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Phosphoglycerate kinase and triosephosphate isomerase from the hyperthermophilic bacterium *Thermotoga maritima* form a covalent bifunctional enzyme complex

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Dedicated to Professor Max A.Lauffer on the occasion of his 80th birthday.

Phosphoglycerate kinase (PGK) from the hyperthermophilic bacterium *Thermotoga maritima* has been purified to homogeneity. A second larger enzyme with PGK activity and identical N-terminal sequence was also found. Surprisingly, this enzyme displayed triosephosphate isomerase (TIM) activity. No other TIM is detectable in *T.maritima* crude extracts. As shown by ultracentrifugal analysis, PGK is a 43 kDa monomer, whereas the bifunctional PGK–TIM fusion protein is a homotetramer of 240–285 kDa. SDS–PAGE indicated a subunit size of 70 kDa for the fusion protein. Both enzymes show high thermostability. Measurements of the catalytic properties revealed no extraordinary results. pH optima, K_m values and activation energies were found to be in the range observed for other PGKs and TIMs investigated so far. The corresponding *pgk* and *tpi* genes are part of the apparent *gap* operon of *T.maritima*. This gene segment contains two overlapping reading frames, where the 43 kDa PGK is encoded by the upstream open reading frame, the *pgk* gene. On the other hand, the 70 kDa PGK–TIM fusion protein is encoded jointly by the *pgk* gene and the overlapping downstream open reading frame of the *tpi* gene. A programmed frameshift may be responsible for this fusion. A comparison of the amino acid sequence of both the PGK and the TIM parts of the fusion protein with those of known PGKs and TIMs reveals high similarity to the corresponding enzymes from different procaryotic and eucaryotic organisms.

Key words: fusion protein/glycolysis/programmed frameshift/thermostability/*Thermotoga maritima*

Introduction

Triosephosphate isomerase (TIM; EC 5.3.1.1) and phosphoglycerate kinase (PGK; EC 2.7.23) are two ubiquitous enzymes of central importance in the major pathways of carbohydrate metabolism, i.e. glycolysis, gluconeogenesis and the oxidative pentose phosphate pathway. PGK catalyzes the phospho group transfer between 1,3 bisphosphoglycerate and ATP, whereas TIM catalyzes the interconversion of dihydroxyacetone phosphate (DHAP) and D-glyceraldehyde 3-phosphate.

The homodimeric isomerases are evolutionarily perfected enzymes that show extremely high catalytic efficiency (Knowles, 1991), working at the diffusional limit *in vivo* (Blacklow *et al.*, 1988). The enzyme is also structurally conserved: highly homologous primary sequences of the enzyme from more than 30 organisms have been determined and the nearly superimposable X-ray crystallographic structures of the isomerases from chicken, trypanosome and yeast have been determined (Lolis *et al.*, 1990; Wierenga *et al.*, 1992). Topologically, TIM is the archetype of a large family of enzymes having an eight stranded α/β-barrel structure (Farber and Petsko, 1990; Brändén, 1991).

The structure of PGK has also been highly conserved throughout evolution (Mori *et al.*, 1986; Watson and Littlechild, 1990). This monomeric enzyme folds into two distinct lobes of approximately equal size. The active site lies deep in the cleft between the two domains, the movement of which relative to each other has been suggested as an essential element in the catalytic mechanism (Banks *et al.*, 1979). Studies of the reversible thermal denaturation of PGK from *Bacillus stearothermophilus* and *Thermus thermophilus* represent one of the few examples of a sound thermodynamic analysis of the strategy of thermal adaptation at the molecular level (Nojima *et al.*, 1977, Nojima and Noda, 1979). Recently, the high resolution X-ray structure of the moderately thermostable PGK from *B.stearothermophilus* was reported (Davies *et al.*, 1993).

