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Gluconeogenesis from pyruvate in the hyperthermophilic archaeon *Pyrococcus furiosus*: involvement of reactions of the Embden-Meyerhof pathway

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Abstract. The hyperthermophilic archaeon *Pyrococcus furiosus* was grown on pyruvate as carbon and energy source. The enzymes involved in gluconeogenesis were investigated. The following findings indicate that glucose-6-phosphate formation from pyruvate involves phosphoenolpyruvate synthetase, enzymes of the Embden-Meyerhof pathway and fructose-1,6-bisphosphate phosphatase.

Cell extracts of pyruvate-grown *P. furiosus* contained the following enzyme activities: phosphoenolpyruvate synthetase (0.025 U/mg, 50 °C), enolase (0.9 U/mg, 80 °C), phosphoglycerate mutase (0.13 U/mg, 55 °C), phosphoglycerate kinase (0.01 U/mg, 50 °C), glyceraldehyde-3-phosphate dehydrogenase reducing either $NADP^+$ or NAD^+ ($NADP^+$: 0.019 U/mg, NAD^+: 0.009 U/mg; 50 °C), triosephosphate isomerase (1.4 U/mg, 50 °C), fructose-1,6-bisphosphate aldolase (0.0045 U/mg, 55 °C), fructose-1,6-bisphosphate phosphatase (0.026 U/mg, 75 °C), and glucose-6-phosphate isomerase (0.22 U/mg, 50 °C). Kinetic properties (V_{max} values and apparent K_m values) of the enzymes indicate that they operate in the direction of sugar synthesis. The specific enzyme activities of phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase ($NADP^+$-reducing) and fructose-1,6-bisphosphate phosphatase in pyruvate-grown *P. furiosus* were by a factor of 3, 10 and 4, respectively, higher as compared to maltose-grown cells suggesting that these enzymes are induced under conditions of gluconeogenesis. Furthermore, cell extracts contained ferredoxin: $NADP^+$ oxidoreductase (0.023 U/mg, 60 °C); phosphoenolpyruvate carboxylase (0.018 U/mg, 50 °C) acts as an anaplerotic enzyme.

Thus, in *P. furiosus* sugar formation from pyruvate involves reactions of the Embden-Meyerhof pathway, whereas sugar degradation to pyruvate proceeds via a modified "non-phosphorylated" Entner-Doudoroff pathway.

Key words: *Pyrococcus furiosus* – Archaea – Hyperthermophiles – Gluconeogenesis – Embden-Meyerhof pathway – Fructose-1,6-bisphosphate aldolase – Fructose-1,6-bisphosphate phosphatase – Glyceraldehyde-3-phosphate dehydrogenase

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The hyperthermophilic archaeon *Pyrococcus furiosus* is an anaerobic chemo-organoheterotrophic organism, which grows on either complex media or on maltose or pyruvate as carbon and energy source (Fiala and Stetter 1986; Schäfer and Schönheit 1991, 1992). In recent studies, the catabolic pathways involved in the fermentation of both maltose and pyruvate have been elucidated (Schäfer and Schönheit 1991, 1992). Maltose is fermented to acetate, CO_2 and H_2, via a novel sugar degradation pathway as concluded from fermentation balances, growth yield data and the determination of enzyme activities in cell extracts (Schäfer and Schönheit 1992). Maltose degradation – via glucose – to pyruvate and $2\ H_2$ proceeds via reactions of a modified "non-phosphorylated" Entner-Doudoroff pathway. This part of the fermentation is not coupled with net ATP synthesis. Pyruvate is further converted to acetate, CO_2 and H_2 involving pyruvate: ferredoxin oxidoreductase, hydrogenase and acetyl-CoA synthetase (ADP forming). The latter enzyme couples acetate formation from acetyl-CoA with the phosphorylation of ADP and constitutes the only energy conserving site in maltose and pyruvate fermentation (Schäfer and Schönheit 1991, 1992). Acetyl-CoA synthetase (ADP-forming) represents a novel prokaryotic mechanism of acetate formation and ATP synthesis which is operative in archaea rather than in (eu)bacteria (Schäfer et al. 1992). In (eu)bacteria acetate and ATP are formed by two enzymes, phosphate acetyltransferase and acetate kinase.

Besides the enzymes of the "non-phosphorylated" Entner-Doudoroff pathway, *Pyrococcus* species grown on peptone or maltose have been reported to contain low activities of glyceraldehyde-3-phosphate dehydrogenase and fructose-1,6-bisphosphate aldolase (Zwickl et al. 1990; Mukund and Adams 1991; Schäfer and Schönheit 1992). These enzyme activities of the Embden-Meyerhof

pathway have been suggested to have anabolic rather than catabolic functions. In order to prove this hypothesis we studied the pathway of gluconeogenesis in *P. furiosus*. For this purpose we grew *P. furiosus* on pyruvate as carbon and energy source since under these growth conditions the organism has to synthesize sugars from pyruvate. Sugars are constituents of nucleic acids; furthermore *Pyrococcus* species have been reported to contain an inositol phosphate as thermostabilizing compound (Scholz et al. 1992) and glycolipids in the cell envelope (Fiala and Stetter 1986) and in the cytoplasmic membrane (Lanzotti et al. 1989).

In this communication evidence is presented that glucose-6-phosphate formation from pyruvate in this organism involves phosphoenolpyruvate synthetase, enzymes of the Embden-Meyerhof pathway, fructose-1,6-bisphosphate phosphatase, and hexosephosphate isomerase. Furthermore, the presence of a ferredoxin: $NADP^+$ oxidoreductase involved in the generation of NADPH and phosphoenolpyruvate carboxylase as an anaplerotic reaction are described.

Materials and methods

Source of material

Pyruvate kinase and 3-phosphoglycerate were from Sigma (Deisenhofen, Germany). Yeast extract was from Difco (Stuttgart, Germany). If not mentioned otherwise, all other chemicals were reagent grade and obtained from E. Merck (Darmstadt, Germany); enzymes and coenzymes were from Boehringer (Mannheim, Germany). N_2 (5.0) was from Messer-Griesheim (Berlin, Germany). Ferredoxin from *Clostridium pasteurianum* was prepared according to the method of Schönheit et al. (1978). *Pyrococcus furiosus* VC1 (DSM 3638) was from the Deutsche Sammlung von Mikroorganismen (Braunschweig, Germany).

