Biochemical adaptations of two sugar kinases from the hyperthermophilic archaeon *Pyrococcus furiosus*

Corné H. VERHEES*[1], Denise G. M. KOOT*, Thijs J. G. ETTEMA*, Cor DIJKEMA†, Willem M. DE VOS* and John VAN DER OOST*

*Laboratory of Microbiology, Department of Agrotechnology and Food Sciences, Wageningen University, The Netherlands, and †Laboratory of Biophysics, Wageningen University, Hesselink van Suchtelenweg 4, 6703 CT Wageningen, The Netherlands

The hyperthermophilic archaeon *Pyrococcus furiosus* possesses a modified Embden–Meyerhof pathway, including an unusual ADP-dependent glucokinase (ADP-GLK) and an ADP-dependent phosphofructokinase. In the present study, we report the characterization of a *P. furiosus* galactokinase (GALK) and its comparison with the *P. furiosus* ADP-GLK. The pyrococcal genes encoding the ADP-GLK and GALK were functionally expressed in *Escherichia coli*, and the proteins were subsequently purified to homogeneity. Both enzymes are specific kinases with an optimal activity at approx. 90 °C. Biochemical charac-

terization of these enzymes confirmed that the ADP-GLK is unable to use ATP as the phosphoryl group donor, but revealed that GALK is ATP-dependent and has an extremely high affinity for ATP. There is a discussion about whether the unusual features of these two classes of kinases might reflect adaptations to a relatively low intracellular ATP concentration in the hyperthermophilic archaeon *P. furiosus*.

Key words: adenine nucleotide, ADP-dependent glucokinase, ATP-dependent galactokinase.

INTRODUCTION

During the catabolic conversion of carbohydrates, sugar molecules are activated by specific kinases to a phosphorylated form (sugar + ATP → sugar-P + ADP). The universal energy carrier of biological systems and the preferred phosphoryl group donor in most kinase reactions is ATP. However, glucose can also be phosphorylated by polyphosphate or by phosphoenolpyruvate (PEP) as a part of the phosphotransferase systems, and fructose 6-phosphate by PP_i instead of ATP [1–3]. Sugar kinases of the central catabolic pathways can be classified into at least four different monophyletic enzyme families [4] (http://www.scop.mrc-lmb.cam.ac.uk/scop/). Glucokinases (GLKs)/hexokinases (HKs) generally belong to the HK family. Phosphofructokinases (PFKs) belong to the PFKA family, or to the ribokinase (PFKB) family. Galactokinases (GALKs) are classified under the GALK family. For HK, PFKA and PFKB families, three-dimensional structures are available [5–7], but for GALK, it is yet to be solved.

Two sugar kinases have been identified in the hyperthermophilic archaeon *Pyrococcus furiosus* that differ considerably from the canonical glycolytic kinases by being dependent on ADP rather than ATP [8]. The ADP-dependent GLK has been purified from *P. furiosus* cell extracts and the protein was biochemically characterized [9,10]. The gene encoding the ADP-dependent PFK from *P. furiosus* was expressed in *Escherichia coli* and the protein was studied in detail. Analyses of the primary structure revealed that the ADP-GLK and ADP-PFK belong to the same enzyme family [11]. Recently [12], the crystal structure of the ADP-GLK from *Thermococcus litoralis* revealed a similar fold as the ATP-dependent ribokinase family.

An intriguing question is why *P. furiosus* contains ADP-dependent kinases such as ADP-GLK and ADP-PFK in its

central metabolic pathway. A plausible reason is that the ADP-dependent kinases enable *P. furiosus* to recover more easily after periods of starvation. As soon as glucose becomes available, phosphorylation of glucose can proceed due to the high ADP level under these conditions. An alternative explanation could be the fact that ADP is more stable than ATP at elevated temperatures, with half-lives of 750 and 115 min respectively at 90 °C [13]. However, several hyperthermophilic species with similar optimum growth temperatures ($T_{opt} \geqslant 80$ °C), such as *Thermotoga maritima* ($T_{opt} = 80$ °C) or *Desulfurococcus amylolyticus* ($T_{opt} = 90$ °C), are known to use ATP in the phosphorylation of sugars [14]. But it cannot be ruled out that the intracellular ATP concentration is relatively low in *P. furiosus*, either because of a distinct physiology or because of the even more extreme optimum growth temperature ($T_{opt} = 100$ °C).

Genome analysis revealed that an orthologue of a *GALK* gene is present in *P. furiosus* (http://www.genome.utah.edu). The ATP-dependent GALK catalyses the first step in galactose metabolism [15]. This eucaryal and bacterial enzyme has been extensively studied, but the archaeal GALK has never been described or studied. Hereditary deficiency of GALK in man results in galactosemia, galactosuria and cataracts in early childhood [16]. Interestingly, the putative GALK from *P. furiosus* is very similar to the bacterial and eucaryal GALK. Similar to the genetic composition of some bacterial GALK sequences, the *P. furiosus* sequence is clustered on the genome with other genes that encode enzymes involved in galactose metabolism, e.g. α-galactosidase and galactose-1-phosphate uridylyltransferase. In the present study, we describe that the *P. furiosus* GALK is ATP-dependent, implying that ADP- and ATP-dependent sugar kinases co-exist in this hyperthermophilic archaeon. A comparison of the characteristics of the *E. coli*-produced kinases from *P. furiosus*, the

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ATP-dependent GALK and the ADP-GLK reveals distinct adaptations of sugar kinases to function optimally at extreme temperatures.

