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ORIGINAL PAPER

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Role of two different glyceraldehyde-3-phosphate dehydrogenases in controlling the reversible Embden–Meyerhof–Parnas pathway in *Thermoproteus tenax*: regulation on protein and transcript level

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Abstract The hyperthermophilic archaeum *Thermoproteus tenax* uses a variant of the Embden–Meyerhof–Parnas (EMP) pathway as the main route for carbohydrate metabolism. This variant is characterized by a reversible nonallosteric PP_i-dependent phosphofructokinase and two glyceraldehyde-3-phosphate dehydrogenases differing in cosubstrate specificity, phosphate dependence, and allosteric behavior. Although the nonphosphorylating NAD^+-dependent glyceraldehyde-3-phosphate dehydrogenase (GAPN; E.C. 1.2.1.8) fulfills exclusively catabolic purposes, the phosphorylating $NADP^+$-dependent glyceraldehyde-3-phosphate dehydrogenase ($NADP^+$-GAPDH; E.C. 1.2.1.13) exhibits anabolic features. The gene encoding the $NADP^+$-GAPDH was cloned, sequenced, and expressed in *Escherichia coli*. The deduced protein sequence displayed 47%–53% sequence identity to archaeal phosphorylating GAPDHs. The kinetic parameters of the $NADP^+$-GAPDH showed a clear preference for the reductive reaction with a 5-fold-higher specific activity in the reductive reaction as compared to the oxidative reaction and a 20-fold-lower K_m for 1,3-bisphosphoglycerate as compared to glyceraldehyde-3-phosphate. Contrary to GAPN, the enzyme is not allosterically regulated. The coding gene overlaps by 1 bp with a preceding open reading frame coding for 3-phosphoglycerate kinase (PGK; E.C. 2.7.2.3). Northern analyses identified mono- and bicistronic messages of both genes in an equimolar ratio. Transcript levels and specific activity of $NADP^+$-GAPDH and PGK were 3- to 4-fold higher under autotrophic conditions as compared to heterotrophic conditions, whereas transcript abundance and specific activity of GAPN remained constant in autotrophically and heterotrophically grown cells. The different regulation of the two counteracting glyceraldehyde-3-phosphate dehydrogenases is discussed with respect to the flux control of the *T. tenax*-specific EMP variant.

Key words Archaea · Gene organization · Carbohydrate metabolism · Phylogeny · Thermoadaptation

Introduction

To study carbohydrate metabolism and its regulation in hyperthermophilic *Archaea*, we focused on the crenarchaeote *Thermoproteus tenax*. This organism is able to grow lithoautotrophically as well as organoheterotrophically in the presence of complex organic substrates such as glucose, starch, and amylose (Zillig et al. 1981), providing an opportunity to obtain insight into the regulatory response of its central metabolism to changing trophic conditions. For degradation of carbohydrates, *T. tenax* possesses two different pathways, a nonphosphorylative Entner–Doudoroff (ED) pathway and a variant of the classical Embden–Meyerhof–Parnas (EMP) pathway (Siebers and Hensel 1993; Siebers et al. 1997). Labeling experiments with 1-[^{13}C]glucose revealed that the EMP pathway is the prominent route of degradation during growth on glucose (Siebers et al. 1997; Selig et al. 1997). The EMP variant of *T. tenax* is characterized by a reversible PP_i-dependent phosphofructokinase (PFK), which is not regulated allosterically (Siebers et al. 1998), and by the presence of two glyceraldehyde-3-phosphate dehydrogenases differing in cosubstrate specificity (Hensel et al. 1987).

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The nicotinamide adenine dinucleotide (NAD^+) -dependent glyceraldehyde-3-phosphate dehydrogenase was recently identified as nonphosphorylating GAPDH (GAPN; E.C. 1.2.1.8), belonging to the superfamily of aldehyde dehydrogenases (Brunner et al. 1998). Because of its nonphosphorylating, irreversible mode of catalysis, i.e., oxidizing glyceraldehyde-3-phosphate (GAP) directly to 3-phosphoglycerate (3-PG), the function of GAPN is confined to glycolysis. The enzyme exhibits a complex allosteric regulation, being activated by AMP, ADP, and glucose 1-phosphate as well as fructose 6-phosphate and inhibited by NADP(H) and NADH, thus indicating that the enzyme plays a central role in controlling the catabolic carbon flux through the EMP pathway and compensates the lacking regulatory potential of the reversible nonallosteric PP_i-dependent PFK.

The $NADP^+$-dependent glyceraldehyde-3-phosphate dehydrogenase ($NADP^+$-GAPDH; E.C. 1.2.1.13), on the other hand, is strictly phosphate dependent. Contrary to all other known archaeal phosphorylating GAPDHs using both NAD(H) and NADP(H) as cosubstrates, the enzyme of *T. tenax* is characterized by an absolute specificity for NADP(H) (Hensel et al. 1987). As indicated by N-terminal sequencing of the protein (Hensel et al. 1987), however, the enzyme belongs, despite its differing cosubstrate specificity, to the typical archaeal GAPDHs. The homology of these enzymes to the classical GAPDHs from Bacteria and Eukarya has been seriously doubted because of their low sequence similarity to the latter (Doolittle et al. 1990). As shown only recently by three-dimensional structure analysis of the GAPDH of *Sulfolobus solfataricus* and *Methanothermus fervidus* (Isupov et al. 1999; Charron et al. 2000), however, the phosphorylating archaeal GAPDH exhibits high structural similarity to the eucaryal or bacterial enzymes identifying the archaeal GAPDHs as homologues of the classical GAPDHs, thus finally confirming the common origin of the phosphorylating GAPDHs of all three domains (Hensel et al. 1989).