Growth of Pyrococcus furiosus

The organism was routinely grown at 95 °C in an open fermenter system as described by Schäfer and Schönheit (1991): The 1.5 l medium contained 0.1% yeast extract and either pyruvate (20 mM) or maltose (10 mM) as carbon and energy sources. The medium was continuously flushed with N_2 (100%). Cells were harvested in the late-log phase (2×10^8 cells/ml) under strictly anaerobic conditions as described by Schäfer and Schönheit (1991).

Preparation of cell extracts

Cell extracts of pyruvate- or maltose-grown *P. furiosus* were prepared under strictly anaerobic conditions as described previously (Schäfer and Schönheit 1991). The buffer systems used during cell breakage were different depending on the particular enzyme activity to be analyzed (see below). The buffers were made anaerobic by repeatedly evacuating and gassing with N_2 (100%). The cell extracts (20–30 mg protein/ml) were stored under N_2 on ice for several hours or at −20 °C for not longer than 48 h. Protein was determined by the Biuret method according to Bode et al. (1968) using bovine serum albumin as a standard.

Preparation of phosphate free cell extracts

The phosphate content of cell extracts was determined by the modification of the Fiske-Subbarow method as described by Josse (1966). Protein was precipitated from the cell extract (3 ml) with 0.3 ml 3 M trichloroacetic acid. After 10 min on ice the samples were centrifuged and the phosphate content was determined in the supernatant. The cell extracts (about 30 mg protein/ml) contained about 1 mM phosphate.

Phosphate-free cell extracts were prepared as follows: two ml of cell extract were supplemented with 5 mM potassium phosphate and 0.4 μCi $H_3{}^{32}PO_4$. The sample was desalted by passing over a Sephadex G-25 column (16 × 160 mm) equilibrated with 100 mM Tris/HCl pH 8.0 plus 20 mM $MgCl_2$. Phosphate separation from the protein fraction was followed by measuring radioactivity of the eluted fractions by liquid scintillation counting. More than 99.9% of the phosphate in the extracts (< 1 mM) was removed. This procedure was also used to remove nucleotides and 2,3-bisphosphoglycerate. Sephadex-treated cell extracts were used for measuring phosphoenolpyruvate synthetase, glyceraldehyde-3-phosphate dehydrogenase, fructose-1,6-bisphosphate phosphatase and phosphoglycerate mutase.

Determination of enzyme activities

All enzyme assays were performed anaerobically at 50 °C–90 °C in stoppered glass cuvettes filled with 0.7–1 ml assay mixture as described previously (Schäfer and Schönheit 1991). The assay mixtures contained 0.3–3 mg protein. Coupled enzymatic assays containing auxiliary enzymes (e.g. lactate dehydrogenase, pyruvate kinase, endolase, glucose-6-phosphate dehydrogenase) were performed between 50 and 60 °C. The auxiliary enzymes were generally added shortly before reaction start and it was ensured that the enzymes were not rate-limiting. One unit (1 U) of enzyme activity is defined as 1 μmol substrate consumed or product formed per minute.

Phosphoenolpyruvate synthetase (EC 2.7.9.2)

Phosphoenolpyruvate (PEP) synthetase was measured as pyruvate and ATP dependent formation of PEP according to Eyzaguirre et al. (1982; Method II). The ATP dependent pyruvate consumption by PEP synthetase was followed by coupling the reaction to the supply of pyruvate from lactate catalyzed by an excess of lactate dehydrogenase (LDH).

Phosphoglycerate mutase (EC 2.7.5.3)

Phosphoglycerate mutase activity was assayed at 55 °C in both directions:

(A) The 3-phosphoglycerate dependent formation of 2-phosphoglycerate was assayed according to Jansen et al. (1982) by coupling the reaction to the oxidation of NADH via enolase, pyruvate kinase and lactate dehydrogenase. The effect of 2,3-bisphosphoglycerate on the activity was determined by adding 1 mM of the corresponding cyclohexylammonium salt to the assay.

(B) The 2-phosphoglycerate–dependent formation of 3-phosphoglycerate was assayed by coupling the reaction to the oxidation of NADH via phosphoglycerate kinase, pyruvate kinase and lactate dehydrogenase. The assay mixture contained 100 mM Tris/HCl pH 8.0, 5 mM 2-phosphoglycerate, 5 mM phosphoenolpyruvate, 0.5 mM ATP, 10 mM $MgCl_2$, 0.4 mM NADH, 4.5 U phosphoglycerate kinase, 2.5 U pyruvate kinase, 7.5 U lactate dehydrogenase and the Sephadex-treated cell extract (0.5–1.0 mg). In kinetic studies concentration of 2-phosphoglycerate was varied from 1.25–12.5 mM. The reaction was started by the addition of either cell extract, 2-phosphoglycerate or 3-phosphoglycerate kinase.

Phosphoglycerate kinase (EC 2.7.2.3)

Phosphoglycerate kinase was assayed at 50 °C as 3-phosphoglycerate- and ATP-dependent formation of either 1,3-bisphosphoglycerate (A) or of ADP (B):

(A) The formation of 1,3-bisphosphoglycerate was assayed according to Jansen et al. (1982) by coupling the reaction to the

oxidation of NADH via glyceraldehyde-3-phosphate dehydrogenase.

(B) The formation of ADP was assayed by coupling the reaction to the oxidation of NADH via pyruvate kinase and lactate dehydrogenase. The assay mixture contained 100 mM Tricine/KOH pH 8.2, 20 mM $MgCl_2$, 3 mM ATP, 10 mM 3-phosphoglycerate, 5 mM phosphoenolpyruvate, 0.4 mM NADH, 10 U pyruvate kinase and 15 U lactate dehydrogenase and extract (0.26–2.6 mg protein in 100 mM Tris/HCl pH 8.0, 20 mM $MgCl_2$). The reaction was started by the addition of cell extract.

Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.13)

Glyceraldehyde-3-phosphate dehydrogenase was assayed at 50 °C in both directions according to Zeikus et al. (1977):

(A) In the direction of 1,3-bisphosphoglycerate formation the reduction of NAD^+ or $NADP^+$ was measured. The assay mixture contained 100 mM Tris/HCl pH 8.0, 2 mM fructose-1,6-bisphosphate, 20 mM potassium arsenate or 20 mM potassium phosphate, 0.9 U fructose-1,6-bisphosphate aldolase, 2.5 mM NAD^+ or $NADP^+$ and phosphate-free cell extract (0.5–2.0 mg protein, prepared in 100 mM Tris/HCl pH 8.0, 20 mM $MgCl_2$). In kinetic studies the concentrations of NAD^+ (0.31–6.4 mM), of $NADP^+$ (0.05–3.2 mM) and of phosphate (2.5–25 mM) were varied.