EXPERIMENTAL

Materials

ADP (monopotassium salt, $< 0.2\%$ ATP), ATP (disodium salt), GDP (dilithium salt), glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate: NADP oxidoreductase, EC 1.1.1.49; yeast), GTP (dilithium salt), PEP (tricyclohexylammonium salt), lactate dehydrogenase (EC 1.1.1.27; pig heart), phosphoglucose isomerase (D-glucose-6-phosphate ketol isomerase, EC 5.3.1.9; yeast), phosphomannose isomerase (D-mannose-6-phosphate ketol isomerase, EC 5.3.1.8; yeast) and pyruvate kinase (EC 2.7.1.40; rabbit muscle) were obtained from Roche Molecular Biochemicals (Monza, Italy). CDP (sodium salt), D-galactose, 2-deoxy-D-glucose, kanamycin A (monosulphate, $< 5\%$ kanamycin B), NADP (sodium salt) and NADH (disodium salt) were purchased from Sigma (Bornem, Belgium). D-glucose, D-fructose, D-glucosamine and D-mannose were obtained from Merck (Darmstadt, Germany). All chemicals were of analytical grade.

Organisms and growth conditions

P. furiosus (DSM 3638) was obtained from the German Collection of Microorganisms (DSM Braunschweig, Germany) and was routinely grown at 90 °C, as described previously [17]. *E. coli* XL1 Blue (Stratagene, Europe, Amsterdam, The Netherlands) was used as a host for the construction of pET9d derivatives (Novagen). *E. coli* BL21(DE3) (Stratagene) was used as an expression host. Both strains were grown in Luria–Bertani medium with kanamycin (50 μg/ml) in a rotary shaker at 37 °C.

Cloning of the sugar kinase genes in *E. coli*

Based on the N-terminal sequence [9], the putative *ADP-GLK* gene (GenBank® accession nos AF127910 and E14588 [10], NCBI) was identified as described previously [11]. The following primer set was designed to amplify this open reading frame (ORF) by PCR: BG451 (5′-GCGCG**CCATGG**CACCCACTTG-GGAGGAGCTTTA-3′, sense) and BG452 (5′-GCGCG**GGAT-CC**TTAGAGAGTGAATGAAAACTCACCAA-3′, antisense), with *Nco*I and *Bam*HI restriction sites in boldface.

An orthologue of a classical GALK (GenBank® accession no. AF195244, NCBI) was identified in the *P. furiosus* genome database (http://www.genome.utah.edu). The N-terminus was based on the presence and proper spacing of the ribosomal binding site and annotation from the genome sequence. The following primer set was designed to amplify this ORF by PCR: BG376 (5′-GCGCGCCATGGCAAGTAAAATCACTGTAA-AATCT-3′, sense) and BG377 (5′-GCGCGGGATCCTCAT-ACTCCCACACCATCGGAG-3′, antisense), with *Nco*I and *Bam*HI restriction sites in boldface.

The procedure for cloning of the *GALK* and *ADP-GLK* genes was essentially the same. Chromosomal DNA was isolated from *P. furiosus* as described by Sambrook et al. [18]. The PCR mixture (100 μl) contained 100 ng of *P. furiosus* DNA, 100 ng of each primer, 0.2 mM of dNTPs, *Pfu* polymerase buffer and 5 units of *Pfu* DNA polymerase. The mixture was subjected to 35 cycles of amplification (1 min at 94 °C, 45 s at 60 °C and 3 min 30 s at 72 °C) on a DNA Thermal Cycler (PerkinElmer Life Sciences, Great Shelford, Cambridge, U.K.). The PCR products were digested with *Nco*I–*Bam*HI and cloned into an *Nco*I–*Bam*HI-digested pET9d vector, resulting in pLUW570 and pLUW574 respectively. Sequence analyses of pLUW570 and

pLUW574 were done by the dideoxynucleotide chain-termination method with a Li-Cor automatic sequence system (model 4000L). Sequence data were analysed using the computer program DNASTAR.

Overexpression of the sugar kinase genes in *E. coli*

An overnight culture of *E. coli* BL21(DE3) containing pLUW570 or pLUW574 was used as a 1% inoculum in 1 litre of Luria–Bertani medium with 50 μg/ml of kanamycin. After growth for 16 h at 37 °C, cells were harvested by centrifugation (2200 *g* for 20 min) and resuspended in 10 ml of 50 mM Tris/HCl buffer (pH 7.8). The suspension was passed twice through a French press (100 MPa) and the cell debris was removed by centrifugation (10 000 *g* for 20 min). The resulting supernatant was used for the purification of the *E. coli*-produced sugar kinases.

Purification of the sugar kinases

For the purification of *E. coli*-produced GALK and ADP-GLK, the *E. coli* cell-free extracts were heated for 30 min at 70 °C, and the precipitated proteins were removed by centrifugation. The supernatant containing GALK and ADP-GLK was filtered by a 0.45 μm filter and loaded on to a Q-Sepharose fast-flow column (25 ml; Amersham Pharmacia Biotech, Roosendael, The Netherlands) that was equilibrated with 50 mM Tris/HCl buffer (pH 8.5) and 50 mM Tris/HCl buffer (pH 7.8) respectively. Bound proteins were eluted by a linear gradient of NaCl (0–1 M in Tris/HCl buffer). The GALK and ADP-GLK were eluted at 0.40 and 0.27 M NaCl respectively. Active fractions were pooled and desalted with 50 mM Tris/HCl buffer (pH 7.8), using a Centricon filter with a 10 kDa cutoff. The concentrated extracts were further purified on a Superdex 200 HR 10/30 gel-filtration column (24 ml, Amersham Pharmacia Biotech), equilibrated with 50 mM Tris/HCl (pH 7.8) and 100 mM NaCl. The *E. coli*-produced GALK and ADP-GLK eluted at 15.4 and 12.8 ml respectively. The purified enzymes were desalted in 50 mM Tris/HCl (pH 7.8) as described above. To prevent microbial contamination, all the protein samples contained 0.02% sodium azide and were stored at 4 °C until use.

