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ORIGINAL PAPER

Glutamate kinase from *Thermotoga maritima*: characterization of a thermophilic enzyme for proline biosynthesis

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Abstract Glutamate kinase (GK), an enzyme involved in osmoprotection in plants and microorganisms, catalyses the first and controlling step of proline biosynthesis. The *proB* gene encoding GK was cloned from the hyperthermophilic bacterium *Thermotoga maritima* and overexpressed in *Escherichia coli*, and the resulting protein was purified to homogeneity in three simple steps. *T. maritima* GK behaved as a tetramer, showing maximal activity at 83°C, and was inhibited by ADP and proline. Although *T. maritima* GK exhibited high amino acid similarity to the mesophilic *E. coli* GK, it was less dependent of Mg ions and was not aggregated in the presence of proline. Moreover, it displayed a greater thermostability and higher catalytic efficiency than its mesophilic counterpart at elevated temperatures.

Keywords Amino acid kinase family · PUA domain · proB · Thermophilic enzyme · Proline inhibition

Abbreviations
tmGK *Thermotoga maritima* glutamate kinase
ecGK *Escherichia coli* glutamate kinase

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Introduction

Phosphorylation of glutamate by glutamate kinase (GK) is the key biochemical step of proline biosynthesis in bacteria and plants (Leisinger 1996; Aral and Kamoun 1997). Proline has special properties for bacterial and cellular survival (Smith 1985; Csonka and Hanson 1991; Omori et al. 1992), and is considered to be an osmolyte, a cryoprotectant and an antioxidant, useful in stressful environments, during osmotic challenge and in freezing conditions (Smirnoff and Cumbes 1989; Wondrak et al. 2005; Pavlikova et al. 2008; Takagi 2008). For this reason, considerable research has been conducted to evaluate the biotechnological applications of engineered GK and its homologue in plants, pyrroline 5-carboxylate synthase (P5CS). As a result, crops with higher salinity tolerance (Chen et al. 2007), baker's yeasts with an enhanced capacity for fermentation in frozen dough (Kaino et al. 2008), and a thermophilic microorganism capable of excreting proline into the bacterial medium as a source of amino acid production (Kosuge and Hoshino 1998) have been obtained. Indeed, a deficit of P5CS (OMIM 138250) in humans also produces symptoms attributed to redox imbalance due to proline deficiency (Baumgartner et al. 2005).

Bacterial GK enzymes contain an N-terminal amino acid kinase (AAK) domain (Pfam PF00696), which binds ATP and glutamate, and a C-terminal RNA binding domain (named PUA, PF01472) in approximately 80% of cases (Aravind and Koonin 1999; Perez-Arellano et al. 2007). The presence of the PUA domain, usually found in RNA-modifying enzymes, is somewhat puzzling, but it could be pointing out an additional role of GK as mediator of some cellular responses, given the special functions of proline (Smirnoff and Cumbes 1989; Wondrak et al. 2005; Pavlikova et al. 2008; Takagi 2008).

Thermotoga maritima GK (tmGK) as well as *Escherichia coli* GK (ecGK) have a PUA domain. Both are similar in length, and their amino acid composition (Swiss-Prot P0A7B5 and Q9WYD0) has a 39% of identity and 63% of residue conservation. Differences between the two exist principally in the number of charged and aromatic residues (greater in tmGK), which could be one of the reasons for its thermostability (Vieille and Zeikus 2001).

The 3D structure of GK is known for the mesophilic *E. coli* (Marco-Marin et al. 2007) and *Campylobacter jejuni* enzymes (PDB ID 2AKO), and the *E. coli* enzyme has also been kinetically characterized (Smith 1985; Perez-Arellano et al. 2004; Perez-Arellano et al. 2005; Perez-Arellano et al. 2006). ecGK is a tetrameric enzyme that exhibits competition between one of its substrates, glutamate, and the inhibitor proline (Perez-Arellano et al. 2006).