(B) In the direction of glyceraldehyde-3-phosphate formation the oxidation of NADH and NADPH were measured. The assay mixture contained 100 mM Tris/HCl pH 8.0, 2 mM 3-phosphoglycerate, 2 mM ATP, 5 mM $MgCl_2$, 0.5 mM NADH or NADPH, 4.5 U phosphoglycerate kinase and phosphate-free extract (0.5–1.0 mg protein, prepared in 100 mM Tris/HCl pH 8.0, 20 mM $MgCl_2$). In kinetic studies the concentrations of NADH (0.16–0.53 mM) and NADPH (0.02–0.56 mM) were varied. The reaction was started by the addition of either ATP, 3-phosphoglycerate or phosphoglycerate kinase.

Fructose-1,6-bisphosphate phosphatase (EC 3.1.3.11)

Fructose-1,6-bisphosphate (FBP) phosphatase was assayed by measuring fructose-1,6-bisphosphate-dependent formation of fructose-6-phosphate (A). The amount of fructose-6-phosphate was determined in a separate assay (B) by following the reduction of $NADP^+$ via phosphoglucose isomerase and glucose-6-phosphate dehydrogenase:

(A) The assays were performed at 80 °C in 10 ml serum bottles (6 separate bottles) filled with 1 ml assay mixture containing 100 mM Tricine/KOH pH 8.2, 10 mM fructose-1,6-bisphosphate, 20 mM $MgCl_2$ and Sephadex-treated (AMP-depleted) cell extract (0.26–2.6 mg protein, in 100 mM Tris/HCl pH 8.2, 20 mM $MgCl_2$). The gas phase was 100% N_2. In a kinetic study the FBP concentration was varied from 0.5–10 mM. The temperature dependence of the reaction was measured between 60 and 90 °C. The effects of $MgCl_2$ and of AMP on the reaction were tested by adding 20 mM $MgCl_2$ or 1–2 mM AMP to the assay mixtures. The reaction was started by the addition of FBP. In 10 min time intervals the reaction was stopped by rapidly cooling the assay mixtures down to 0 °C on ice.

(B) The amount of fructose-6-phosphate formed was determined at 37 °C in an assay mixture containing 100 mM Tricine/KOH pH 8.2, 0.4 mM $NADP^+$, 3.5 U phosphoglucose isomerase and 1.4 U glucose-6-phosphate dehydrogenase and 20 to 100 µl stopped assay mixture. The reaction was started by the addition of glucose-6-phosphate dehydrogenase.

Phosphoenolpyruvate carboxylase (EC 4.1.1.31)

Phosphoenolpyruvate (PEP) carboxylase was assayed at 50 °C according to Jansen et al. (1982) by coupling the phosphoenolpyruvate- and bicarbonate-dependent formation of oxaloacetate with the oxidation of NADH via malate dehydrogenase.

Ferredoxin: $NADP^+$ oxidoreductase (EC 1.18.1.2)

Ferredoxin: $NADP^+$ oxidoreductase activity was assayed at 60 °C in the presence of endogeneous pyruvate: ferredoxin oxidoreductase by measuring the pyruvate-, CoA- and ferredoxin-dependent reduction of $NADP^+$. The assay mixture contained 100 mM Tris/HCl pH 8.0, 10 mM pyruvate, 0.2 mM CoA, 6.5 µM ferredoxin from *Clostridium pasteurianum* and extract (0.28–0.56 mg protein, Sephadex-treated in 100 mM Tris/HCl pH 8.0, 20 mM $MgCl_2$). In kinetic studies the concentrations of either ferredoxin (0.65–13 µM) and $NADP^+$ (0.05–2.6 mM) were varied. The reaction was started by the addition of either ferredoxin, $NADP^+$, pyruvate, CoA or cell extract and the reduction of $NADP^+$ was followed at 365 nm. In few experiments NAD^+ (1 mM) was used as electron acceptor instead of $NADP^+$. The reaction was started by the addition of either ferredoxin, $NADP^+$, pyruvate, CoA or cell extract.

Enolase (EC 4.2.1.11), triosephosphate isomerase (EC 5.3.1.1), fructose-1,6-bisphosphate aldolase (EC 4.1.2.13), glucose-6-phosphate isomerase (EC 5.3.1.9), glucose isomerase (EC 5.3.1.18) and pyruvate: ferredoxin oxidoreductase (EC 1.2.7.1) were measured as described previously (Schäfer and Schönheit 1991, 1992).

Results

Pyrococcus furiosus was grown on pyruvate as carbon and energy source (in the presence of 0.1% yeast extract). The cells grew at 95 °C with a doubling time of about 1 h up to densities of 2×10^8 cells/ml (= 0.05 mg protein/ml). Enzymes required for gluconeogenesis starting from pyruvate were investigated in these cells. For comparison we measured gluconeogenetic enzyme activities in cells of *P. furiosus* grown on maltose. The organism grew on maltose (in the presence of 0.1% yeast extract) at 95 °C with a doubling time of 75 min up to cell densities of about 2×10^8 cells/ml.

Phosphenolpyruvate synthetase

Cell extracts of pyruvate-grown *P. furiosus* catalyzed the Mg^{2+}- and ATP-dependent consumption of pyruvate with a specific activity of 0.05 U/mg at 50 °C (Table 1). The reaction was neither dependent on phosphate nor on bicarbonate. These properties indicate that a PEP synthetase is operative in *P. furiosus* catalyzing the following reaction: pyruvate + ATP + H_2O → PEP + AMP + P_i. The presence of pyruvate-phosphate dikinase (pyruvate + P_i + ATP → PEP + AMP + PP_i), which is phosphate-dependent, could be excluded: addition of phosphate (10 mM) to phosphate-free cell extracts (< 1 µM) did not stimulate pyruvate consumption. Since the reaction also proceeded in the absence of bicarbonate, pyruvate conversion to oxaloacetate catalyzed by pyruvate carboxylase, pyruvate + ATP + HCO_3^- → oxaloacetate + ADP + P_i, could be excluded as well.