Determination of standard enzyme activity

GALK activity was determined by measuring the oxidation of NADH in a coupled assay with pyruvate kinase from rabbit muscle and lactate dehydrogenase from pig heart. One unit was defined as the amount of enzyme required to convert 1 μmol of galactose/min. The standard assay was performed at 50 °C. At this temperature, the rabbit and pig enzymes remained active and the *P. furiosus* enzyme was sufficiently active to measure its activity. The standard assay mixture contained 100 mM Tris/HCl (pH 7.8), 2 mM EDTA, 10 mM MgCl$_2$, 0.2 mM NADH, 15 mM D-galactose, 5 mM ATP, 2 mM PEP, 2 units of pyruvate kinase, 4 units of lactate dehydrogenase and 5–50 μl of enzyme preparation. NADH was monitored spectrophotometrically (ϵ_{340} 6220 M$^{-1} \cdot$ cm^{-1}). The auxiliary enzymes were present in excess to ensure that the detected NADH oxidation corresponded to the GALK activity. ADP-GLK activity at 50 °C was determined as described by Kengen et al. [9]. Protein concentrations were determined with Coomassie Brilliant Blue G-250 as described previously [19].

Substrate specificity

For the determination of the substrate specificity of GALK, the standard enzyme assay was used. Instead of D-galactose, either D-glucose, D-fructose, D-mannose, 2-deoxy-D-glucose or

D-glucosamine was added as substrate. The divalent cation requirement was tested by adding 10 mM each of $MnCl_2$, $CaCl_2$, $ZnCl_2$ or $CoCl_2$, instead of $MgCl_2$ to the standard assay mixture. Phosphoryl group donor specificity of GALK was determined by HPLC. The assay mixture contained 100 mM Tris/HCl buffer (pH 7.8), 2 mM EDTA, 10 mM $MgCl_2$, 10 mM galactose and 10 mM of a phosphoryl group donor (either ATP, ADP, GTP, PEP or PP_i). After incubation for an appropriate time at 50 °C, the reaction was stopped on ice and analysed by HPLC. To test whether GALK phosphorylates galactose into galactose-1-phosphate, ^{13}C-/^{31}P-NMR spectra of the conversion of [1-^{13}C]-galactose by the purified GALK were recorded at 76.47 MHz [^{13}C] and 125.5 MHz [^{31}P] on an AMX300 spectrometer (Bruker, Germany) using a 10 mm (outer diameter) probe. The incubation was continued for 12 min at 80 °C, and 1 min spectra were recorded. The presence of α-galactose-1-phosphate was confirmed by spiking with commercial α-galactose-1-phosphate (Sigma). Substrate specificity of the ADP-GLK was determined as described by Kengen et al. [9].

Molecular-mass determination

The molecular masses of GALK and ADP-GLK were determined on a Superdex 200 HR 10/30 gel-filtration column (24 ml; Amersham Pharmacia Biotech) using 50 mM Tris/HCl buffer (pH 7.8) with 100 mM NaCl. The column was calibrated using the following standard proteins: bovine pancreas ribonuclease A (13.7 kDa), bovine pancreas chymotrypsinogen A (25 kDa), hen's-egg ovalbumin (43 kDa), bovine pancreas serum albumin (67 kDa), rabbit muscle aldolase (158 kDa) and bovine liver catalase (232 kDa).

Optimum pH

The optimum pH of GALK and ADP-GLK were determined in the standard enzyme assay at 50 °C in 200 mM Tris/maleate buffer over the pH range 3.9–8.4. The pH of the buffer was adjusted at the temperature of incubation.

Optimum temperature

The effect of temperature on the activity of the sugar kinases was determined by incubating an appropriate amount of purified enzyme in 1 ml of crimp-sealed vials containing 200 mM of Tris/maleate buffer (pH 8.5), 20 mM $MgCl_2$ and 20 mM of galactose and glucose respectively. The vials were submerged in an oil bath at temperatures ranging from 30 to 110 °C and preheated for 5 min, and the enzyme reaction was started by injecting 10 μl of 100 mM ATP and ADP respectively. After 1, 3 and 5 min, the reaction was stopped by placing the vials on ice, and the amount of product formed was determined spectrophotometrically at room temperature, by measuring the oxidation of NADH and the reduction of NADP in the standard enzyme assays for GALK and ADP-GLK respectively. Corrections were made for the chemical conversion of ATP in the absence of GALK.

Kinetic parameters

Kinetic parameters of GALK were determined at 50 and 90 °C, in 100 mM MOPS (pH 7.0), by varying the concentration of ATP (0.0005–5 mM) or galactose (0.05–10 mM), in the presence of 5 mM galactose or 2 mM ATP respectively. Kinetic parameters of ADP-GLK were determined at 50 and 90 °C in 200 mM Tris/maleate (pH 7.0), by varying the concentration of ADP (0.02–2 mM) or glucose (0.1–10 mM) in the presence of 15 mM

glucose or 2 mM ADP respectively. At 50 °C a continuous assay was used, whereas at 90 °C a discontinuous assay was used, as described in the Optimum temperature section. Data were analysed by computer-aided direct fit to the Michaelis–Menten curve (TABLECURVE program).

RESULTS AND DISCUSSION

Overexpression and purification of the GALK and ADP-GLK

The ORFs predicted to encode the *P. furiosus* GALK (1059 bp) and ADP-GLK (1368 bp) were PCR-amplified and cloned into pET9d, resulting in plasmids pLUW570 and pLUW574 respectively. DNA sequence analysis of pLUW570 and pLUW574 confirmed the cloning of the correct ORFs into pET9d. SDS/PAGE analysis (results not shown) of a heat-treated cell-free extract of *E. coli* BL21(DE3) harbouring either pLUW570 or pLUW574 revealed an additional band of approx. 38 and 51 kDa respectively, which was in good agreement with the calculated molecular mass of the gene product (39.4 and 51.2 kDa). A heat-treated cell-free extract of *E. coli* BL21(DE3) harbouring pLUW570 was found to contain a thermoactive ATP-dependent GALK activity of 0.7 units/mg, confirming the identity of the gene. In the heat-treated cell-free extract of *E. coli* BL21(DE3) harbouring pLUW574, an ADP-GLK activity of 17.5 units/mg was measured, confirming that the gene indeed encoded an ADP-GLK. In extracts of *E. coli* BL21(DE3) carrying the pET9d vector without insert, the additional protein bands in SDS/PAGE analyses were absent and neither GALK nor ADP-GLK activity was detected.