To analyze the physiological role of the $NADP^+$-GAPDH in *T. tenax* in more detail and to acquire insight into the regulation of the anabolic flux of the EMP pathway of the organism, we investigated the regulation of this enzyme at transcript and protein level.

Materials and methods

Chemicals and plasmids

DL-GAP was prepared from the monobarium salt of the diethyl acetal (Sigma, Deisenhofen, Germany) according to the manufacturer's instructions; all other chemicals (Pro Analysis grade) were from Fluka (Deisenhofen, Germany) or Merck (Darmstadt, Germany). 1,3-bisphosphoglycerate (1,3-BPG) was prepared from glyceraldehyde 3-phosphate by the method of Fufine and Velick (Fufine and Velick 1965). The vectors pBluescript II KS+ (Stratagene, Heidelberg, Germany) and pET 15b (Novagen, Madison, WI, USA) were used for cloning of restriction fragments and heterologous expression, and the vector pSPT 19 (Roche Molecular Biochemicals, Mannheim, Germany) was used for generating antisense mRNA.

Bacterial strains and growth conditions

For cloning and expression experiments, the *Escherichia coli* strains XL1-Blue (Stratagene), DH5α (Life Technologies, Karlsruhe, Germany) and BL21(DE3) (Novagen) were grown under standard conditions (Sambrook et al. 1989). Mass cultures of *Thermoproteus tenax* Kra 1 (DSM 2078) were grown at 86°C in an enameled 100-l fermenter (Braun Biotec International, Melsungen, Germany) in a medium according to Brock et al. (1972) containing, however, only 5 g elemental sulfur and 20 mg yeast extract per liter. For heterotrophic growth, 0.02% glucose was added. Cultures were continuously gassed with 80% H_2/20% CO_2 (autotrophic growth) or 80% H_2/20% N_2 (heterotrophic growth) at a flow rate of 1 l/min and stirred at 250 rpm. Cells were harvested in the late logarithmic phase. After cooling down to 10°C by a plate heat exchanger, sulfur was removed by twofold passage through a folded filter (Schleicher & Schuell, Dassel, Germany) and the cells were concentrated by cross-flow filtration in a Pellicon Acryl system (Millipore, Eschborn, Germany).

Cloning procedures

Genomic DNA from *T. tenax* was prepared as described previously (Weil et al. 1988; Meakin et al. 1991). The gene encoding $NADP^+$-GAPDH was identified using the consensus oligonucleotide 5′-GGRTATGGRACDATWGGNAA-3′, which was 3′-end labeled with digoxigenin according to the manufacturer's instructions (Roche Molecular Biochemicals) and hybridized to genomic DNA immobilized on nylon membranes (Hybond N+; Amersham, Freiburg, Germany) by capillary blotting (Chomczynski 1992). Southern blots were incubated overnight at 37°C and stringently washed for 2 ×15 min at 37°–40°C in 0.5×SSC. Chemiluminescence detection (CDP-Star; Tropix, Bedford, MA, USA) performed according to Engler-Blum et al. (1993) revealed strong hybridization signals with a 2.3-kb *Cla*I and a 5.8-kb *Hin*dIII fragment, which were cloned and sequenced with the aid of an Automated Laser Fluorescent DNA Sequencer (Amersham).

Sequence handling and phylogenetic analysis

For sequence analysis and computer alignments the programs GENMON, version 4.4 (GBF, Braunschweig) and CLUSTAL W (Thompson et al. 1994) were used. The source of sequence information was GenBank. Phylogenetic trees were calculated with PROTPARS and NEIGHBOR-JOINING/ UPGMA as part of the PHYLIP program package, version 3.5c (Felsenstein 1996).

Table 1. Molar fraction of D-$GAP_{aldehyde}$ in aqueous solution at different temperatures

Temperature (°C)	15	20	25	30	35	40	45	50	60	70	80	90
D-$GAP_{aldehyde}$ (%)	3.0	3.7	4.5	5.4	6.5	7.7	9.2	10.6	14.1	18.3	23.1	28.3

Values above 45°C were calculated by extrapolation of the D-$GAP_{aldehyde}$/ D-GAP_{diol} equilibrium constants in the linear van't Hoff plot