Important insights into protein evolution and thermostability have been provided by the completion of the genome sequence of *Thermotoga maritima* (Nelson et al. 1999) and the results achieved with structural genomics (Lesley et al. 2002). To further this knowledge, in addition to the comparison of the 3D structure of mesophilic and thermophilic variants and bioinformatic analyses based on protein sequence conservation, an in-depth understanding of their biochemical parameters is essential.

In this sense, the paradigmatic AAK-family enzyme *N*-acetylglutamate kinase (NAGK), which is homologous to the GK AAK domain and catalyses the same reaction except for the use of *N*-acetylated glutamate, has been structurally and biochemically characterized from *E. coli* (Ramon-Maiques et al. 2002; Marco-Marin et al. 2003), *Pseudomonas aeruginosa* (Haas and Leisinger 1975a, 1975b; Ramon-Maiques et al. 2006) and *T. maritima* (Fernandez-Murga et al. 2004; Ramon-Maiques et al. 2006). Structurally, the three enzymes are very similar in their dimeric structure, being *P. aeruginosa* and *T. maritima* trimers of dimers (Ramon-Maiques et al. 2002, 2006). However, the thermophilic NAGK, though possessing a greater catalytic efficiency at its optimum temperature, exhibits lower substrate affinities than its mesophilic counterparts.

In the present work, we report the expression, purification and kinetic characterization of tmGK and make comparisons to the ecGK mesophilic variant, with the aim of contributing to a better understanding of thermostability.

Materials and methods

Construction of the GK expression vector (pGKT)

The *proB* gene was PCR-amplified from the genomic DNA of *T. maritima* using the Expand High Fidelity PCR System (from Roche) with the primers 5′-GTGAGGCCATGGTTATGAAAGTCGTTG-3′ and 5′-TGCTGGAGAGGATCCTCAAACACC-3′, which introduce *Nco*I and *Bam*HI sites at the initiator ATG and downstream from the stop codon. The *Nco*I- and *Bam*HI-digested amplified fragment was ligated to the *Nco*I and *Bam*HI sites of plasmid pET15b (Novagen) using T4 ligase (Roche), and *E. coli* DH5α cells (from Clontech) were transformed. Plasmid pGKT was isolated and was shown by restriction analysis and automated DNA sequencing to harbour the full *proB* gene.

Protein expression and purification

E. coli BL21 (DE3) cells (from Novagen) were cotransformed with pGKT and plasmid GroESL (a pACYC184-derived expression plasmid encoding the *E. coli* chaperonins GroEL and GroES). Then they were grown to an A_{600} of 0.4 at 37°C in 1 l LB broth containing 50 μg/ml ampicillin and 20 μg/ml chloramphenicol. The cells were induced overnight with 0.5 mM isopropyl-β-D-thiogalactoside at 25°C and were harvested by centrifugation. Subsequent steps were carried out at 4°C. Cells were suspended in 10 ml buffer A (50 mM Tris–HCl, 1 mM dithiothreitol pH 7.2, glycerol 10%) and then sonicated and centrifuged (30 min, 35,000*g*). The supernatant was incubated for 10 min at 80°C and then chilled and centrifuged again for 15 min at 4°C and 18,000*g*. The supernatant was precipitated with $(NH_4)_2SO_4$ at 50% saturation. The pellet was resuspended in 20 ml buffer A and the solution was subjected, in two batches of 10 ml, to ion-exchange chromatography using an FPLC system (GE Healthcare) fitted with a 5 ml HiTrap column (GE Healthcare) equilibrated and run with buffer A. After washing with 15 ml buffer A, a 30 ml linear gradient of 0–0.5 M NaCl in buffer A was applied. Enzyme-rich fractions were eluted at approximately 0.3 M NaCl. They were then pooled and concentrated with Amicon filter devices (Millipore) and kept at −20°C.