GALK and ADP-GLK could be produced up to 20 and 10 % of total soluble cell protein respectively, after growth for 16 h at 37 °C. Both enzymes were purified to apparent homogeneity (> 95 %; results not shown) by two successive chromatographic steps. The molecular masses of GALK and ADP-GLK were determined by gel-filtration chromatography to be approx. 32 and 89 kDa respectively, suggesting that GALK is a monomer and ADP-GLK is a dimer. The dimeric structure of the *E. coli*-produced ADP-GLK is in good agreement with that of the native ADP-GLK, which has a molecular mass of 93 kDa [9]. Classical GALKs in general occur as monomers or dimers [16,20,21], which agrees well with the determined monomeric structure of the *P. furiosus* GALK.

Comparison of the primary structure

Orthologues of the *P. furiosus* GALK were identified in a wide range of bacteria and eucarya, with a high degree of identity (*E. coli*; 32 % identity P06976 and human; 31 % identity NP_000145). No orthologues could be identified in any of the archaeal or hyperthermophilic bacterial genomes, except for *P. horikoshii* (PH0369 putative GALK, 77 % identity), *T. maritima* (TM1190 putative GALK, 41 % identity) and *T. neapolitana* (putative GALK, 41 % identity). The presence of a GALK orthologue in both *P. furiosus* and *P. horikoshii*, and the absence of this gene in all other available archaeal genomes, including *P. abyssi*, is an example of a gain of genetic information in these *Pyrococci* sp., probably the result of horizontal gene transfer [22]. Analysis of the primary structure of the *P. furiosus* GALK revealed the presence of all typical GALK motifs. These motifs are conserved in most, if not in all, eucaryal and bacterial GALKs, and are located at the N-terminal domain of these enzymes. The presence and location of these motifs are conserved in the GALK of *P. furiosus* as well. Motif 2 consists of a conserved glycine/serine-rich region, which is probably involved in the binding of ATP [23] (Figure 1). This motif is present in
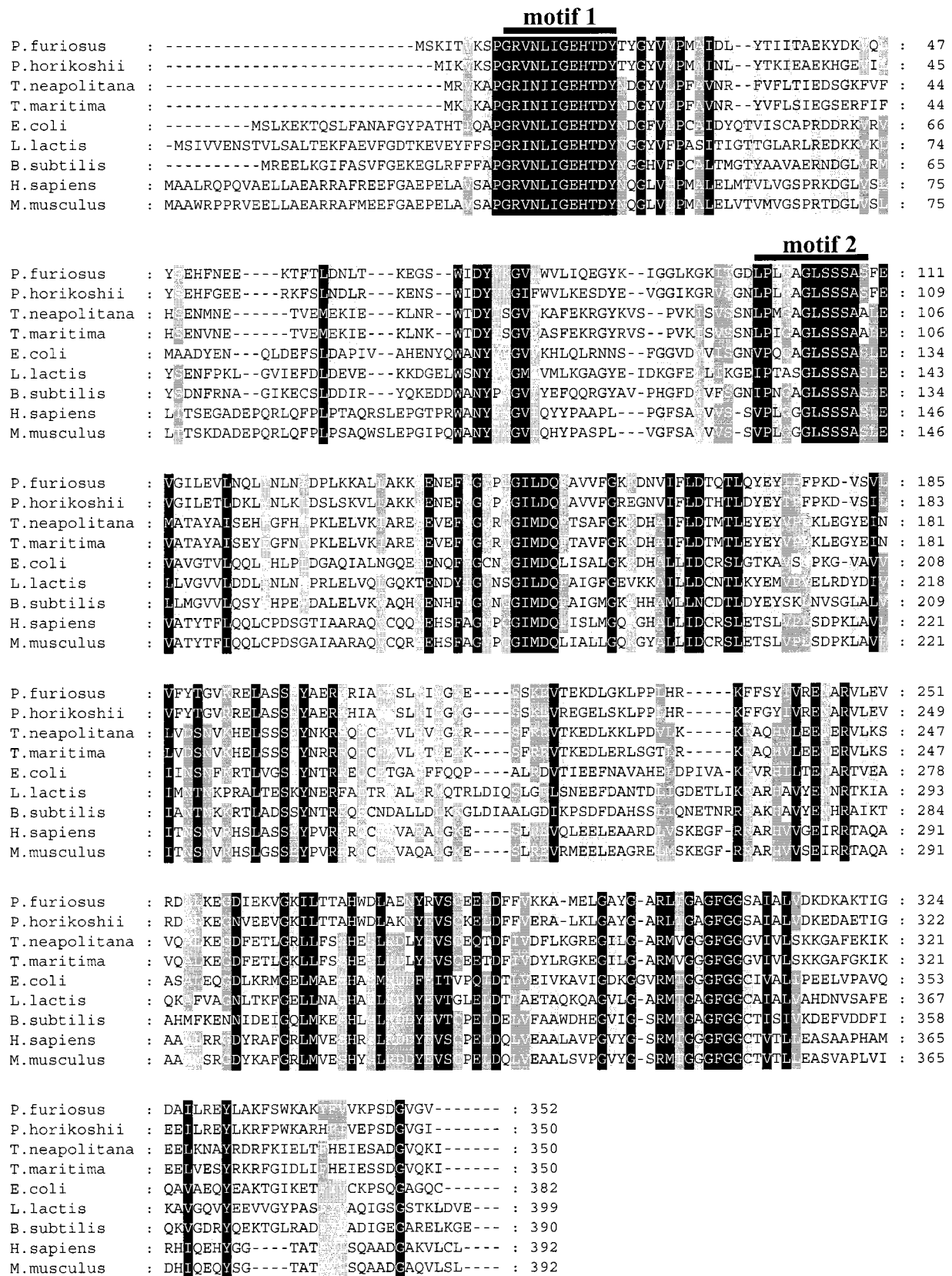
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                                                     motif 1
P.furiosus    : --------------------------MSKIT KSPGRVNLIGEHTDYTYGYV PM IDL--YTIITAEKYDK Q : 47
P.horikoshii  : --------------------------MIK  KSPGRVNLIGEHTDYTYGYV PM INL--YTKIEAEKHGE I  : 45
T.neapolitana : --------------------------MR   KAPGRINIIGEHTDY DGYV PF VNR--FVFLTIEDSGKFVF  : 44
T.maritima    : --------------------------MK   KAPGRINIIGEHTDY DGYV PF VNR--YVFLSIEGSERFIF  : 44
E.coli        : ---------MSLKEKTQSLFANAFGYPATHT QAPGRVNLIGEHTDY DGFV PC IDYQTVISCAPRDDRK R  : 66
L.lactis      : -MSIVVENSTVLSALTEKFAEVFGDTKEVEYFFSPGRINLIGEHTDY GGYVFPAS ITIGTTGLARLREDKK K : 74
B.subtilis    : ----------MREELKGIFASVFGEKEGLRFFFAPGRVNLIGEHTDY GGHVFPC LTMGTYAAVAERNDGL R : 65
H.sapiens     : MAALRQPQVAELLAEARRAFREEFGAEPELA SAPGRVNLIGEHTDY QGLV PM LELMTVLVGSPRKDGL S  : 75
M.musculus    : MAAWRPPRVEELLAEARRAFMEEFGAEPELA SAPGRVNLIGEHTDY QGLV PM LELVTVMVGSPRTDGL S  : 75