Northern blot analyses

Total RNA from *T. tenax* was isolated with TRIzol (Life Technologies) according to the manufacturer's protocol and separated by denaturing gel electrophoresis. The gels contained 1% agarose, 2% formaldehyde, and 1×MOPS [(*N*-2-morpholino) propane sulfonic acid] buffer (20 mM MOPS, 5 mM Na acetate, 1 mM ethylenediaminetetraacetic acid [EDTA], pH 7.0). After capillary transfer to positively charged nylon membranes (Roche Molecular Biochemicals), blots were hybridized with digoxigenin-labeled, gene-specific antisense mRNA. Antisense mRNA was generated by in vitro transcription from a T7 promoter after cloning internal gene fragments of GAPN (1,125 bp; positions 379–1,503), $NADP^+$-GAPDH (1,009 bp; positions 11–1,019), and 3-phosphoglycerate kinase (PGK) (1,066 bp; positions 133–1,198) into vector pSPT 19 (Roche Molecular Biochemicals). Hybridization proceeded for 4–16 h at 60°C in DIG Easy Hyb (Roche Molecular Biochemicals) containing 50 ng labeled probe per milliliter. Chemiluminescence detection was performed according to Engler-Blum et al. (1993). Transcript lengths were determined using a RNA molecular size standard (Roche Molecular Biochemicals). Densitometric quantification of the hybridization signals was performed using an Ultroscan XL Enhanced Laser Densitometer (Pharmacia).

Determination of free aldehyde of D-GAP in aqueous solution at elevated temperatures

In aqueous solution, aldehydes such as GAP exist in two states, the free aldehyde and the diol form. Only the free aldehyde of D-GAP (D-$GAP_{aldehyde}$) is the substrate for GAPDH (Trentham et al. 1969). For calculating the affinity of the *T. tenax* GAPDHs to their true substrate at temperatures near the growth optimum of the organism, the temperature dependence of the D-$GAP_{aldehyde}$/ D-GAP_{diol} equilibrium in aqueous solution was determined. The determinations were performed enzymatically according to Trentham et al. (1969), using a stopped-flow apparatus (SX.18MV, Applied Photophysics, Surrey, UK) thermostatically controlled over a temperature range of 15° to 45°C (the upper temperature limit of the equipment). One syringe contained 2.4 mg/ml rabbit muscle GAPDH (Sigma Aldrich, Deisenhofen, Germany) activated according to Souza and Radi (1998) in assay buffer (200 mM triethanolamine, 30 mM sodium arsenate, 1 mM EDTA, 2 mM NAD^+, pH 8.6), and the other contained 2.8–5.6 mM DL-GAP (or D-GAP, respectively) in the same buffer. Kinetics of NADH formation were followed at 340 nm (light path, 0.2 cm) over 200 ms. The portion of D-$GAP_{aldehyde}$ at temperatures higher than 45°C was calculated from the extrapolated van't Hoff plot of the D-$GAP_{aldehyde}$/ D-GAP_{diol} equilibrium (Table 1).

Preparation of cell-free extracts and enzyme assays

Frozen cells were thawed in 3 ml buffer A (100 mM Tris/HCl, pH 8.0; 250 mM β-mercaptoethanol) per gram wet cells, passed three times through a French press at 200 MPa, and centrifuged (100,000 *g*, 30 min, 4°C). After heat precipitation (90°C, 30 min), extracts were centrifuged again (100,000 *g*, 20 min, 4°C) and dialyzed overnight against 1 l buffer containing 100 mM Tris/HCl, pH 8.0, and 25 mM β-mercaptoethanol. $NADP^+$-GAPDH was assayed in 100 mM Tris/HCl buffer containing 150 mM K_2HAsO_4 (pH 7.0 at 70°C; total volume, 1 ml) in the presence of 5 mM $NADP^+$ and 4 mM DL-GAP in the oxidative direction (70°C) and in the presence of 0.2 mM NADPH and 0.5 mM 1,3-BPG in the reductive direction (45°C). 3-Phosphoglycerate kinase (PGK; E.C. 2.7.2.3) activity was determined using the phosphorylating GAPDH from *Methanothermus fervidus* as auxiliary enzyme at 70°C (Fabry et al. 1988). The assay (total volume, 1 ml) contained 250 mM KCl, 100 mM MOPS, 10 mM $MgCl_2$, 15 mM 3-PG, and 0.2 mM NADPH (pH 7.0 at 70°C). To avoid background activity caused by the presence of oxygen, all stock solutions were prepared in an anaerobic tent and cuvettes were filled under argon atmosphere. GAPN was assayed as described previously (Brunner et al. 1998).

Purification of $NADP^+$-GAPDH and N-terminal sequencing

Purification of $NADP^+$-GAPDH from *T. tenax* was performed according to Hensel et al. (1987). For N-terminal sequencing, the protein obtained after chromatography on Blue Sepharose CL6B (Pharmacia) was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970), transferred to ProBlott membranes (Applied Biosystems) by semidry electrotransfer (Jungblut et al. 1990), and analyzed by Edman degradation in a gas-phase Sequencer 473 A (Applied Biosystems).

Heterologous expression of the $NADP^+$-GAPDH and purification of the enzyme

The *gap* gene was cloned into pET 15b after PCR amplification using the mutagenic primers 5′-CTCAGAAGTTCTG-

GTCATGAAGGTCGC-3′ and 5′-CCAGTTTATAGGCC TGATCATCTATTGTAT-3′. The sequence of the expression clone was confirmed by sequencing of both strands.