Enzyme activity assays

GK activity was assayed at various temperatures by measuring ADP release in the presence of 20 mM ATP, 0.15 M Na glutamate and 20 mM $MgCl_2$, as previously described (Perez-Arellano et al. 2005, 2006). The reaction was terminated after 10 min by adding an equal volume of 10% (w/v) trichloroacetic acid, and the ADP produced was measured at 340 nm by the decrease in absorbance of the neutralized supernatant using 2.5 mM phosphoenol pyruvate, 0.04 mg/ml pyruvate kinase, 0.25 mM NADH and 0.025 mg/ml lactate dehydrogenase (Bergmeyer et al. 1986). When ATP was varied, glutamate was kept at 0.15 M. When glutamate was varied, 20 mM ATP and 20 mM $MgCl_2$ were used. To evaluate inhibition by ADP,

phosphate was determined at the end of the 10-min incubation period using malachite green and 2 mM MgATP (Perez-Arellano et al. 2005). To assess the formation of γ-glutamyl phosphate through conversion to γ-glutamyl hydroxamate, 0.2 M hydroxylamine (neutralized) was included in the assay, and hydroxamate was determined colorimetrically at 535 nm at the end of the 10 min incubation at 37°C (Krishna and Leisinger 1979). One enzyme unit makes 1 µmol ADP/min. The GraphPad Prism (GradPad Software, San Diego, California) was used to fit data to hyperbolic kinetics, sigmoidal kinetics ($v = V_{\max} \times [S]^{\mathrm{H}}/[S]^{\mathrm{H}}_{0.5} + [S]^{\mathrm{H}}$) or sigmoidal inhibition ($v = v^{[I]=0} \times (1 - [I]^{\mathrm{H}}/[I]^{\mathrm{H}}_{0.5} + [I]^{\mathrm{H}})$), where H is the Hill coefficient, $v^{[I]=0}$ is the velocity without proline and $I_{0.5}$ is the proline concentration giving 50% of inhibition.

Analytical gel filtration chromatography

A Superdex 200 H/R 10/30 column connected to an AKTA FPLC system (both from GE Healthcare) was used at a flow rate of 0.5 ml/min at 23°C to monitor the A_{280} of the eluate. The solution used for dissolving the protein and for equilibrating and running the column was 50 mM Tris–HCl, pH 7.2, 1 mM dithiothreitol, 0.15 M NaCl and, when indicated, 10 mM proline.

The following protein standards (with their masses in kDa in brackets) were employed: ovalbumin (42.7), *E. coli* acetylglutamate kinase (54.3), bovine serum albumin (66.4), *E. coli* aspartokinase III (97.1), yeast alcohol dehydrogenase (146.8), aldolase (156.8), c-globulin (160), *Pseudomonas aeruginosa* acetylglutamate kinase (190.5), β-amylase (223.8), ferritin (440), and bovine thyroglobulin (669).

Other methods

Circular dichroism was performed in the far-UV region (195–250 nm) at 25°C on a Jasco 810 spectropolarimeter, using 3 µM enzyme (expressed as the concentration of the enzyme polypeptide) in a 0.2 ml solution of 25 mM Tris–HCl pH 7.2 containing 0.5 mM dithiothreitol. The optical path was 0.1 cm and the integration time was 4 s. Ten scans for each sample were averaged and the background spectrum of the buffer was subtracted.

MALDI-TOF mass spectrometry analysis was carried out by the proteomic service of CIPF using a 4700 Proteomics analyser (Applied Biosystems).

Protein concentration was quantified in a spectrophotometer at 595 nm using the Bradford reagent (Bio-Rad) and BSA as a standard (Bradford 1976).

Proteins were analysed by SDS-PAGE using 10–12% polyacrylamide gels according to the procedure described by Laemmli (1970). Densitometric quantization of protein gels was carried out with the Sigma Gel software package (Sigma) to assess protein purity.

Results and discussion

Cloning, expression and purification of tmGK

The *proB* gene (1.06 kb) encoding tmGK was amplified by PCR from *T. maritima* genomic DNA and cloned into the pET15b expression vector, resulting in pGKT. The *T. maritima proB* gene expressed from pGKT yielded scanty amounts of insoluble protein when overexpressed in *E. coli* BL21 (DE3) cells under standard induction conditions. However, when expressed in cells that also overexpressed the chaperonins GroES and GroEL on being induced at ≤25°C, production increased and a fraction of the product was soluble. The *E. coli* lysates were heated to 80°C to remove the majority of the endogenous proteins, while tmGK remained soluble and active. Ammonium sulphate precipitation and a single ion-exchange chromatography step were used to isolate tmGK to 90% purity (SDS-PAGE estimate) with a yield of approximately 1 mg/l of the initial culture (Fig. 1a).