                                                              motif 2
P.furiosus    : Y EHFNEE----KTFT DNLT---KEGS--WIDY GV WVLIQEGYK--IGGLKGK GDLPL AGLSSSA FE : 111
P.horikoshii  : Y EHFGEE----RKFS NDLR---KENS--WIDY GIFWVLKESDYE--VGGIKGR GNLPL AGLSSSA FE : 109
T.neapolitana : H ENMNE------TVEMEKIE---KLNR--WTDY SGV KAFEKRGYKVS--PVK S SNLPM AGLSSSAA E : 106
T.maritima    : H ENVNE------TVEMEKIE---KLNK--WTDY SGV ASFEKRGYRVS--PVK S SNLPI AGLSSSAA E : 106
E.coli        : MAADYEN---QLDEFS DAPIV--AHENYQWANY GV KHLQLRNNS--FGGVD V GNVPQ AGLSSSA LE : 134
L.lactis      : Y ENFPKL--GVIEFD DEVE---KKDGEL WSNY GM VMLKGAGYE-IDKGFE L KGEIPTAS GLSSSA LE : 143
B.subtilis    : Y DNFRNA--GIKECS DDIR---YQKEDD WANYP GV YEFQQRGYAV-PHGFD VF GNIPN AGLSSSA LE : 134
H.sapiens     : L TSEGADEPQRLQFP PTAQRSLEPGTPR WANY GV QYYPAAPL---PGFSA V S-SVPL GGLSSSA LE : 146
M.musculus    : L TSKDADEPQRLQFP PSAQWSLEPGIPQ WANY GV QHYPASPL---VGFSA V S-SVPL GGLSSSA LE : 146

P.furiosus    : VGILEV NQL NLN DPLKKAL AKK ENEF G P GILDQ AVVFGK DNVIFLDTQTLQYEY FPKD-VSV : 185
P.horikoshii  : VGILET DKL NLK DSLSKVL AKK ENEF G P GILDQ AVVFGREGNVIFLDTHTLDYEY FPKD-VSI : 183
T.neapolitana : MATAYA SEH GFH PKLELVK ARE EVEF G R GIMDQ TSAFGK DH IFLDTMTLEYEY KLEGYE N : 181
T.maritima    : VATAYA SEY GFN PKLELVK ARE EVEF G R GIMDQ TAVFGK DH IFLDTMTLEYEY KLEGYE N : 181
E.coli        : VAVGTV QQL HLP DGAQIALNGQE ENQF GCN GIMDQ LISALGK DH LLIDCRSLGTKA S PKG-VAV : 208
L.lactis      : LLVGVV DDL NLN PRLELVQ GQKTENDY G NSGILDQ AIGFGEVKK ILLDCNTLKYEM ELRDYDI : 218
B.subtilis    : LLMGVV QSY HPE DALELVK AQH ENHF G N GIMDQ AIGMGK HH MLLNCDTLDYEYSK NVSGLAL : 209
H.sapiens     : VATYTF QQLCPDSGTIAARAQ CQQ EHSFAG P GIMDQ ISLMGQ GH LLIDCRSLETSL SDPKLAV : 221
M.musculus    : VATYTF QQLCPDSGAIAARAQ CQR EHSFAG P GIMDQLIALLGQ GYA LLIDCRSLETSL SDPKLAV : 221

P.furiosus    : VFYTGV RELASS YAER RIA SL I G E----S VTEKDLGKLPP HR-----KFFSY VRE ARVLEV : 251
P.horikoshii  : VFYTGV RELASS YAER HIA SL I G G----S VREGELSKLPP HR-----KFFGY VRE ARVLEV : 249
T.neapolitana : LV SV HELSSS YNKR Q C VL V G R----F VTKEDLKKLPD K-----K AQ LBE ERVLKS : 247
T.maritima    : LV SV HELSSS YNRR Q C VL T E K----F VTKEDLERLSGT R-----K AQ VLBE ERVLKS : 247
E.coli        : II SF RTLVGS YNTR E C TGA FFQQP----AL DVTIEEFNAVAHE DPIVA-K VR LTE ARTVEA : 278
L.lactis      : IM T KPRALTESKYNERFA TR AL R QTRLDIQ LG LSNEEFDANTD GDETLIK AR AVYE NRTKIA : 293
B.subtilis    : IA T K RTLADSSYNTR Q CNDALLD K GLDIAALGDIKPSDFDAHSS QNETNRR AK AVYE HRAIKT : 284
H.sapiens     : IT SV HSLASS YPVR R C VA A G E----L VQLEELEAARD SKEGF-R AR VGEIRRTAQA : 291
M.musculus    : IT SV HSLGSS YPVR R C VAQA G E----L VRMEELEAGRE SKEGF-R AR VVSEIRRTAQA : 291