Expression in *E. coli* BL21(DE3) was performed using standard procedures (Sambrook et al. 1989). For purification, 10 g of *E. coli* cells (wet weight) were resuspended in 100 mM Tris/HCl (pH 8.0) containing 30 mM β-mercaptoethanol and passed three times through a French press cell at 150 MPa. After centrifugation (50,000 *g*, 30 min, 4°C), 0.5 vol buffer was added, and the suspension was heat precipitated (90°C, 30 min) and centrifuged again (20,000 *g*, 20 min, 4°C). Cell-free extracts were dialyzed against 100 mM Tris/HCl (pH 8.0) containing 7.5 mM dithiothreitol and separated by chromatography on hydroxylapatite High Resolution (Fluka) as described previously (Hensel et al. 1987). Active fractions were pooled, dialyzed against 50 mM HEPES/KOH (pH 7.5) containing 300 mM KCl, and concentrated to a volume of 7 ml by microfiltration (Centricon 30; Amicon, Witten, Germany). Homogeneous enzyme preparations were obtained by gel filtration on a HiLoad 26/60 Superdex Prepgrade column (Amersham Pharmacia) equilibrated in dialysis buffer. The average recovery of homogeneous protein ranged from 0.3 to 0.5 mg/g cells of *E. coli* (wet weight). Protein solutions (1 mg/ml) were stored at 4°C under N_2 atmosphere. The predicted N-terminal amino acid sequence (MKVAIV) was confirmed by Edman degradation in a gas-phase Sequenator 473 A (Applied Biosystems).

Results and discussion

The NADP⁺-GAPDH gene and gene organization

Hybridization experiments with a consensus oligonucleotide directed against a highly conserved region of the cosubstrate-binding domain of archaeal phosphorylating GAPDHs gave strong signals with a 2.3-kb *Cla*I and a 5.8-kb *Hin*dIII genomic fragment. Subsequent sequencing led to the identification of the gene encoding NADP⁺-GAPDH (*gap*). The deduced protein sequence of the reading frame showed 47%–53% identity with known archaeal phosphorylating GAPDHs and corresponds exactly with the N-terminal amino acid sequence of the NADP⁺-GAPDH (MKVAIVGYGTIGKRVA) isolated from *T. tenax* in this study. However, it deviates from the formerly published protein sequence (Hensel et al. 1987) at some positions, probably as a result of impurities of the earlier preparation.

The *gap* gene (1,029 bp) is preceded by a second open reading frame (1,203 bp) encoding a protein that showed high similarity to archaeal 3-phosphoglycerate kinases (PGK), 35%–45% identity. The coding regions of both reading frames overlap by 1 bp, resembling the *pgk-gap* gene cluster in *Sulfolobus solfataricus,* which displays a 8-bp overlap of both genes (Jones et al. 1995). A corresponding juxtaposition of both genes in the same order could also be found in the sequenced genomes of other Crenarchaeota such as *Pyrobaculum aerophilum* (Fitz-Gibbon et al. 1997) and *Aeropyrum pernix* (Kawarabayasi et al. 1999) but, surprisingly, not in Euryarchaeota.

Downstream of the *gap* gene, three additional open reading frames were detected (Fig. 1). The first (*βglc*) is orientated in the same direction as *gap* and *pgk* and encodes a protein with significant similarity to family 1 type β-glucosidases (approximately 32% identity to archaeal β-glycosidases). Further downstream, two open reading frames followed [*rs17*, *rs19* (truncated), in the opposite direction]. Their deduced protein sequences showed significant similarity to ribosomal proteins of the small subunit (approximately 43% identity to archaeal RS17 or 52% identity to archaeal RS 19, respectively). The complete sequence of the 5,847-bp *Hin*dIII fragment is available in the EMBL database under accession number Y10126.

Phylogenetic analyses with NADP⁺-GAPDH and PGK

The deduced protein sequence of the NADP⁺-GAPDH displayed 47%–53% sequence identity to known archaeal

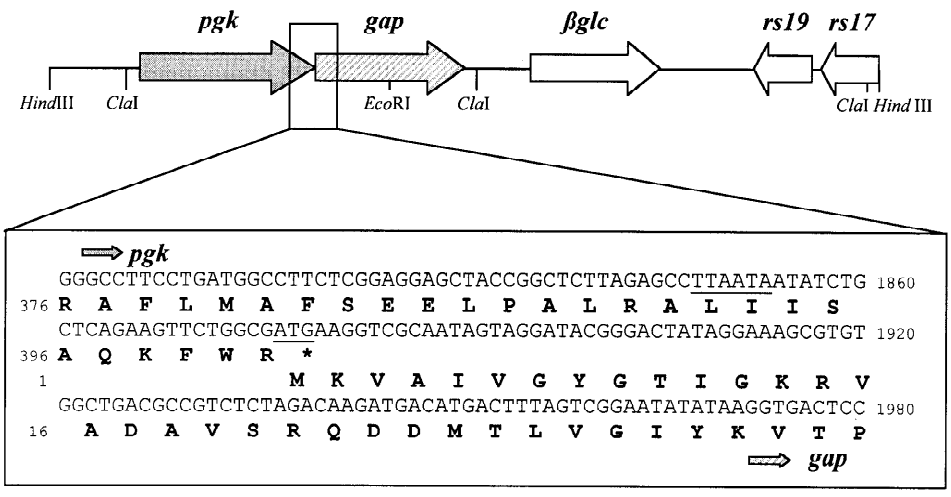

Fig. 1. The 5.8-kbp *Hin*dIII genomic fragment of *Thermoproteus tenax* harboring *pgk* and *gap*. *Arrows* represent open reading frames and their orientation. The enlargement shows the overlapping region of *pgk* and *gap* with the encoded protein sequence in *bold*. The putative BRE site, promoter box A element, and the start codon of *gap* are *underlined*. *Asterisk* indicates the stop codon of *pgk*