Biochemical characterization

The apparent Mr was estimated by SDS-PAGE to be 42 kDa, which was similar to the Mr calculated from the amino acid sequence (38.524 kDa). The mass fingerprinting of the isolated protein determined by MALDI-TOF mass spectrometry agreed with the gene-deduced sequence (results not shown). In addition, both the CD spectrum of tmGK, with a minimum at 222 nm and a maximum at 196 nm (Fig. 1b), and size exclusion chromatography experiments (Fig. 1c) suggested the appropriate folding of the enzyme.

tmGK was eluted from a Superdex 200 column as a single peak at a position close to that expected for a tetramer (Fig. 1c). No substantial change in the elution pattern was observed when high proline concentration (10 mM) was included in the running buffer (Fig. 1c). Conversely, gel filtration analysis of ecGK revealed the aggregation of the enzyme in the presence of 0.2 mM proline, shown by a dramatic left shift in the elution pattern. However, this effect was not observed with the C-terminal truncated variant, which lacked the PUA domain (Perez-Arellano et al. 2005). Given the flat and elongated 3D structure of ecGK and the varying effects of proline on the aggregation of truncated and full-length versions of this enzyme, it was concluded that the PUA domain was implicated in the formation of an oligomer in the presence of proline by permitting the spatial apposing

of tetramers through their PUA domains (Perez-Arellano et al. 2005; Marco-Marin et al. 2007). In spite of this and the fact that tmGK has a PUA domain, the enzyme was not aggregated in the presence of proline. These findings point to a different overall organization of the thermophilic enzyme, which would appear to have a more globular and compact shape. The determination of the 3D structure of tmGK, which we are currently pursuing, will allow us to understand this new circumstance.

Purified tmGK yielded colour when the classic hydroxylamine-coupled GK enzyme activity assay was performed (Leisinger 1996; Perez-Arellano et al. 2005), which means that the enzyme produces γ-glutamyl phosphate. Due to the lability of the γ-glutamyl phosphate (Seddon et al. 1989), the amount detected was around 10% that of the ADP produced (data not illustrated). The same effect was observed for ecGK (Perez-Arellano et al. 2005).

This enzyme is relatively stable in a wide range of pHs (from 6.5 to 9.0), and specifically uses ATP, and not GTP, UTP or CTP. In addition, ADP release by the tmGK enzyme proved to be strictly dependent on, and highly specific for, L-glutamate, and did not occur (detection limit, 1% of the release with L-glutamate) when 80 mM L-glutamate was replaced by the same concentration of either L-aspartate, L-glutarate, γ-amino butyrate or L-norvaline (data not illustrated).

tmGK behaved towards proline analogues in a similar manner to ecGK. The enzyme required a concentration of 20 mM (~150 times greater than $I_{0.5}$ for proline) of *trans*-hydroproline, *cis*-hydroproline, methyl-proline or acetyl-carboxylate acid to be totally inhibited, whereas in the case of 3,4 dehydroproline, total inhibition was observed at 2 mM (data not illustrated). These results prove that proline is a highly specific inhibitor of tmGK.