P.furiosus    : RD KE DIEKVGKIL TTAHWDLAE YRVS EE DFF KKA-MELCAYG-ARL GAGFGGSAIAL DKDKAKTIG : 324
P.horikoshii  : RD KE NVEEVGKIL TTAHWDLAK NYIVS KE DFF ERA-LKLCAYG-ARL GAGFGGSAIAL DKEDAETIG : 322
T.neapolitana : VQ KE DFETLGRLLFS HE L LY IVS EQTDF DFLKGREGILG-ARMVGGGFGGGVIVLSKKGAFEKIK : 321
T.maritima    : VQ KE DFETLGKLLFS HE L LY IVS EETDF DYLRGKEGILG-ARMVGGGFGGGVIVLSKKGAFGKIK : 321
E.coli        : AS EQ DLKRMGELMAE HA M IF ITVPQ DT VEIVKAVIGDKGGVRM GGGFGGCIVAL PEELVPAVQ : 353
L.lactis      : QK FVA NLTKFGELLNA HA L IY VTGLE DT AETAQKQACVLG-ARM GAGFGGCAIAL AHDNVSAFE : 367
B.subtilis    : AHMFKENN IDEIGQLMKE HL L IY VT PE DE FAAWDHECVIG-SRM GAGFGGCTISL KDEFVDDFI : 358
H.sapiens     : AA RR DYRAFGRLMVE HR L IY IVS PE DQ EAALAVPCVYG-SRM GGGFGGCTVTL EASAAPHAM : 365
M.musculus    : AA SR DYKAFGRLMVE HY L IY IVS PE DQ EAALSVPCVYG-SRM GGGFGGCTVTL EASVAPLVI : 365

P.furiosus    : DA ILREY LAKFSWKAK VKPSDGVGV------- : 352
P.horikoshii  : EE ILREY LKRFPWKARH VEPSDGVGI------- : 350
T.neapolitana : EELKNAY RDRFKIELT HEIESADGVQKI------ : 350
T.maritima    : EELVESY RKRFGIDLI HEIESSDGVQKI------ : 350
E.coli        : QAVAEQY EAKTGIKET CKPSQGAGQC------ : 382
L.lactis      : KAVGQVY EEVVGYPAS AQIGSGSTKLDVE--- : 399
B.subtilis    : QKVGDRY QEKTGLRAD ADIGEGARELKGE--- : 390
H.sapiens     : RHIQEHY GG----TAT SQAADGAKVLCL---- : 392
M.musculus    : DHIQEQY SG----TAT SQAADGAQVLSL---- : 392
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Figure 1   Multiple sequence alignment of the deduced amino acid sequence of the *P. furiosus* GALK with sequences of GALKs from bacteria and eucarya

Sequences were deduced from: *P. furiosus* (AAG28454), *P. horikoshii* PH0369 (NP_142343), *T. neapolitana* (O85253), *T. maritima* TM1190 (P56838), *E. coli* (P06976), *L. lactis* (Q9R7D7), *Bacillus subtilis* (P39574), *Homo sapiens* (NP_000145), *Mus musculus* (AAF78226). Accession nos are given in parentheses. Gaps introduced for optimal alignment are marked by hyphens. Completely conserved regions are indicated as black boxes. Highly conserved regions are shaded grey. Conserved motifs are indicated in bars above the alignment. Motif 1: G-R-x-N-[LIV]-I-G-[DE]-H-x-D-Y; GALK signature (PS00106). Motif 2: [LIVM]-[PK]-x-[GSTA]-x(0,1)-G-L-[GS]-S-S-[GSA]-[GSTAC]; GHMP kinases putative ATP-binding domain (PS00627).

GALK, homoserine kinase, mevalonate kinase and phospho-mevalonate kinase. The functions of motif 1 are unknown (Figure 1).

The *P. furiosus* ADP-GLK is unrelated to classsical GLKs/HKs and showed high similarity with ADP-GLKs and ADP-PFKs from several *Pyrococcus* sp. and from *Methanococcus jannaschii* [11]. Recently [24], the functional presence of homologues has been identified in several methanogens, and homologues (with unidentified functions) have been identified in higher eukaryotes. In contrast with GALK, ADP-GLK is phylogenetically unrelated to its canonical counterparts and has probably evolved independently. The specific function of ADP-GLK and ADP-PFK, therefore, might have been invented in the archaea as an adaptation to function optimally under extreme conditions.

Biochemical characteristics and physiology of GALK and ADP-GLK

Two distinct kinases, i.e. ATP and ADP-dependent, are potentially present in *P. furiosus* for galactose and glucose conversion respectively. The presence of enzyme activities of GALK (0.001 units/mg; C. H. Verhees, unpublished work), ADP-GLK (0.4 units/mg) and ADP-PFK (0.2 units/mg) [9] could be demonstrated in extracts of *P. furiosus* grown on starch. Moreover, the presence of both ADP-GLK and GALK transcripts has been established by reverse transcriptase–PCR and primer extension (C. H. Verhees, unpublished work). As expected, ATP-dependent phosphorylation of glucose and galactose could not be detected in *P. furiosus* extracts.