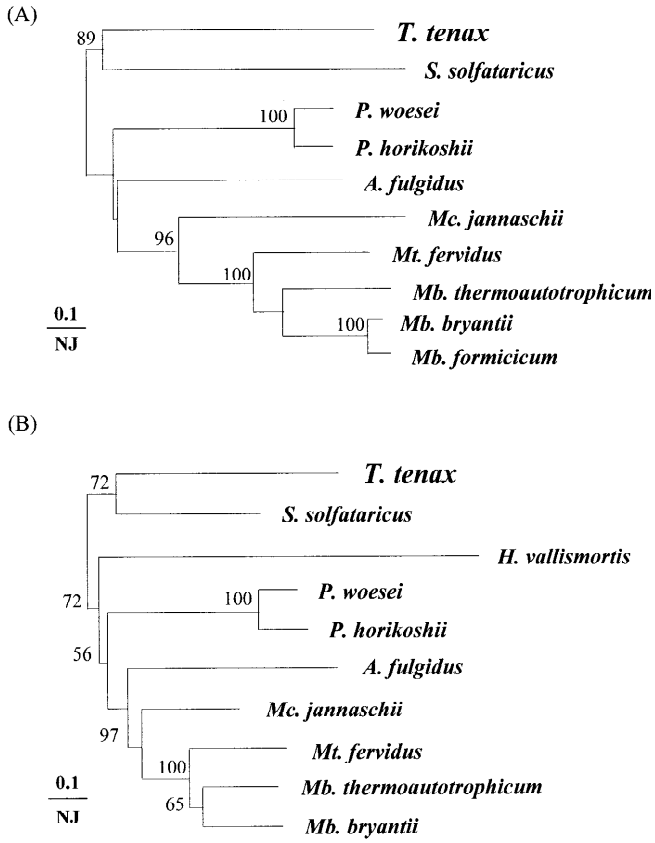

Fig. 2. Phylogenetic tree of archaeal glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (**A**) and 3-phosphoglycerate kinase (3-PGK) (**B**). Both trees are based on distance matrix analyses (neighbor-joining method) of protein sequences from 10 representatives. *Numbers* at nodes indicate bootstrap values greater than 50 for 100 replicates (neighbor-joining/parsimony). *Bar* corresponds to 0.1 nonsynonymous substitution per site. *T.*, *Thermoproteus*; *S.*, *Sulfolobus*; *P.*, *Pyrococcus*; *A.*, *Archaeoglobus*; *Mc.*, *Methanococcus*; *Mt.*, *Methanothermus*; *Mb.*, *Methanobacterium*; *H.*, *Haloarcula*

GAPDH sequences, characterizing the *T. tenax* enzyme as a true homologue of archaeal phosphorylating GAPDHs. Similar to other archaeal phosphorylating GAPDHs, the *T. tenax* enzyme showed only a low similarity (12%–16% identity) to eucaryal and bacterial GAPDHs. Phylogenetic analyses of archaeal phosphorylating GAPDHs resulted in the same tree topology as deduced from 16S rRNA comparisons (Fig. 2A), giving no hints for an evolutionary diversification into various paralogous lineages as observed for bacterial GAPDHs (Cerff 1995).

Respective analyses with the deduced PGK sequence exhibiting 35%–45% sequence identity with other known archaeal PGKs mainly confirmed the tree topology for GAPDH or 16S rRNA, respectively (Fig. 2B). Deviating from the "conventional" topology, however, the *Haloarcula vallismortis* PGK branches off as the earliest diverging line within the Euryarchaeota, thus possibly representing a second, paralogous lineage within the archaeal PGK phylogeny, as recently proposed by Brinkmann and Martin (1996).

Table 2. Kinetic parameters of nonphosphorylating NAD^+-dependent glyceraldehyde-3-phosphate dehydrogenase (GAPN) and $NADP^+$-dependent glyceraldehyde-3-phosphate dehydrogenase ($NADP^+$-GAPDH) of *Thermoproteus tenax*

Parameters	Assay temperature (°C)	GAPN	$NADP^+$-GAPDH
K_m for substrates (μM)			
D-GAP[a]	45	20	200
	70	20	450
1,3-BPG[b]	45	–	10
K_m for cosubstrates (μM)			
NAD^+[c]	45	1,000	–
	70	3,100	–
$NADP^+$[d]	45	–	100
	70	–	150
V_{max} (U/mg)			
D-GAP[a]	45	3	2
	70	36	17
1,3-BPG[b]	45	–	10