PEP synthetase activity linearily increased with protein concentration (0.25–2.0 mg in the assay) and with time for at least 20 minutes. The dependence of the rate on the ATP and Mg^{2+} concentration followed Michaelis-Menten kinetics. From Lineweaver-Burk plots apparent K_m values of 0.07 mM for ATP and of 10 mM for Mg^{2+} were determined.

Table 1: Specific enzyme activities and apparent K_m values of enzymes involved in gluconeogenesis (glucose-6-phosphate formation) from pyruvate in cell extracts of pyruvate-grown *Pyrococcus furiosus*. PEP = phosphoenolpyruvate, FBP = fructose-1,6-bisphosphate, GAP = glyceraldehyde-3-phosphate, DHAP = dihydroxyacetone phosphate, F-6-P = fructose-6-phosphate, P_i = inorganic phosphate, -P- = phospho, Fd = Ferredoxin from *Clostridium pasteurianum*, ox = oxidized, red = reduced. Numbers in brackets refer to calculated specific activities at 100 °C assuming a constant Q_{10} value of the reaction rates of 2.0 in a temperature range from 50–100 °C

Enzyme activity	Test reaction	Temperature [°C]	Specific activity [U/mg]	Apparent K_m [mM]
PEP-Synthetase	Pyruvate + ATP → PEP + AMP + P_i	50	0.05 (1.6)	0.07 (ATP) 10 ($MgCl_2$)
Enolase	2-P-Glycerate → PEP	80	0.9 (3.5)	n.d.
Phosphoglycerate mutase	3-P-Glycerate → 2-P-Glycerate	55	0.13 (3.0)	6.3 (3-P-Glycerate)
	2-P-Glycerate → 3-P-Glycerate	55	0.05 (1.2)	0.3 (2-P-Glycerate) 1.7 ($MgCl_2$)
Phosphoglycerate kinase	3-P-Glycerate + ATP → 1,3-Bis-P-Glycerate + ADP	50	0.011 (0.35)	2.0 (3-P-Glycerate) 2.7 (ATP)
Glyceraldehyde-3-phosphate dehydrogenase[2]	GAP → 1,3-Bis-P-Glycerate	50	0.019 (0.62)	0.05 ($NADP^+$) 2.7 (Pi)
	1,3-Bis-P-Glycerate → GAP	50	0.060 (1.9)	0.025 (NADPH)
Triosephosphate isomerase	GAP → DHAP	50	1.4 (44)	n.d.
Fructose-1,6-bisphosphate aldolase	FBP → GAP + DHAP	55	0.0045 (0.1)	2.0 (FBP)
Fructose-1,6-bisphosphate phosphatase	FBP → F-6-P + P_i	75	0.026 (0.075)[2]	0.5 (FBP) 3.3 ($MgCl_2$)
Glucose-6-phosphate Isomerase	F-6-P → G-6-P	50	0.22 (7.0)	n.d.
Glucose Isomerase	Fructose → Glucose	50	0.065 (2.1)	n.d.
PEP-Carboxylase	PEP + HCO_3^- → Oxaloacetate + P_i	50	0.018 (0.58)	2.0 (PEP)
Fd: NADP oxidoreductase	Fd_{red} + $NADP^+$ → Fd_{ox} + NADPH + H^+	60	0.023 (0.37)	0.0013 (Fd) 0.22 ($NADP^+$)

[2] See also Table 2

[b] For FBP phosphatase a Q_{10} value of 1.5 has been determined

The enzyme was O_2-sensitive. No activity could be measured in aerobic 100 mM Tris/HCl pH 8.0. The activity could, however, partially be restored by incubation (5 min) with dithioerythritol (DTE) (22% activity by 10 mM DTE; 65% by 30 mM or 50 mM DTE). PEP synthetase activity of *P. furiosus* – like that of *M. thermoautotrophicum* – was not cold labile, a property found for the enzymes of various organisms (see in Cooper and Kornberg 1974). Storage of the cell extracts on ice for several hours or freezing at −20 °C did not significantly affect the enzyme activity.

Enolase

Cell extracts of pyruvate-grown *P. furiosus* contained enolase activity catalyzing 2-phosphoglycerate-dependent PEP formation at a rate of 0.9 U/mg (at 80 °C). Enolase has also been shown to be present in maltose-grown cells (2.5 U/mg at 80 °C) where it is involved in sugar degradation via the modified, "non-phosphorylated" Entner-Doudoroff pathway (Schäfer and Schönheit 1992). The apparent K_m for 2-phosphoglycerate was 1.5 mM.

Phosphoglycerate mutase

P. furiosus contained phosphoglycerate mutase activity (3-phosphoglycerate ⇌ 2-phosphoglycerate). The reaction was measured in both directions (Table 1). Sephadex-treated cell extracts catalyzed 3-phosphoglycerate-dependent formation of 2-phosphoglycerate with a specific activity at 55 °C of 0.10 U/mg. Phosphoglycerate mutase activity was slightly stimulated (30%) by the addition of the cosubstrate 2,3-bisphosphoglycerate (1 mM), which was included in the assay. No stimulation was observed in crude extracts not depleted of 2,3-bisphosphoglycerate. The rate dependence of the reaction on the 3-phosphoglycerate followed Michaelis-Menten kinetics in a concentration range between 0.56–20 mM; the rate decreased at concentrations higher than 20 mM indicating substrate inhibition (35% inhibition at 40 mM). From the slope of the linear part of the Lineweaver-Burk plot an apparent K_m of 6.3 mM for 3-phosphoglycerate was determined. The reaction was also dependent on Mg^{2+} with an apparent K_m value of 1.7 mM Mg^{2+}. Cell extracts also catalyzed 2-phosphoglycerate-dependent 3-phosphoglycerate formation at 55 °C with a rate of 0.05 U/mg and an apparent K_m value of 0.3 mM for 2-phosphoglycerate (in the presence of 1 mM 2,3-bisphosphoglycerate).

The finding that the apparent K_m for 2-phosphoglycerate is by a factor of 21 smaller than the apparent K_m for 3-phosphoglycerate whereas the V_{max} values were similar indicates that phosphoglycerate mutase *in vivo* operates in the direction of 3-phosphoglycerate formation, i.e. sugar synthesis.