The present study involves PGK and TIM from the hyperthermophilic bacterium *Thermotoga maritima*, the enzyme inventory of which has been shown to extend to the extreme of intrinsic protein stability reported so far (Jaenicke, 1993). Making use of the broad database accumulated for a set of representative enzymes from mesophiles, thermophiles and hyperthermophiles, one may come closer to a solution of the problem of how structure, function and energetics of proteins have adapted to the wide temperature range in the biosphere (Jaenicke, 1991). More specifically, the characterization of the two enzymes under scrutiny here will add to our understanding of the evolution of carbohydrate metabolism at a deep branch, close to the root of the phylogenetic tree (Woese, 1993). At the present state of 16S rRNA analyses, *T.maritima* and *Aquifex pyrophilus* are the only hyperthermophilic members of the bacterial domain of the phylogenetic tree (Huber *et al.*, 1986, 1992). *Thermotoga* grows fermentatively, using simple and complex carbohydrates, with lactate, acetate, CO_2 and H_2 as the main products of its metabolism. *Thermotoga maritima* is thought to use

the conventional Embden–Meyerhof–Parnas pathway (Blamey and Adams, 1994). One of its key enzymes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), has been studied extensively (Wrba *et al.*, 1990; Rehaber and Jaenicke, 1992; Tomschy *et al.*, 1993) and its high resolution crystal structure has recently been solved (Korndörfer *et al.*, 1995). Here we report the isolation and characterization of PGK and TIM from *T.maritima* and the surprising result that PGK occurs in two forms, the expected monomeric monofunctional enzyme and a tetrameric, bifunctional protein that exhibits both PGK and TIM activity. *Thermotoga maritima* does not contain a monofunctional TIM. Our results suggest that both proteins are encoded by two genes with an overlapping reading frame. Synthesis of PGK requires termination of translation at the stop codon at the end of the *pgk* gene, whereas the amino acid sequence of the PGK–TIM fusion protein is determined by the two overlapping *pgk* and *tpi* genes, as a consequence of a ribosomal frameshift upstream of the monomeric PGK stop codon.

Results

Purification of PGK and PGK–TIM fusion protein

In cell-free extracts of *T.maritima* cells grown under standard conditions (Huber *et al.*, 1986), two well-separated peaks with PGK activity were eluted on gel permeation chromatography (Sephacryl S300HR, 1.6×100 cm) (Figure 1). Calibration with standard proteins revealed molecular masses of ~400 and 50 kDa respectively. The PGKs known so far are monomeric enzymes with molecular masses in the range 42–49 kDa (Scopes, 1973; Suzuki and Imahori, 1974), in accordance with the lower molecular weight fraction, peak II. Monitoring TIM activity in the fractions from the same gel permeation column (Figure 1), TIM is found to co-elute with the PGK activity in peak I, at ~ 400 kDa. It is obvious from Figure 1 that there is only one peak showing TIM activity and that this peak elutes at a molecular weight much higher than the homodimeric TIMs, with masses of 43–60 kDa, known so far (Noltmann, 1972).

To exclude the possibility that the two peaks of PGK activity in the crude extract belong to one and the same enzyme in different states of oligomerization, re-chromatography and purification of each of the two enzymes were undertaken. Repeated chromatography did not split peak I, nor were any transitions from one peak fraction to the other observed (data not shown). PGK I and PGK II exhibit different binding behaviour on most of the chromatographic columns in the purification scheme (Table I). Optimum separation of the two enzymes was accomplished using hydrophobic chromatography on phenyl-Sepharose (Figure 2). The purification of the two PGKs was followed by SDS–PAGE and is illustrated in Figure 3. After elution of the PGKs from the phenyl-Sepharose column, the total PGK activity was found to have doubled, perhaps the result of elimination of inhibitory factors.

In connection with the question of whether the occurrence of two PGK fractions might be artifactual, it is important to note that in each purification step the TIM activity co-elutes with the high molecular weight PGK (peak I, Figure 2). In the pure fractions of the 'large PGK', the TIM activity was found to exceed 500 U/mg at 40°C. Despite the high catalytic efficiency of TIM (Knowles, 1991), a specific catalytic activity of this order cannot be present as an impurity. No protein band was observed between 25 and 30 kDa on SDS–PAGE after overloading the gel with 100 μg of peak I PGK, as one would expect for a homodimeric TIM. Furthermore, protein sequencing revealed only one N-terminal sequence (see below). Consequently the 'large PGK' must be a fusion protein of PGK with TIM, termed the PGK–TIM fusion protein. The yield of purified PGK and PGK–TIM fusion protein, starting from 60 g (wet weight) cells, was *c.* 25 and 20 mg respectively.