[a]Assay conditions: 20 μM–2 mM D-GAP, 20 mM NAD^+ (4 mM $NADP^+$)
[b]20 μM–0.5 mM 1,3-BPG (1,3-bisphosphoglycerate), 0.5 mM NADPH
[c]4 mM DL-GAP, 0.5–20 mM NAD^+
[d]4 mM DL-GAP, 5 μM–2 mM $NADP^+$

Characterization of the recombinant $NADP^+$-GAPDH

To describe the properties of the $NADP^+$-GAPDH of *T. tenax* in more detail, the *gap* gene was expressed in *E. coli*. The purified recombinant enzyme exhibits kinetic properties very similar to those of the formerly described $NADP^+$-dependent GAPDH purified from *T. tenax* (Hensel et al. 1987). Like the latter, the recombinant enzyme catalyzes the reversible interconversion of D-GAP and 1,3-BPG, using $NADP^+$ exclusively as cosubstrate and exhibiting a clear preference for the reductive reaction (5-fold-higher specific activity in the reductive direction as compared to the oxidative reaction) (Table 2). NAD^+ has no influence on the activity of the enzyme. Because only minor deviations from the formerly described enzyme were observed with respect to K_m values of 1,3-BPG and $NADP^+$, the *gap* gene product obviously corresponds with the originally described $NADP^+$-GAPDH isolated from *T. tenax*.

The enzymic description of the $NADP^+$-GAPDH was complemented by the parameters of GAP saturation determined in the present study. The enzyme was specific for D-GAP; L-GAP did not act as substrate or competitive inhibitor. The enzyme showed classical Michaelis–Menten kinetics with D-GAP. For determining the K_m value for GAP under thermophilic conditions, the portion of the free aldehyde of D-GAP (D-$GAP_{aldehyde}$), which represents the physiologically relevant state of the substrate binding directly to GAPDH, was determined at elevated temperatures. Considering the temperature dependence of the $GAP_{aldehyde}$-GAP_{diol} equilibrium (see Table 1), the K_m values for D-

$GAP_{aldehyde}$ were determined to be 200 μM at 45°C and 450 μM at 70°C, respectively, indicating approximately 20-fold-lower affinity to D-$GAP_{aldehyde}$ as compared to the substrate of the reductive direction [$K_{m\ 1,3\text{-}BPG}$, 10 μM (45°C)] (Table 2). Thus, both the higher V_{max} of the reductive reaction (10 U/mg compared to 2 U/mg of the oxidative reaction, 45°C) and the lower K_m value for 1,3-BPG account for the anabolic function of the enzyme. As compared to the $NADP^+$-GAPDH, the GAPN showed a significantly higher affinity to D-$GAP_{aldehyde}$, reflected by a K_m value of only 20 μM at 45°C and 70°C (Table 2).

The activity of the $NADP^+$-GAPDH proved to be insensitive toward the presence of various metabolites and intermediates. No influence on activity was observed assaying the enzyme at half-saturating substrate and cosubstrate concentrations with 1 mM and 10 mM AMP, ADP, ATP, cAMP, NADH, dihydroxyacetone phosphate, fructose-6-phosphate, fructose-1,6-phosphate, fructose-1-phosphate, glucose-6-phosphate, glucose-1-phosphate, gluconate, phosphoenolpyruvate, pyruvate, and citrate. Thus, in contrast to GAPN, the $NADP^+$-GAPDH is obviously not allosterically regulated by heterotropic effectors.

Analyses of *gap* and *gapN* transcripts and their abundance in autotrophically and heterotrophically grown cells

As shown by Northern analyses documented in Fig. 3, *gapN* is transcribed as a monocistronic message of approximately 1.6 kb. In contrast to that, using RNA probes derived either from *gap* or *pgk*, two transcript species of approximately 2.4 and 1.1 kb (*gap*) or of 2.4 and 1.3 kb (*pgk*), respectively, could be detected. Because antisense *gap* and *pgk* RNA hybridized with the 2.4-kb transcript and additionally with a smaller RNA species corresponding in size with *gap* or *pgk*, respectively, we conclude that bi- and monocistronic messages are generated from both genes. The ratio between mono- and bicistronic messages was approximately 1:1 and was not influenced by growth conditions. Although we cannot decide at the moment whether the shorter transcripts are generated by mRNA processing or by alternative transcription termination and initiation, the overlap of both reading frames and the presence of a putative BRE and promoter box A motif within *pgk* 32 or 22 bp 5′ to the *gap* start codon, respectively (see Fig. 1), favors the idea of an alternative transcription termination at the beginning of *gap* and a concomitant transcription start at the end of *pgk*.

Quantifications of Northern blot signals from cells grown on either CO_2 (autotrophic conditions) or glucose (heterotrophic conditions) revealed that the abundance of *gap* and *pgk* mRNA (considering both mono- and bicistronic messages) was 3- to 4-fold higher under autotrophic conditions as compared to heterotrophic conditions, whereas the level of *gapN* transcripts remained constant (Fig. 4). Concomitant determination of enzyme activities in crude extracts of autotrophically and heterotrophically grown cells showed a similar response to growth conditions as observed for the transcript levels: as demonstrated in Fig. 4, the specific activity of GAPN proved to be similar under either condition (mean value, 0.042 U/mg), whereas $NADP^+$-GAPDH and PGK displayed a 3- to 4-fold-higher specific activity under autotrophic conditions as compared to heterotrophic conditions ($NADP^+$-GAPDH, 0.035 U/mg versus 0.012 U/mg; PGK, 0.011 U/mg versus 0.003 U/mg).