Phosphoglycerate kinase

Cell extracts contained phosphoglycerate kinase catalyzing the 3-phosphoglycerate- and ATP-dependent

formation of 1,3-bisphosphoglycerate and ADP with a specific activity of 0.011 U/mg at 50 °C (Table 1). At 60 °C the specific activity was 0.022 U/mg indicating a Q_{10} of the reaction rate of 2. The rate dependence on the 3-phosphoglycerate and ATP concentration followed Michaelis-Menten kinetics; apparent K_m values of 2.0 mM for 3-phosphoglycerate and of 2.7 mM for ATP were calculated. Phosphoglycerate kinase activity was dependent on Mg^{2+} which was saturated at 5 mM concentration. Maltose-grown cells of *P. furiosus* contained phosphoglycerate kinase at about 1/3 of the activity of pyruvate-grown cells, suggesting that the enzyme is induced under conditions of gluconeogenesis (Table 3).

Glyceraldehyde-3-phosphate dehydrogenase

Cell extracts contained glyceraldehyde-3-phosphate dehydrogenase, which catalyzes the phosphate- or arsenate-dependent oxidation of glyceraldehyde-3-phosphate with $NADP^+$ and NAD^+ and the reduction of 1,3-bisphosphoglycerate with NADPH and NADH. The kinetic properties of glyceraldehyde-3-phosphate dehydrogenase activity are summarized in Table 2.

The specific rate of glyceraldehyde-3-phosphate oxidation determined in the presence of arsenate was 0.019 U/mg with $NADP^+$ and 0.009 U/mg with NAD^+ as electron acceptor (at 50 °C). In the presence of phosphate the rate of $NADP^+$ reduction was 0.0045 U/mg. The apparent K_m value for $NADP^+$ was 0.05 mM, which was measured both in the presence of arsenate and phosphate. The apparent K_m for NAD^+ was 1.6 mM. Both the higher rate of $NADP^+$ reduction and the lower apparent K_m for $NADP^+$ as compared to NAD^+ indicate that $NADP^+$ is the preferred cofactor of glyceraldehyde-3-phosphate dehydrogenase (see Table 2).

The specific rate of the reduction of 1,3-phosphoglycerate with NADPH was 0.060 U/mg and 0.035 U/mg with NADH. Apparent K_m values for NADPH and NADH were 0.025 mM and 1.4 mM, respectively.

The finding that V_{max}/apparent K_m of 1,3 bisphosphoglycerate reduction with NADPH (in the presence of phosphate) is about 32-fold higher than of glyceraldehyde-3-phosphate oxidation with $NADP^+$ indicates that the enzyme is operating in vivo in the direction of glyceraldehyde-3-phosphate formation.

Glyceraldehyde 3-phosphate dehydrogenase was also present in cell extracts of maltose-grown *P. furiosus*. The specific activity of glyceraldehyde-3-phosphate oxidation with $NADP^+$ was 0.002 U/mg (at 50 °C) and, thus, was about 10% of the activity of pyruvate-grown cells (Table 2). The same differences in enzyme activities were observed with NAD^+ as electron acceptor. These findings indicate that glyceraldehyde-3-phosphate dehydrogenase was induced 10-fold under growth conditions which require gluconeogenesis.

Table 2: Specific activities and apparent K_m values of glyceraldehyde-3-phosphate dehydrogenase for NAD^+/$NADP^+$ or NADH/NADPH in cell extracts of pyruvate-grown *Pyrococcus furiosus*. GAP = glyceraldehyde-3-phosphate; 1,3-BPG = 1,3-bisphosphoglycerate. Numbers in brackets refer to calculated specific enzyme activities at 100 °C assuming a constant Q_{10} value of 2.0 in a temperature range from 50–100 °C

Test direction	Cofactor	Arsenate	Phosphate	Specific activity [U/mg at 50 °C]	Apparent K_m [mM]	V_{max}/K_m
GAP oxidation	NAD^+	Arsenate	–	0.009 (0.29)	1.6	0.0056
	$NADP^+$	Arsenate	–	0.019 (0.62)	0.05	0.38
	$NADP^+$	–	Phosphate	0.0045 (0.144)	0.06	0.075
1,3-BPG reduction	NADH	–	–	0.035 (1.1)	1.4	0.025
	NADPH	–	–	0.060 (1.9)	0.025	2.4

Table 3. Specific enzyme activities in *Pyrococcus furiosus* grown either on pyruvate or on maltose as carbon and energy source. The enzyme activities given were determined at the temperatures shown in Table 1. GAP = Glyceraldehyde-3-phosphate, PEP = Phosphoenolpyruvate

Enzyme activity	Specific activity in pyruvate-grown cells [U/mg]	Specific activity in maltose-grown cells [U/mg]	% Activity in maltose-grown cells
PEP-Synthetase	0.05	0.05	100
Enolase	0.9	2.5	280
Phosphoglycerate mutase	0.13	0.13	100
Phosphoglycerate kinase	0.011	0.004	36
GAP dehydrogenase	0.009 (NAD^+)	0.0015 (NAD^+)	16
	0.019 ($NADP^+$)	0.002 ($NADP^+$)	10
Triosephosphate isomerase	1.4	1.4	100
Fructose-1,6-phosphate aldolase	0.0045	0.003	66
Fructose-1,6-bisphosphate phosphatase	0.026	0.007	27
Glucosephosphate isomerase	0.22	0.85	390
Glucose isomerase	0.065	0.4	615

Triosephosphate isomerase

Cell extracts of pyruvate-grown *P. furiosus* contained high activities of triosephosphate isomerase catalyzing glyceraldehyde-3-phosphate-dependent dihydroxyacetone phosphate formation at a rate of 1.4 U/mg (at 50 °C).

Fructose-1,6-bisphosphate aldolase

Cell extracts of pyruvate-grown *P. furiosus* contained fructose-1,6-bisphosphate (FBP) aldolase catalyzing the FBP-dependent formation of dihydroxyacetone phosphate. The specific activity of 0.0045 U/mg measured at 55 °C corresponds to a activity of 0.1 U/mg at 100 °C (Table 1). From the rate dependence on the FBP concentration an apparaent K_m of 2 mM was calculated. Maltose-grown cells also contained fructose-1,6-bisphosphate aldolase, however, with a lower specific activity (0.003 U/mg at 55 °C, Table 3).