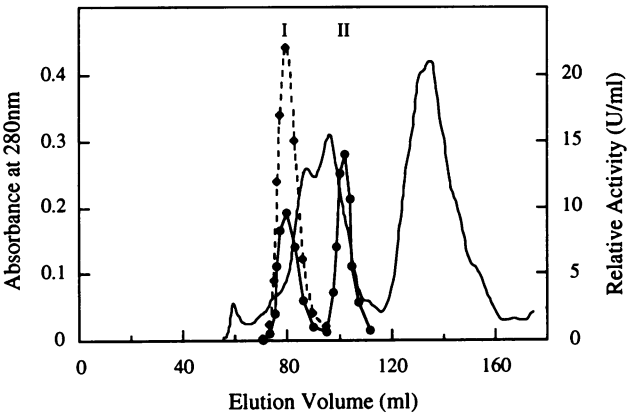

Fig. 1. Gel-permeation chromatography of *T.maritima* cell-free crude extract on a Sephacryl S300 HR column (1.6×100 cm). The crude extract was produced as described in Materials and methods. Absorbance at 280 nm (——); relative activity of PGK (—●—); relative activity of TIM (– – ● – –).

N-terminal sequencing

For both purified enzymes, PGK and the PGK–TIM fusion protein, a 30 residue N-terminal sequence was determined by Edman degradation. The sequence was identical for both proteins (Figure 4).

Molecular properties of PGK and PGK–TIM fusion protein from T.maritima

The molecular masses and the subunit composition of the purified enzymes were determined by SDS–PAGE, gel permeation chromatography and sedimentation analysis. SDS–PAGE on 5–25 % (w/v) acrylamide gradient gels yielded 43 ± 3 kDa for PGK and 71 ± 3 kDa for the PGK–TIM fusion protein. To disrupt disulfide bonds, the samples were heated with 2.5% SDS under reducing conditions (0.1 M 2-mercaptoethanol) for 15 min at 100°C prior to SDS–PAGE analysis. The difference in the subunit structure between the two enzymes is ~27 kDa, in accordance with the known average molecular mass of the TIM subunit from other sources. Thus it is obvious that the PGK part and the TIM part of the PGK–TIM fusion protein must be covalently linked. Sedimentation velocity experiments showed that both enzymes sediment as single symmetrical boundaries with sedimentation coefficients $s^0_{20,w}$ = 3.4 S for PGK and $s^0_{20,w}$ = 8.6 S for the PGK–TIM fusion protein. Sedimentation equilibrium experiments provided clear evidence for the monomeric structure of PGK: the molecular mass was determined to be 45 ± 4 kDa, in good agreement with the result

Table I. Purification of PGK and the PGK–TIM fusion protein from *T.maritima*[a]

Fraction	Volume (ml)	Total protein (mg)	Total units (U)	Sp. act. (U/mg)	Purification factor	Recovery (%)
Crude extract	116	2800	5300	2	n.d.	n.d.
Ammonium sulfate precipitate (40–70%)	76	1900	4900	3	n.d.	n.d.
PGK–TIM fusion						
Phenyl-Sepharose eluate	210	320	6000	9	1	100
Q-Sepharose eluate	85	80	4000	45	5	67
Blue Sepharose	14	21	3800	181	20	63
Superdex 200 pg	22	20	3800	190	21	63
PGK						
Phenyl-Sepharose eluate	250	180	11000	61	1	100
Q-Sepharose	110	28	8600	300	5	78
Superdex 75 pg	29	25	8300	330	5	75

[a]Crude extract was obtained from 60 g cells. PGK activity was determined under standard conditions at 40°C.
n.d., not determined.