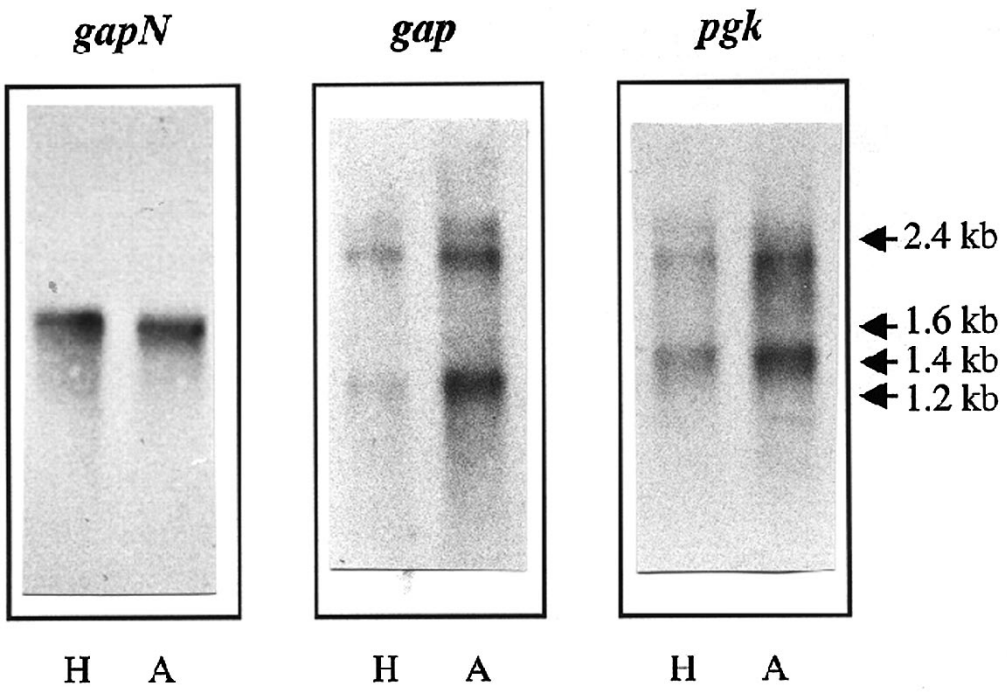

Fig. 3. Northern blot analyses of *gapN*, *gap*, and *pgk* in cells grown on glucose (*H*) or CO_2 (*A*), respectively; 10 μg of total RNA was loaded per lane. The size of the hybridization bands in kilobases (kb) is shown to the *right*

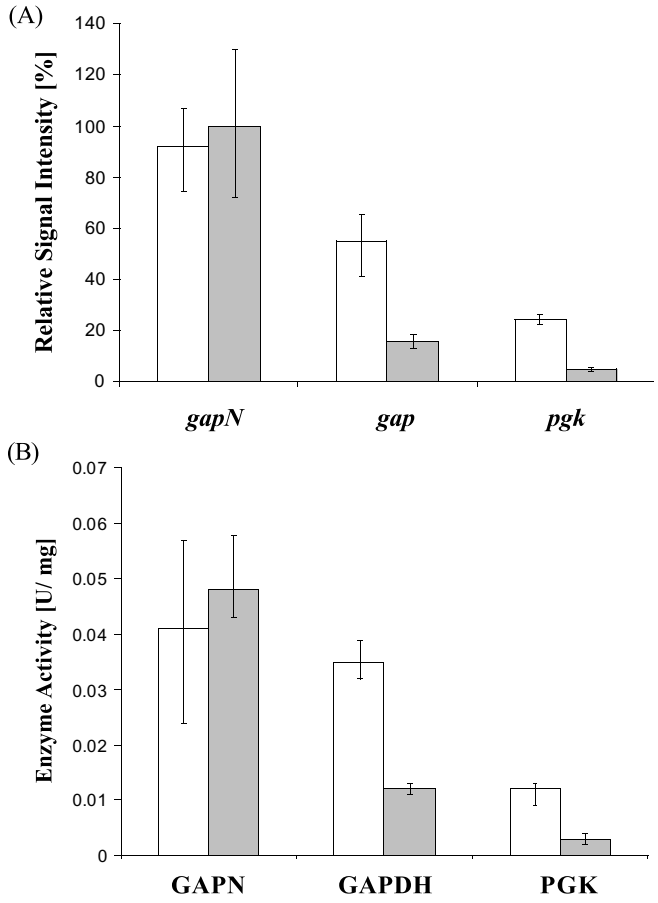

Fig. 4. mRNA abundance of *gapN*, *gap*, and *pgk* (**A**) and specific activity of the encoded enzymes (**B**) in autotrophically (*light columns*) and heterotrophically grown cells (*dark columns*). *Bars* indicate error margins (n=6)

This parallelism of transcript abundance and specific activity of NADP$^+$-GAPDH and PGK suggests that the observed changes in specific activities of these enzymes are caused by changed transcript levels.