Fructose-1,6-bisphosphate phosphatase

Cell extracts contained fructose-1,6-bisphosphate (FBP) phosphatase activity with a specific activity of 0.026 U/mg protein at 75 °C (Table 1). The rate dependence on the FBP and $MgCl_2$ concentration followed Michaelis-Menten kinetics; apparent K_m values for FBP of 0.5 mM and for $MgCl_2$ of 3.3 mM were determined. The temperature dependence of the enzyme activity was tested between 60 °C and 90 °C. Plots of the logarithms of the specific activities versus the reciprocal temperatures (Arrhenius plot) were linear in a range from 60 °C–80 °C. From the slope a Q_{10} value of 1.5 and an activation energy of 41 kJ/mol were calculated. AMP, an allosteric effector for FBP phosphatase in various organisms did not affect FBP phosphatase activity in *P. furiosus* at concentrations up to 2 mM when Sephadex-treated, AMP-depleted, cell extracts were tested.

Maltose-grown cells contained FBP phosphatase with a specific activity of 0.007 U/mg at 75 °C, which is about 25% of the activity of pyruvate-grown cells (Table 3). This indicates that the enzyme is induced under conditions of gluconeogenesis.

Glucose-6-phosphate isomerase

Cell extracts contained glucose-6-phosphate isomerase catalyzing the fructose-6-phosphate dependent glucose-6-phosphate formation at a rate of 0.22 U/mg (at 50 °C).

Ferredoxin: $NADP^+$-oxidoreductase

The kinetic properties of glyceraldehyde-3-phosphate dehydrogenase indicated that in vivo the enzyme catalyzes the reduction of 1,3-phosphoglycerate with NADPH as electron donor. A reaction generating NADPH during pyruvate catabolism has not been identified so far. *P. furiosus* has been shown to contain pyruvate: ferredoxin oxidoreductase (Schäfer and Schönheit 1991). Its specific activity was 0.4 U/mg when measured at 60 °C with ferredoxin from *Clostridium pasteurianum*) as the electron acceptor. From the rate dependence on the ferredoxin concentrations an apparent K_m of about 4 µM was calculated.

The following experiments indicate that *P. furiosus* contains ferredoxin: $NADP^+$ oxidoreductase forming NADPH at the expense of pyruvate oxidation to acetyl-CoA (Table 1): cell extracts (< 0.56 mg protein/assay) catalyzed the pyruvate- and CoA-dependent reduction of $NADP^+$ with a rate of 0.023 U/mg (60 °C) provided that ferredoxin (6.5 µM) had been added to the assay mixture. This reaction is ascribed to the combined action of pyruvate : ferredoxin oxidoreductase and ferredoxin: $NADP^+$ oxidoreductase. The rate dependence of pyruvate-dependent $NADP^+$ reduction on both the concentration of clostridial ferredoxin and of $NADP^+$ followed Michaelis-Menten kinetics. Apparent K_m values of 1.3 µM for ferredoxin and of 0.22 mM for $NADP^+$ were determined. When higher protein concentrations (> 1 mg) were used in the assay system, $NADP^+$ was reduced at a low rate (0.012 U/mg) in the absence of added ferredoxin which is probably due to the presence of endogenous ferredoxin in the *P. furiosus* extracts. Cell extracts also catalyzed the pyruvate dependent reduction of NAD^+ at a rate of 0.004 U/mg at 60 °C which is about 15% of the rate obtained with $NADP^+$.

Phosphoenolpyruvate carboxylase

We also looked for enzymes involved in oxaloacetate formation as an anaplerotic sequence in the anabolism of *P. furiosus*. Cell extracts of pyruvate grown cells contained phosphoenolpyruvate carboxylase with a specific activity of 0.018 U/mg at 50 °C. From the rate dependence on the phosphoenolpyruvate concentration, an apparent K_m of 2.0 mM was calculated. Pyruvate carboxylase activity could not be detected in cell extracts of *P. furiosus*.

Discussion

In the present communication we report on enzyme activities in pyruvate-grown cells of *P. furiosus*, which are involved in sugar (glucose-6-phosphate) formation from pyruvate. We have found phosphoenolpyruvate (PEP) synthetase activity, all enzymes of the Embden-Meyerhof pathway catalyzing PEP conversion to fructose-1,6-bisphosphate (FBP), fructose-1,6-bisphosphate phosphatase and hexosephosphate isomerase indicating that sugars are synthesized from pyruvate according to the pathway depicted in Fig. 1: Furthermore, we could detect ferredoxin: $NADP^+$ oxidoreductase activity generating NADPH which is the electron donor for glyceraldehyde-3-phosphate dehydrogenase, and PEP carboxylase as an anaplerotic enzyme.

PEP synthetase has been demonstrated in both archaea and bacteria performing gluconeogenesis; e.g. in *Methanobacterium thermoautotrophicum* (Eyzaguirre et al. 1982), in *Enterobacteriaceae* (see in Cooper and Kornberg 1974) and *Alcaligenes species* (Frings and Schlegel 1971). Pyruvate phosphate dikinase (see in Cooper and Kornberg 1974) has been demonstrated e.g.