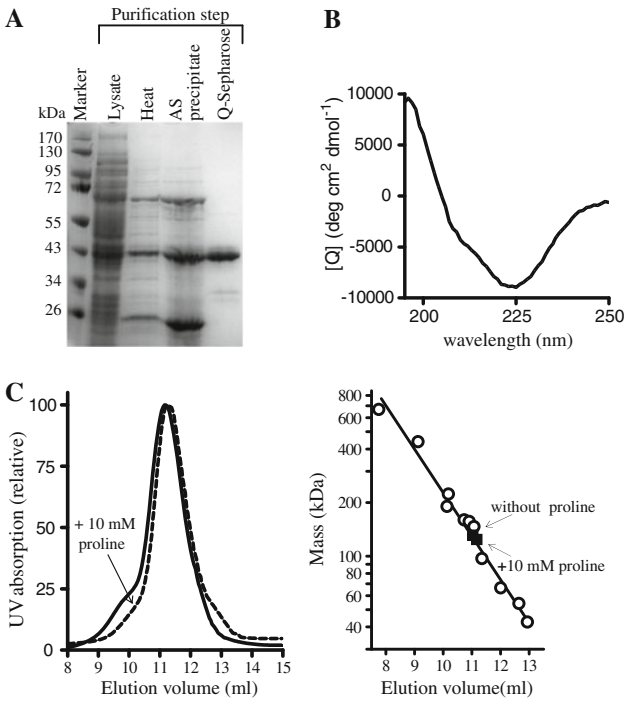

Fig. 1 Purification and biochemical characterization of tmGK. **a** Analysis of tmGK, by SDS-PAGE, at various steps of the purification procedure. Molecular weight marker proteins (Fermentas) are indicated on the *left*. **b** CD spectra of tmGK at 20°C in 25 mM Tris–HCl pH 7.2 containing 0.5 mM dithiothreitol. **c** Gel filtration chromatography of tmGK with or without proline and semilogaritmic plot of molecular mass versus elution volume. The protein standards used and the enzyme samples are shown as *open circles* and *filled squares*, respectively

Determination of kinetic parameters of tmGK

Kinetic parameters of tmGK were determined for the substrates and the inhibitor at 37 and 75°C. Similar to ecGK, tmGK exhibited values of the Hill coefficient close to 1 when assayed at 37°C (Table 1). At a higher temperature (75°C), a small increase in sigmoidicity and a very slight change in the $S_{0.5}$ accompanied a marked improvement in the catalytic efficiency of tmGK (Table 1). As shown in Fig. 2a and b, the value for $S_{0.5}^{Glu}$ increased with the proline concentration as expected for competition between proline and glutamate. In accordance with this, the dependence of tmGK activity on proline concentration varied with the concentration of glutamate present in the assay, while the sigmoidicity of the inhibition increased (Fig. 2c). As shown in Fig. 2b and d proline triggered minor alterations of $S_{0.5}^{ATP}$ whereas it produced a considerable decrease in V_{max} for ATP.

Both the mesophilic and thermophilic GKs exhibited competition between proline and glutamate and displayed

Table 1 Kinetic parameters for *E. coli* and *T. maritima* GK

Enzyme	Assay temperature (°C)	ATP			Glutamate			Proline	
		V_{max} (U/mg)	$S_{0.5}$ (mM)	*H*	V_{max} (U/mg)	$S_{0.5}$ (mM)	*H*	$I_{0.5}$ (mM)	*H*
*tm*GK	37	81 ± 6	3.3 ± 0.6	1.1 ± 0.1	80 ± 2	23 ± 1	0.9 ± 0.06	0.14 ± 0.01	1.2 ± 0.1
*tm*GK	75	726 ± 58	5.3 ± 0.9	1.3 ± 0.2	680 ± 29	39 ± 5	1.5 ± 0.2	0.19 ± 0.01	1.6 ± 0.1
ecGK[a]	37	127 ± 1	2 ± 0.1	1	163 ± 3	82 ± 4	1.1 ± 0.1	0.15 ± 0.01	2.1 ± 0.2

[a] Data taken from Perez-Arellano et al. (2006)

the same apparent low affinity for proline analogues. Therefore, they would appear to share the same inhibitory mechanism, in spite of tmGK being a thermophilic enzyme. It has been postulated that proline binds in ecGK (PDB code 2J5T) near the $\beta 4\alpha$E loop that surrounds the glutamate site, partially overlapping it (Marco-Marin et al. 2007). This sequence is mostly invariant in the corresponding region of tmGK.