Regulation and supposed physiological role of NADP$^+$-GAPDH and GAPN in *T. tenax*

The enzymic properties of NADP$^+$-GAPDH and GAPN of *T. tenax* are indicative of their physiological functions. Thus, in the case of the phosphorylating NADP$^+$-GAPDH, a 20-fold-lower K_m value for 1,3-BPG as compared to GAP and an approximately 5-fold-higher specific activity in the reductive reaction (see Table 2) clearly indicate its preference for the gluconeogenic reaction. The anabolic function of the enzyme is further supported by its higher specific activity and the higher transcript level of its coding gene under autotrophic conditions. Contrary to that, the nonphosphorylating NAD$^+$-dependent GAPN catalyzes the irreversible oxidation of GAP to 3-PG and thus fulfills explicitly catabolic purposes. Differing also from NADP$^+$-GAPDH, the specific activity of GAPN as well as the transcript level of its coding gene (*gapN*) are virtually not affected by the trophic conditions. To avoid futile cycling – especially under autotrophic conditions in which the anabolic NADP$^+$-GAPDH is preferentially expressed – the activity of the enzyme is allosterically controlled (Brunner et al. 1998). As we know from in vitro measurements, the enzyme is strongly inhibited by NADP$^+$ and NADPH, the cosubstrates of the phosphorylating NADP$^+$-GAPDH, and by an high energy charge of the cell. Thus, under productive anabolic conditions, we would not expect any significant activity of the enzyme, which could reoxidize GAP to 3-PG. It is active, however, under conditons in which energy conservation is necessary, i.e., at low energy charge and in the presence of available energy resources, as indicated by its activation by AMP and ADP or fructose 6-phosphate, an early intermediate of glycolysis, and glucose 1-phosphate, an intermediate of glycogen metabolism. Its allosteric properties not only allow it to react immediately to a change of carbon source (from CO_2 to carbohydrate or vice versa) by inverting the carbon flux but also to immediately switch on its catabolic activity even under autotrophic conditions, if the energy charge falls below the lower threshold for anabolism and if carbohydrates for generation of energy are available. Thus, the activities of both GAPDHs are controlled on different levels, allowing a short-term and a long-term adaptation of the EMP pathway in *T. tenax*.

Strikingly, also in the catabolic EMP pathways of other hyperthermophilic Archaea (*Pyrococcus furiosus, Thermococcus celer, Thermococcus litoralis, Desulfurococcus amylolyticus*) (Mukund and Adams 1995; Selig et al. 1997), GAP is oxidized by an irreversible reaction catalyzed, however, by ferredoxin-dependent GAP oxidoreductases (GAPOR). However, more detailed information about the regulation of the enzymes in dependence of growth conditions is available only for *P. furiosus* (van der Oost et al. 1998). In contrast to *T. tenax*, the irreversible GAP oxidizing enzyme (GAPOR) of *P. furiosus* is not allosterically regulated, but the level of transcripts of its coding gene depends on trophic conditions; also, the phosphorylating GAPDH of this organism shows higher activity under anabolic growth conditions than under catabolic conditions, although the transcript level of the coding gene remains constant under either condition.

Interestingly, all modifications of the EMP pathway described so far in Archaea (Selig et al. 1997) are restricted to hyperthermophiles, suggesting that these variants are related to life at high temperature. Thus, under the aspect of thermoadaptation, the involvement of an irreversibly working, nonphosphorylating enzyme for catalyzing the conversion of GAP to 3-PG (GAPN or GAPOR) would provide at least two advantages for the thermophilic metabolism: (i) avoidance of the extremely heat-labile intermediate 1,3-BPG and (ii) an enforced unidirectional carbon flux, which could decrease the pools of labile intermediates above the reaction step, such as GAP and dihydroxyacetone phosphate (DHAP), and thus reduce the velocity of their heat destruction.

An indication for a low GAP concentration in *T. tenax* is the unusually high affinity of GAPN for D-$GAP_{aldehyde}$ (K_m: 20 μM; see Table 2). Also, the properties of the pyruvate kinase (PK), another enzyme of the catabolic EMP variant of *T. tenax*, can be interpreted in terms of metabolic thermoadaptation and fit well into the supposed strategy of minimizing pools of labile intermediates. This enzyme is not regulated by heterotropic effectors as usually found for PKs of mesophilic organisms but only exhibits positive binding cooperativity with phosphoenol pyruvate (PEP) and divalent metal ions, which, obviously, do not allow the enzyme to exert its common function as a stringent throttle valve at the last step of the catabolic pathway. As a consequence, an accumulation of intermediates below the GAPN reaction including PEP, another labile EMP compound, can be avoided. On the other hand, the low K_m for 1,3-BPG of $NADP^+$-GAPDH (10 μM) suggests that, for anabolism, low intermediate concentrations are also adjusted in the hyperthermophilic cell. However, for a better understanding of the specific features of the EMP variants in hyperthermophilic Archaea, complete knowledge about the properties of all enzymes involved in the pathway, their influence on carbon flux, and the pool size of intermediates is necessary.

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