closer to the apparent K_m values reported from the bacterial and archaeal PORs (0.4 mM and 63 μM for pyruvate and CoA, respectively; Table I).

The ability of the bifunctional ThPDC/POR and TmPDC/POR to catalyse non-oxidative decarboxylation of 2-ketoisovalerate (KDC) was also investigated. The enzyme from *T. hypogea* showed KDC activity of ~10% of PDC activity (0.14 U/mg). *Thermotoga maritima* enzyme was also able to catalyse the non-oxidative decarboxylation of 2-ketoisovalerate with an activity of ~13% (0.18 U/mg) of the reaction with pyruvate (PDC, 1.4 U/mg). However, neither of these enzymes was able to catalyse the oxidative decarboxylation of 2-ketoisovalerate also known as VOR (data not shown). It has previously been reported that TmPOR is unable to catalyse the oxidative decarboxylation of 2-oxoglutarate, indolyl pyruvate and phenyl pyruvate (*23*); however, the ability of the enzyme to carry out oxidative decarboxylation of 2-ketoisovalerate was not examined at that time.

To determine the relatedness of the bifunctional PDC/POR of *T. maritima* and *T. hypogea* with PORs from other microorganisms, the nucleotide sequences of ~4.9 kb including the PDC/POR-encoding genes of the genome of *T. hypogea* were determined. Four genes corresponding to subunits α, β, γ and δ with 391, 324, 190 and 100 amino acid residues were sequenced, which showed similar gene arrangements as others from *Thermotogales*. The calculated molecular weights for the ThPOR were 43.8, 35.9, 21.2 and 11.6 kDa for subunits α, β, γ and δ, respectively. The sequenced genes were organized in the *porG-porD-porA-porB* order, which is similar to all *por* open reading frames in *Thermotogales* (*44*).

A phylogenetic analysis of ThPDC/POR and TmPDC/POR sequences indicated their close relatedness to each other and to other bacterial and archaeal PORs. Each subunit was orthologous to the corresponding subunits in other *Thermotogales* as well as other keto acid ferredoxin oxidoreductases from hyperthermophilic archaea. The closest homologues of the ThPDC/POR seem to be the PORs from *T. lettingae* and *T. thermarum* (Supplementary Fig. S4). The amino acid sequences of TmPDC/POR subunits are very similar to the ones from *Thermotoga* sp. strain RQ2, *T. petrophila* and *T. naphthophila*. The *Thermotogales* POR subunit sequences were divergent from the *Thermococcales* counterparts, manifested by their separate clustering in the phylogenetic tree (Supplementary Fig. S4). The pairwise comparison of the amino acid sequences of the whole length PORs from *T. hypogea* and *T. maritima* indicated an overall 76% amino acid sequence identity. The close relatedness of PORs as demonstrated here by sequence analysis, suggests the presence of such bifunctionality in other hyperthermophilic bacteria.

It was found that TmPDC/POR contained 15 conserved cysteine residues per tetrameric structure, which were all conserved within the PORs of *Thermotogales* and other hyperthermophilic PORs (44 and present study). The subunits PorA, PorB, PorG and PorD contain 1, 4, 2 and 8 conserved cysteine residues, respectively. However, there was an extra cysteine residue in ThPDC/POR subunit alpha (a total of two cysteine residues), which was also conserved within some (but not all) *Thermotogales*, including *T. lettingae*, *T. thermarum* and *Kosmotoga oleriae*.

Unlike the archaeal hyperthermophiles that contain at least four types of the 2-keto acid ferredoxin oxidoreductases (namely POR, VOR, KGOR and IOR), mesophilic and hyper/thermophilic bacteria generally carry a less diverse repertoire of 2-ketoacid oxidoreductases. The PORs of both *T. maritima* and *T. hypogea* showed specificity towards pyruvate and could not use 2-ketoisovalerate as substrate in the oxidation direction.

The PDC to POR activity ratios of the enzymes from hyperthermophilic bacteria *T. hypogea* and *T. maritima* were consistently much lower (<10 times) than those reported from the enzymes of hyperthermophilic archaea *P. furiosus* (*17*) and *Thermoc. guaymasensis* (*16*). However, there are higher POR activity levels (at least 4–fold) in *Thermotogales* (hyper/thermophile bacteria) than those of their archaeal counterparts (*Thermococcales*), of which the physiological implications are not well understood.

An iron-containing ADH with higher catalytic efficiency in acetaldehyde and butyraldehyde reduction compared with oxidation was characterized in *T. hypogea* (*11*), suggesting its physiological role to be in the production of alcohols from aldehydes. However, no aldehyde-producing activity was known in *T. hypogea* or any other hyper/thermophilic bacterium. Here, the PDC activities of both ThPDC/POR and TmPDC/POR were described for the first time, which are most likely involved in aldehyde production in the hyperthermophilic bacteria. Based on the high sequence similarity levels between the bifunctional enzymes studied here and other PORs, it is proposed that such bifunctionality is a general property of the hyper/thermophilic PORs. CFE of *T. hypogea* contained ~0.2 U/mg of ADH activity (*11*) compared with specific PDC activity in CFE of ~0.08 U/mg, suggesting that the production of ethanol is limited by the availability of acetaldehyde, the substrate of the ADH.

Supplementary Data

Supplementary Data are available at *JB* Online.

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Conflict of Interest

None declared.

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