On the contrary to that observed with ecGK, the thermophilic enzyme does not require free Mg for its activity. Moreover, only one of the four residues of ecGK which form the negatively charged hole where magnesium binds is conserved in tmGK. These data are consistent with an inhibitory effect of high concentrations of Mg, an effect that is accentuated at elevated temperatures. Conversely, proline inhibition was not found to depend on temperature (Fig. 3b). ADP was also shown to be an inhibitor of tmGK (Fig. 3c). This inhibition was lower at 75°C than at 37°C, which is in accordance with the extraordinary catalytic rate observed at these temperatures, at which there is also a higher rate of ADP production.

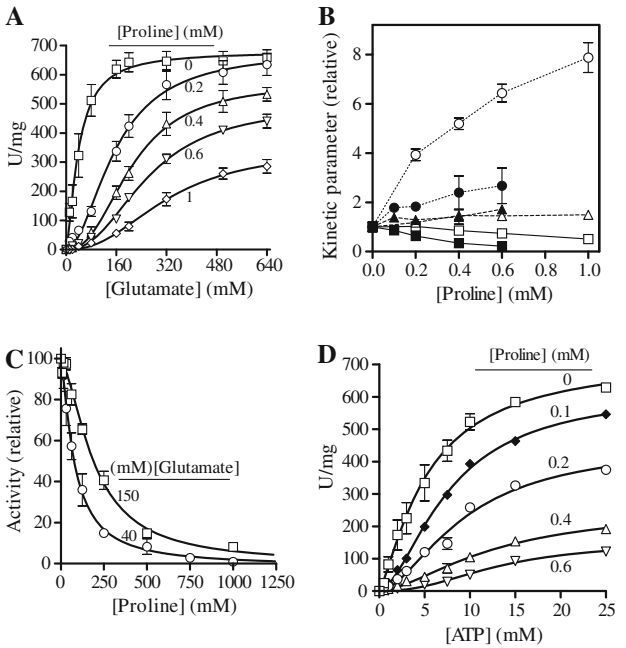

Fig. 2 Effect of proline concentration on the kinetic constants of tmGK at 75°C. **a** Glutamate saturation curves in the presence of 0, 0.2, 0.4, 0.6, and 1 mM proline. **b** Variation of kinetic parameters for ATP (*filled symbols*) and glutamate (*open symbols*) as a function of proline concentration. V_{max} is represented by *squares*, the Hill coefficient by *triangles* and the $S_{0.5}$ by *circles*, and all are expressed relative to values in the absence of proline (see Table 1). **c** Influence of glutamate concentration on proline inhibition. Activity is shown as a percentage of the activity in the absence of proline at 40 mM glutamate (323 ± 23 U/mg) and at 150 mM glutamate (593 ± 61 U/mg). Data were fitted to sigmoidal inhibition kinetics for $I_{0.5}$ and H values of 79 ± 8 and 1.3 ± 0.1 and 194 ± 12 μM and 1.6 ± 0.1 for 40 and 150 mM glutamate, respectively. **d** ATP saturation curves in the presence of 0, 0.1, 0.2, 0.4, and 0.6 mM proline

Thermostability and effect of temperature on kinetics

The highest catalytic activity of the enzyme was exhibited at 83°C, close to the optimal growth temperature for *T. maritima* cells, which is 80°C (Fig. 4a). While at 37°C its specific activity (80 U/mg) was close to that of ecGK, at its optimum temperature tmGK activity increased approximately one order of magnitude. An Arrhenius plot of activity versus the reciprocal of absolute temperature (Fig. 4a, inset) did not reveal any break in the 30–83.5°C temperature range that might reflect a drastic phase transition. The linearity of the plot suggests no change in the functional conformation throughout all temperatures (Vieille and Zeikus 2001).