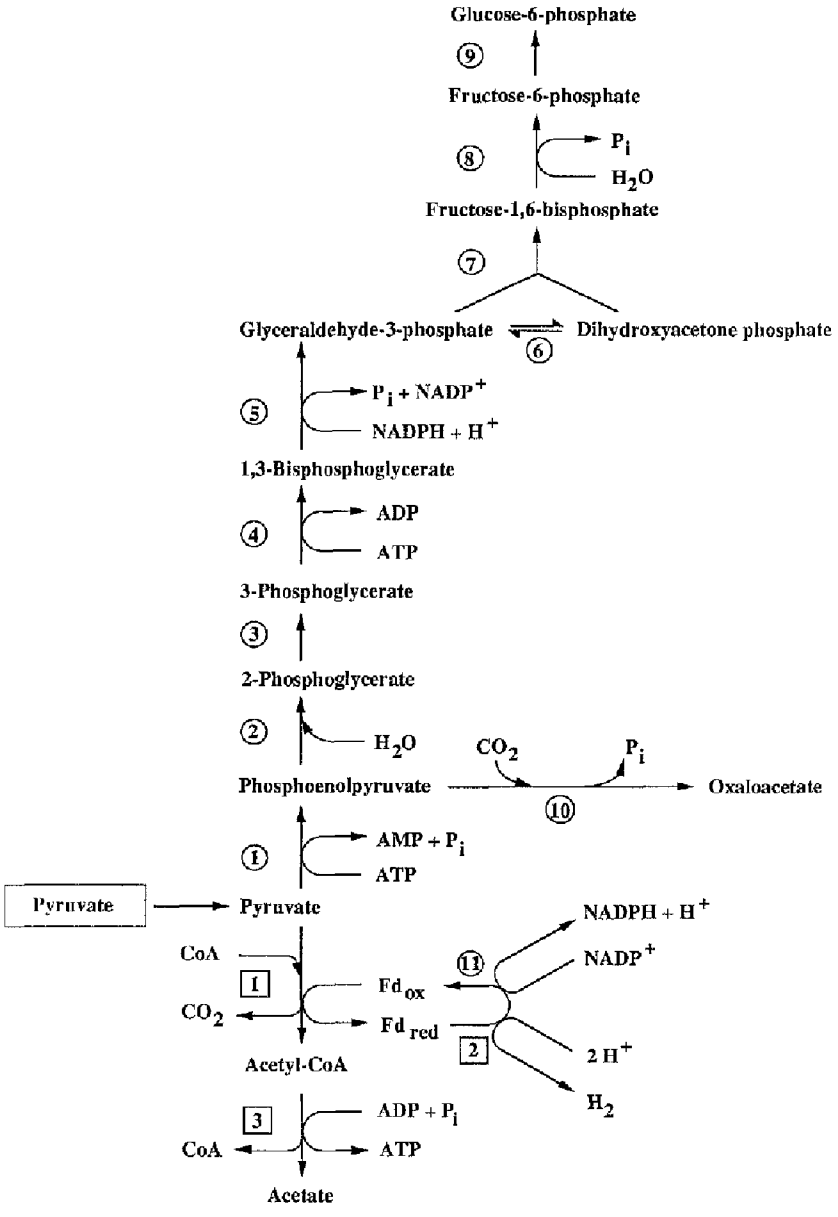

Fig. 1. Pathway of gluconeogenesis (glucose-6-phosphate formation) from pyruvate in *Pyrococcus furiosus*. The scheme includes reactions catalyzing the formation of NADPH and oxaloacetate, and the catabolic reactions involved in pyruvate fermentation to acetate, CO_2 and H_2. The numbers in circles refer to the anabolic enzymes involved: ① phosphoenolpyruvate synthetase; ② enolase; ③ phosphoglycerate mutase; ④ phosphoglycerate kinase; ⑤ glyceraldehyde-3-phosphate dehydrogenase ($NADP^+$-specific); ⑥ triosephosphate isomerase; ⑦ fructose-1,6-bisphosphate aldolase; ⑧ fructose-1,6-bisphosphate phosphatase; ⑨ hexosephosphate isomerase; ⑩ phosphoenolpyruvate carboxylase; ⑪ ferredoxin:$NADP^+$ oxidoreductase. The numbers in boxes refer to the catabolic enzymes involved (Schäfer and Schönheit 1991): [1] pyruvate: ferredoxin oxidoreductase; [2] hydrogenase; [3] acetyl-CoA synthetase (ADP-forming)

in some phototrophs (Buchanan 1974), in *Propionibacterium shermanii* (Evans and Wood 1971), and in *Acetobacterium woodii* (Eden and Fuchs 1983).

All enzymes of the Emden-Meyerhof pathway catalyzing phosphoenolpyruvate (PEP) conversion to fructose-1,6-bisphosphate (FBP) are present in cells grown on pyruvate as well as on maltose. However, kinetic and regulatory properties clearly show that in vivo the enzymes are involved in gluconeogenesis rather than in sugar degradation. The effects are most pronounced with glyceraldehyde-3-phosphate dehydrogenase. The enzyme of *P. furiosus* couples with both $NADP^+$ and NAD^+ as electron acceptor. The apparent K_m for $NADP^+$ (0.05 mM) was 32 fold-lower than for NAD^+ (1.6 mM) indicating that $NADP^+$ is the physiological cofactor of the glyceraldehyde-3-phosphate dehydrogenase. This dual cofactor ($NADP^+$, NAD^+) specificity of glyceraldehyde-3-phosphate dehydrogenase with preference for $NADP^+$ has also been described in several other archaea, e.g. *Methanobacterium thermoautotrophicum* (Zeikus et al. 1977), *Methanothermus fervidus* (Fabry and Hensel 1987) and *Pyrococcus woesei* (Zwickl et al. 1990). The enzymes of the latter two organisms have been purified. Two different glyceraldehyde-3-phosphate dehydrogenases — one specific for $NADP^+$ and the other specific for NAD^+ — have been purified from the sulfur-dependent archaeon *Thermoproteus tenax* (Hensel et al. 1987). Generally, glyceraldehyde-3-phosphate dehydrogenases from autotrophic and heterotrophic eubacteria and eucaryotes are specific for either NAD^+ or $NADP^+$ (for a discussion see in Hensel et al. 1987). Also, other dehydrogenases in archaea show a dual cofactor specificity (see in Danson 1988).

The following findings suggest a biosynthetic role of glyceraldehyde-3-phosphate dehydrogenase in *P. furiosus* (Tables 2 and 3): (i) The value of V_{max}/apparent K_m

of 1,3-phosphoglycerate reduction with NADPH was 32-fold higher than that of glyceraldehyde-3-phosphate oxidation with $NADP^+$ showing that the enzyme is kinetically controlled to operate in the direction of gluconeogenesis. (ii) The specific activity of glyceraldehyde-3-phosphate dehydrogenase was 10-fold higher in pyruvate-grown cells than in maltose-grown cells indicating that the enzyme was induced during growth on pyruvate, when gluconeogenesis is required.

Kinetic and regulatory properties of other enzymes also indicate a function in gluconeogenesis: (i) The apparent K_m of phosphoglycerate mutase for 2-phosphoglycerate (0.3 mM) was by a factor of 21 lower than that for 3-phosphoglycerate (6.3 mM) indicating that the enzyme catalyzes in vivo the formation of 3-phosphoglycerate. (ii) The specific activity of both phosphoglycerate kinase and FBP phosphatase was about 4-fold higher in pyruvate-grown cells as compared to maltose-grown cells. (iii) FBP phosphatase was not affected by AMP. AMP is a negative allosterical effector of FBP phosphatases of those organisms, in which the Embden-Meyerhof pathway is involved in both sugar catabolism and gluconeogenesis. Under these conditions FBP phosphatase has to be regulated in order to prevent a futile cycle of the phosphofructokinase/FBP phosphatase couple (see in Pontremoli and Horecker 1971; Daldal and Fraenkel 1983). AMP regulation of FBP phosphatase has not been observed in organisms which do not contain phosphofructokinase, e.g. in the obligate lithoautotroph *Methanobacterium thermoautotrophicum* (Fuchs et al. 1983) or in *Alcaligenes* and *Nocardia* species (see in Abdelal and Schlegel 1974; Amachi and Bowien 1979), which degrade sugars via the Entner-Doudoroff pathway rather than by the Embden-Meyerhof pathway. Thus, the absence of AMP regulation of FBP phosphatase in *P. furiosus* is in accordance with the operation of a modified "non-phosphorylated" Entner-Doudoroff pathway in sugar degradation rather than of the Embden-Meyerhof pathway (Schäfer and Schönheit 1992).