Growth experiments in (fed) batch fermentations have shown that *P. furiosus* is able to ferment the monosaccharide-like glucose, disaccharide-like maltose and cellobiose, and polysaccharide-like starch [13,25]. However, the organism was unable to ferment galactose or galactose-containing polysaccharides, such as carrageenan, galactomannan, *ara*-galactan and stractan. Nevertheless, growth has been observed on lactose (4-*O*-β-D-galactopyranosyl-D-glucose) and melibiose (6-*O*-α-D-galactopyranosyl-D-glucose) as the carbon source. Unfortunately, only the glucose moiety of these substrates is completely fermented (C. H. Verhees, unpublished work). Similar observations were made for *Streptococcus thermophilus* and *Lactococcus bulgaricus* [26,27]. Small mutations in the *galK* promoter of *S. thermophilus* have been suggested to give rise to this deficiency in galactose fermentation. Because candidates for all the gene-encoding enzymes involved in the degradation of galactose are present in *P. furiosus* (PF0445, PF0441, PF1788, PF0588) and the constitutive activity of a key enzyme of galactose metabolism, i.e. GALK, could be measured in cell extracts of *P. furiosus*, it is concluded that the induction of these genes is hampered due to used growth conditions or promoter mutations.

The purified GALK was found to have a specific activity of 0.96 units/mg at 50 °C at its optimum pH of 5.0 in a Tris/maleate buffer, and retained > 50 % of its optimal activity between pH 4.5 and 8.5 (results not shown). Classical GALKs from bacteria and eucarya generally have a more neutral or even alkali optimum pH, e.g. *E. coli* (pH 7.8) [28], *Saccharomyces cerevisiae* (pH 8.3) [21] and *Vicia faba* (pH 7.3) [29]. The *P. furiosus* GALK is the first archaeal and thermoactive GALK known at present, and showed maximal activity at approx. 90 °C (Figure 2). The second most thermoactive GALK studied is the one from *Tetrahymena thermophila* with an optimum temperature of 41 °C [20]. For its activity, the *P. furiosus* GALK required divalent cations, with the highest activity in the presence of Mg^{2+} followed by Mn^{2+}. The enzyme was very specific for its substrate, since the enzyme under the tested conditions could phosphorylate only galactose, and ATP was the only suitable phosphoryl group donor for the enzyme (Table 1). ^{13}C-NMR showed that GALK converted a mixture of α- and β-[1-^{13}C]galactose (being in anomeric equilibrium) into only α-[1-^{13}C]galactose 1-phosphate. This was confirmed by ^{31}P-NMR on spiking with α-galactose-1-phosphate (results not shown). It was thus determined that a single phosphate from ATP was transferred to the C_1 position of galactose producing α-galactose-1-phosphate and ADP. In contrast with the ADP-GLK, the *P. furiosus* GALK shows the same
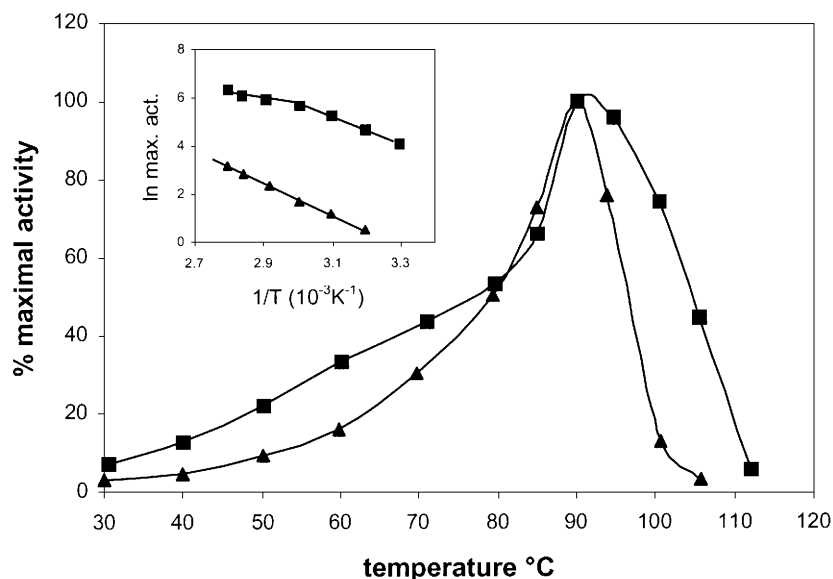

Figure 2   Dependence of GALK and ADP-GLK activity on temperature

Activity was determined as described in the Eperimental section. ▲, GALK and ■, ADP-GLK activities (100 %) correspond to 33.5 and 844 units/mg respectively. Inset, Arrhenius plot indicating a break point at 60 °C for ADP-GLK.

Table 1   Substrate specificity and cation dependence of *E. coli*-produced GALK and ADP-GLK from *P. furiosus*

Standard enzyme assays were performed, except that cations, carbon substrates and phosphoryl group donors were varied, as described in the Experimental section. GALK and ADP-GLK activities (100%) correspond to a specific activity of 2.6 and 139 units/mg respectively. n.m., not measured.

Sugar	Relative activity (%)		Divalent cation	Relative activity (%)		Phosphoryl group donor	Relative activity (%)	
	GALK	ADP-GLK		GALK	ADP-GLK		GALK	ADP-GLK
D-Glucose	< 0.3	100	Mg^{2+}	100	88	ATP	100	< 0.3
D-Galactose	100	< 0.3	Mn^{2+}	37	100	ADP	< 0.3	100
D-Fructose	< 0.3	< 0.3	Co^{2+}	35	59	GTP	60	n.m.
D-Mannose	< 0.3	2	Ca^{2+}	7	15	GDP	n.m.	< 0.3
2-Deoxy-D-glucose	< 0.3	8	Zn^{2+}	< 0.3	60	CDP	n.m.	66
D-Glucosamine	< 0.3	< 0.3				PP_i	< 0.3	n.m.
						PEP	< 0.3	< 0.3

Table 2   Comparison of GALK and ADP-GLK from *P. furiosus* with other GALKs and ATP-dependent HKs and GLKs