The temperature stability of the purified enzyme was tested between 70 and 90°C. The enzyme retained approximately 100, 90 and 65% of its activity when heated for 45 min at 70, 80 and 85°C, respectively. At 90°C, tmGK displayed around 40% of the activity observed after

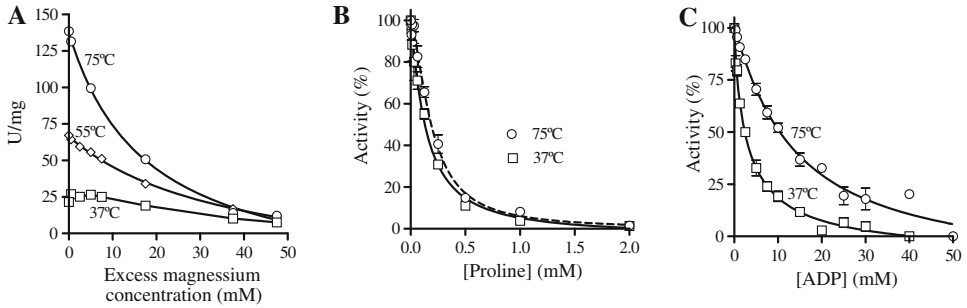

Fig. 3 Effect of temperature on the inhibition of enzyme activity by **a** excess of magnesium concentration other than the one needed for producing the MgATP complex; **b** proline; and **c** ADP. **a** Enzyme was assayed at 2 mM MgATP and 0.15 M glutamate. Data at 75 and 55°C were fitted to hyperbolas, with K_i of 17.8 and 37.5 mM, respectively. **b**, **c** Activities are represented as a percentage of the activity of the enzyme in the absence of the inhibitor (593 ± 61 U/mg at 75°C and 67 ± 4 U/mg at 37°C). Data were fitted to sigmoidal inhibition kinetics for **b** $I_{0.5}^{Pro}$ and H values of 0.19 ± 0.01 and 1.6 ± 0.1 mM for 75°C and of 0.14 ± 0.01 and 1.2 ± 0.1 mM for 37°C and **c** $I_{0.5}^{ADP}$ and H of 3.1 ± 0.4 and 0.9 ± 0.07 mM for 75°C and of 13.7 ± 1.8 and 1.1 ± 0.1 mM for 37°C, respectively

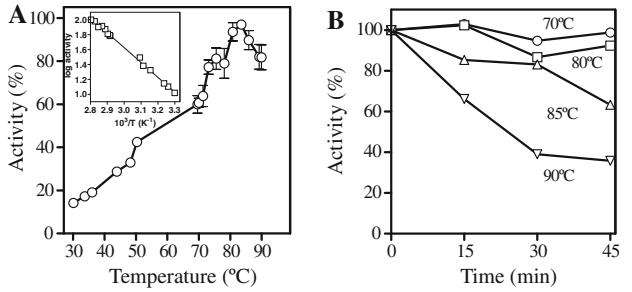

Fig. 4 Effect of temperature on **a** the activity and **b** the stability of tmGK. **a** Activity at each temperature is represented as a percentage of the activity at 83.5°C (689 ± 43 U/mg). The *inset* gives the Arrhenius plot in the temperature range of 30–83.5°C. **b** Purified tmGK (~1 mg/ml) was incubated at the indicated temperatures in 50 mM Tris–HCl, pH 7.2, 10% glycerol, 1 mM dithiothreitol and samples were taken after the indicated periods of time for assays of enzyme activity at 37°C. This activity is represented as a percentage of the activity before incubation (67 ± 4 U/mg)

the same period of time (Fig. 4b). In contrast, ecGK lost all its activity after 15 min at 60°C (Perez-Arellano et al. 2005).

In our case, both ecGK and tmGK behaved similarly, exhibiting equivalent kinetic parameters (Table 1). However, NAGK, another *T. maritima* enzyme from the same AAK family (Fernandez-Murga et al. 2002, 2004; Fernandez-Murga and Rubio 2008), and very similar to GK, displayed lower substrate affinities than its mesophilic counterparts.

tmGK works at a very wide range of temperatures, and its kinetic constants are not affected by temperature. Hence, the thermophilic property of this enzyme would seem to consist of a higher catalytic rate accompanied by the same affinity for substrates. However, NAGK shows different kinetic parameters depending on temperature (Fernandez-Murga et al. 2004).

Thus, even though GK and NAGK are closely related, there are specific aspects that determine the thermostability features of each protein.

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