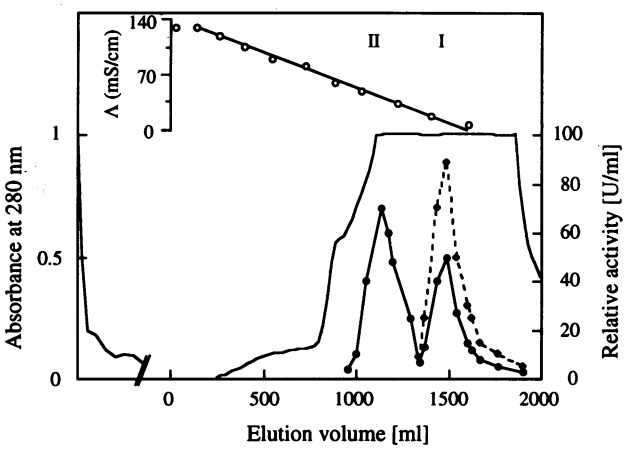

Fig. 2. Elution profile of PGK and PGK–TIM fusion protein obtained by hydrophobic interaction chromatography on phenyl-Sepharose FF. The 35–70% ammonium sulfate precipitate was dissolved in 50 mM Tris–HCl buffer, pH 7.5, plus 1 mM EDTA and 30% saturated ammonium sulfate and applied to a 160 ml phenyl-Sepharose FF (high sub) column (5.0×8 cm). After washing, two different peaks with PGK activity were eluted by applying a linear gradient of 30–0% ammonium sulfate in a total volume of 1.6 l. The first peak (PGK) elutes at a conductivity of ~40 mS/cm, the second one (PGK–TIM fusion protein) at 5 mS/cm. Absorbance at 280 nm (——); relative activity of PGK (—●—); relative activity of TIM (– – ● – –); conductivity (—○—).

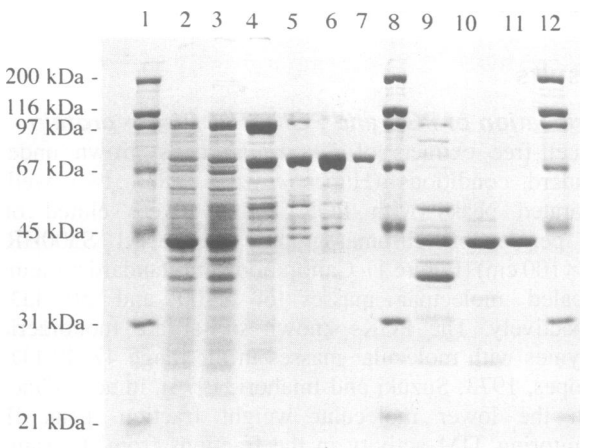

Fig. 3. SDS–PAGE illustrating the purification scheme for PGK and PGK–TIM fusion protein from *T.maritima*. Lanes 1, 8 and 12: molecular mass standards; myosin, 200 kDa; β-galactosidase, 116 kDa; phosphorylase, 97 kDa; bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; trypsin inhibitor, 21.5 kDa; lysozyme, 14 kDa; aprotinin, 6.5 kDa. Lane 2: crude extract. Lane 3: 35–70% ammonium sulfate precipitate after resuspension and dialysis against low-salt buffer. Lanes 4–7: combined fractions of PGK–TIM fusion protein after hydrophobic chromatography, anion exchange chromatography, affinity chromatography and gel permeation chromatography respectively. Lanes 9–11: combined fractions of PGK after hydrophobic chromatography, anion exchange chromatography and gel permeation chromatography respectively.

from native gel permeation chromatography. High speed sedimentation equilibrium experiments with the PGK–TIM fusion protein yielded 260 ± 20 kDa, suggesting that the native protein is a homotetramer. The corresponding analytical gel permeation chromatography resulted in a substantially higher molecular mass of 370 ± 30 kDa. Varying the salt concentration of the equilibration buffer from 0.1 to 1.0 M NaCl led to a further shift of the apparent molecular mass to 400 kDa. The difference in the molecular mass derived from size exclusion chromatography and sedimentation equilibrium may reflect a large hydrodynamic volume of the protein. This assumption is supported by dynamic light scattering experiments which yield $D_{20,w} = 2.7\times10^{-7}$ cm²/s (M.Ott, unpublished result). Combining the above $s^0_{20,w}$ value with this result, the tetrameric quaternary structure is confirmed: $M_{s,D}$ = 286 kDa.