Various reductive biosynthetic reactions, including the reduction of 1,3-bisphosphoglycerate, require NADPH as electron donor. *P. furiosus* contains high activities of both pyruvate: ferredoxin oxidoreductase and ferredoxin: $NADP^+$ oxidoreductase, which can generate NADPH at the expense of pyruvate oxidation. Ferredoxin: $NADP^+$ oxidoreductases are widely distributed in phototrophic and heterotrophic eubacteria (Gottschalk 1986; Thauer et al. 1977); however, to our knowledge, this is the first report on the presence of such an enzyme in archaea. For distribution of pyruvate: ferredoxin oxidoreductases in archaea see Schäfer et al. (1992).

P. furiosus contains PEP carboxylase activity as anaplerotic enzyme catalyzing the formation of oxaloacetate. PEP carboxylase has been detected before in other archaea, such as *M. thermoautotrophicum* (Jansen et al. 1982) and *Thermoproteus neutrophilus* (Schäfer et al. 1986).

So far, gluconeogenesis in archaea has been investigated in methanogens and extremely halophilic organisms. Gluconeogenesis in the lithoautotrophic *M. thermoautotrophicum* has been studied in detail by Fuchs and coworkers (for literature see Fuchs and Stupperich 1982, 1986): In this organism acetyl-CoA is formed from 2 CO_2 via the acetyl-CoA/carbon monoxide dehydrogenase pathway and pyruvate is formed by pyruvate synthase. From kinetic properties of some enzymes involved in pyruvate conversion to glucose-6-phosphate (Eyzaguirre et al. 1982; Zeikus et al. 1977; Fuchs et al. 1983), it was followed that sugar formation operates unidirectional. The same pathway of gluconeogenesis and similar kinetic properties of the enzymes have been described in this study for *P. furiosus*.

Sugar metabolism has also been studied with extreme halophilic archaea, in particular with *Halobacterium*, *Haloarcula* and *Haloferax* species (Tomlinson et al. 1974; Altekar and Rangaswamy 1992; Danson 1988). There is evidence that glucose is converted in these organisms to pyruvate via a modified partially phosphorylated Entner-Doudoroff pathway involving 2-keto-3-deoxygluconate kinase and 2-keto-3-deoxy-phosphogluconate aldolase. The findings that *H. halobium* contains FBP aldolase, FBP phosphatase and hexosephosphate isomerase after growth on complex media containing citrate rather than on sugars (Danson 1988; Hochstein 1988) indicate that these enzymes are involved in gluconeogenesis. FBP aldolase has also been found in other halophiles and has been purified from *Haloarcula vallismortis* (Krishnan and Altekar 1991). Recently, it has been reported that a modified Embden-Meyerhof pathway is operating in fructose catabolism in *Haloarcula* and *Haloferax*. These organisms contain ketohexokinase and fructose-1-phosphate kinase catalyzing fructose conversion to FBP (Altekar and Rangaswamy 1991, 1992).

In summary, all archaea investigated so far, use the reverse Embden-Meyerhof pathway for gluconeogenesis. This is also true for all (eu)bacteria and eukarya.

The question why gluconeogenesis from pyruvate occurs via the Embden-Meyerhof pathway rather than by the reversal of the Entner-Doudoroff pathway or its modifications can be explained as follows: The free energy change associated with glucose conversion to pyruvate is conserved to a different extent in the form of ATP: 2 mol ATP are formed in the Embden-Meyerhof pathway, 1 mol ATP in the Entner-Doudoroff pathway or its partially phosphorylated version. Sugar degradation to pyruvate in the "non-phosphorylated" Entner-Doudoroff pathway is not coupled with net ATP synthesis (Danson 1988; Schäfer and Schönheit 1992). Thus, the Embden-Meyerhof pathway shows the highest degree of reversibility among the sugar degradation pathways by conserving most of the free energy in the form of ATP. The Entner-Doudoroff pathway is less reversible and sugar degradation to pyruvate via the "non phosphorylated" Entner-Doudoroff pathway proceeds essentially irreversible. Accordingly, most enzymes of the Embden-Meyerhof pathway operate reversibly; the irreversible pyruvate kinase and phosphofructokinase can be reversed by different enzymes, ATP consuming synthetase and FBP phosphatase. In contrast, the Entner-Doudoroff pathway or the "non phosphorylated" Entner-Doudoroff pathway contain several irreversibly

operating enzymes, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydratase or glucose dehydrogenase, gluconate dehydratase, glyceraldehyde dehydrogenase and glycerate kinase (2-phosphoglycerate forming), respectively. So far, no enzymes have been described which catalyze the reversal of these reactions in vivo.

The hyperthermophilic archaeon *P. furiosus* is considered to be one of the most ancestral organisms and thus exhibits an ancient phenotype (Woese 1987; Woese et al. 1990). Thus, the presence of enzymes of the Embden-Meyerhof pathway in *P. furiosus* indicates that this pathway appeared early in evolution. The finding that all organisms – archaea, (eu)bacteria and eucarya – contain reactions of the Embden-Meyerhof pathway for gluconeogenesis indicates that the pathway has evolved before diversification of the three domains.

All archaea investigated so far lack 6-phosphofructokinase; thus, the "classical" Embden-Meyerhof pathway appears not to be operative as a catabolic pathway within the archaeal domain. It rather seems to be an "invention" of the eukarya and (eu)bacteria.

The "classical" catabolic Embden-Meyerhof pathway is already present in the hyperthermophilic *Thermotoga maritima*, which appears to be the deepest branch-off of the heterotropic (eu)bacteria. It contains 6-phosphofructokinase and all other enzymes of the Embden-Meyerhof pathway in catabolic activities (Schröder and Schönheit, in preparation).

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