Type	Species	Assay temperature (°C)	Sugar	K_m (mM)	V_{max} (units/mg)	k_{cat}/K_m (mM^{-1}·s^{-1})	Phosphoryl group donor	K_m (mM)	V_{max} (units/mg)	k_{cat}/K_m (mM^{-1}·s^{-1})	References
GALK	*P. furiosus*	90	Galactose	0.27	43.2	105	ATP	0.008	41.9	3439	This study
GALK	*E. coli*	37	Galactose	0.70	14	13.8	ATP	0.10	14	96.7	[28]
GALK	*S. cerevisiae*	30	Galactose	0.60	55.8	89.9	ATP	0.15	55.8	360	[21]
GALK	*H. sapiens*	37	Galactose	0.12	81.2	568	ATP	0.35	81.2	195	[16]
GLK	*P. furiosus*	90	Glucose	2.61	1740	570	ADP	0.45	2260	4294	This study
GLK	*E. coli*	37	Glucose	0.78	158	117	ATP	3.76	158	24.3	[30]
GLK	*S. cerevisiae*	25	Glucose	0.03	21.3	631	ATP	0.05	21.3	378	[31]
HK	*H. sapiens*	37	Glucose	0.065	32.1	411	ATP	0.49	32.1	54.5	[32]

substrate preferences as its classical counterparts. GALK showed Michaelis–Menten kinetics at 50 °C. The apparent K_m values of 0.21 ± 0.02 and 0.006 ± 0.001 mM and apparent V_{max} values of 3.66 ± 0.08 and 3.42 ± 0.006 units/mg for galactose and ATP respectively were determined. The apparent K_m values for GALK were not significantly different at 90 °C, being 0.27 ± 0.03 and 0.008 ± 0.002 mM for galactose and ATP respectively, and apparent V_{max} values of 43.2 ± 3.8 and 41.9 ± 3.2 units/mg for galactose and ATP respectively were determined at 90 °C.

The successful heterologous production of the *P. furiosus* ADP-GLK in *E. coli* allowed for comparing the properties of the *E. coli*-produced ADP-GLK with those of the native ADP-GLK at the optimum pH of 7.5 [9,10]. The purified *E. coli*-produced ADP-GLK was found to have a specific activity of 189 units/mg, similar to the native ADP-GLK [9]. The ADP-GLK exhibited a similar optimum temperature as GALK of approx. 90 °C (Figure 2). Interestingly, the ADP-GLK was able to convert D-mannose to some extent, next to D-glucose and 2-deoxy-D-glucose (Table 1). ADP and CDP were potential phosphoryl group donors for ADP-GLK, whereas it could not use ATP. On the other hand, ADP-PFK from *P. furiosus* was able to use ATP to some extent [11]. Divalent cations were required for the activity, with the highest activity in the presence of Mg^{2+} similar to the native ADP-GLK [9,10]. For the *E. coli*-produced ADP-GLK, the apparent K_m values of 1.12 ± 0.10 and 0.078 ± 0.007 mM and apparent V_{max} values of 300 ± 8 and 213 ± 8 units/mg for glucose and ADP respectively were determined at 50 °C, similar to the kinetic parameters of the native enzyme. In general, the characteristics of the *E. coli*-produced ADP-GLK closely resemble those of the native ADP-GLK, although small variations in characteristics occur as well. The affinity of ADP-GLK for both glucose and ADP was lower at 90 °C, resulting in apparent K_m values of 2.61 ± 0.28 and 0.45 ± 0.09 mM and apparent V_{max} values of 1740 ± 210 and 2260 ± 150 units/mg respectively. Thus

the affinity of the ADP-GLK for glucose and ADP decreased by 2- and 6-fold respectively between 50 and 90 °C. This might be explained by assuming that ADP-GLK encounters a conformational change on increasing the temperature, which affects the affinity for ADP. Indeed, a break was observed in the Arrhenius plot for the ADP-GLK at approx. 60 °C (Figure 2), which can be interpreted as a structural change.

The catalytic efficiencies of both sugar kinases, determined at 90 °C, were compared with those from characterized GALKs and GLKs/HKs from mesophiles (Table 2). These data show that the catalytic efficiencies of the pyrococcal sugar kinases for galactose and glucose resemble those of respective sugar kinases from mesophilic bacteria and eukaryotes. On the contrary, the catalytic efficiency of the pyrococcal sugar kinases for the phosphoryl group donor is 10–150-fold higher, when compared with their mesophilic counterparts (Table 2). This difference is caused by the high V_{max} of ADP-GLK (2260 units/mg) and the extremely low K_m for GALK (0.008 mM) at 90 °C. The observation of a high catalytic efficiency for adenine nucleotides and a normal catalytic efficiency for carbohydrates might suggest that the availability of certain nucleotides in the hyperthermophilic *P. furiosus* cells is lower than that in the cells of mesophilic organisms. Interestingly, the ADP-GLK is strongly inhibited (competitively) by AMP (approx. $K_i = 0.06$ mM; C. H. Verhees, unpublished work), and hence the AMP concentration should be kept low during sugar fermentation in *P. furiosus*. The concentration of both ATP and AMP can be kept low by their conversion into ADP by an adenylate kinase (0.045 unit/mg) [33]. The ADP produced can subsequently serve as an energy carrier for glycolysis. In this scenario, the high affinity of GALK for ATP as well as the ADP dependence of the glycolytic kinases (Table 2) could be interpreted as an adaptation to relatively low concentrations of ATP, possibly as a result of thermal instability of ATP. Interestingly, characterization of a glycerol kinase from

the closely related *Pyrococcus* (*Thermococcus*) *kodakaraensis* KOD1 (T_{opt} = 95 °C) revealed that this enzyme also has a relatively high affinity for ATP (K_m = 15.4 μM), when compared with a mesophilic counterpart (K_m = 4 mM) [34]. Studies are in progress to determine the intracellular adenine nucleotide concentrations under different growth conditions in *P. furiosus*, to get more insight into the actual availability of energy carriers under extreme conditions.

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