Catalytic properties

The pH dependencies of the enzyme activities are shown in Figure 5. Evidently, the two pH profiles for the PGK reaction of PGK and the fusion protein do not differ significantly, with an optimum between pH 5.2 and 6.0, in agreement with the pH optimum reported for PGK from *B.stearothermophilus* (Suzuki and Imahori, 1974). In contrast, the pH optima of the enzyme from other sources are shifted to higher pH values: pH 6.0–9.2 for yeast and rabbit (Krietsch and Bücher, 1970), pH 6.0–8.5 for *Thermus thermophilus* (Nojima *et al.*, 1979) and pH 6.7–9.7 for pea seeds (Axelrod and Bandurski, 1953). The PGK activity of both enzymes depends on ionic strength (Figure 6). At an ionic strength of 0.2 M, the

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ATGGAGAAGATGACCATCAGGGATGTGGATCTGAAAGGCAAGAGAGTCATAATGAGAGTGGACTTCAACGTTCCAGTGAAGGATGGGGTC    90
 M  E  K  M  T  I  R  D  V  D  L  K  G  K  R  V  I  M  R  V  D  F  N  V  P  V  K  D  G  V     30

GTTCAGGACGACACGAGAATAAGAGCCGCCCTTCCAACGATAAAGTACGCTCTTGAACAGGGTGCAAAGGTGATACTCCTGTCCCATCTT   180
 V  Q  D  D  T  R  I  R  A  A  L  P  T  I  K  Y  A  L  E  Q  G  A  K  V  I  L  L  S  H  L     60

GGAAGGCCCAAGGGAGAACCTTCACCAGAGTTCAGCCTCGCACCTGTCGCCAAAAGACTCTCTGAGCTTCTTGGAAAAGAAGTGAAGTTT   270
 G  R  P  K  G  E  P  S  P  E  F  S  L  A  P  V  A  K  R  L  S  E  L  L  G  K  E  V  K  F     90

GTTCCCGCTGTTGTCGGTGATGAGGTGAAAAAGGCCGTTGAGGAGCTCAAAGAGGGAGAGGTTCTCCTCCTTGAAAACACCAGATTCCAC   360
 V  P  A  V  V  G  D  E  V  K  K  A  V  E  E  L  K  E  G  E  V  L  L  L  E  N  T  R  F  H     120

CCGGGAGAGACCAAGAACGATCCTGAACTCGCGAAGTTCTGGGCTAGCCTCGCCGATATTCACGTGAACGATGCCTTCGGAACGGCGCAC   450
 P  G  E  T  K  N  D  P  E  L  A  K  F  W  A  S  L  A  D  I  H  V  N  D  A  F  G  T  A  H     150

AGGGCACACGCTTCCAACGTTGGAATCGCACAGTTTATTCCCAGCGTAGCGGGATTCCTCATGGAAAAAGAGATAAAGTTCCTTTCCAAG   540
 R  A  H  A  S  N  V  G  I  A  Q  F  I  P  S  V  A  G  F  L  M  E  K  E  I  K  F  L  S  K     180

GTGACTTATAATCCAGAAAAACCGTACGTTGTGGTTCTTGGAGGAGCAAAGGTATCTGACAAAATCGGCGTCATCACGAACCTCATGGAG   630
 V  T  Y  N  P  E  K  P  Y  V  V  V  L  G  G  A  K  V  S  D  K  I  G  V  I  T  N  L  M  E     210

AAAGCCGACAGAATTCTCATAGGTGGAGCCATGATGTTCACCTTCCTGAAGGCTCTTGGCAAAGAGGTTGGATCGTCCAGGGTTGAAGAA   720
 K  A  D  R  I  L  I  G  G  A  M  M  F  T  F  L  K  A  L  G  K  E  V  G  S  S  R  V  E  E     240

GACAAGATCGACCTCGCAAAAGAACTCCTCGAAAAAGCGAAAGAAAAGGGTGTTGAGATCGTTCTTCCCGTTGATGCCGTTATCGCTCAG   810
 D  K  I  D  L  A  K  E  L  L  E  K  A  K  E  K  G  V  E  I  V  L  P  V  D  A  V  I  A  Q     270

AAGATCGAACCCGGTGTGGAAAAGAAGGTTGTCAGAATCGACGACGGGATACCCGAAGGATGGATGGGCCTCGACATAGGACCCGAGACA   900
 K  I  E  P  G  V  E  K  K  V  V  R  I  D  D  G  I  P  E  G  W  M  G  L  D  I  G  P  E  T     300

ATTGAGCTCTTCAAGCAGAAGCTCTCCGATGCAAAAACCGTTGTCTGGAACGGGCCAATGGGAGTCTTCGAAATAGACGATTTCGCTGAA   990
 I  E  L  F  K  Q  K  L  S  D  A  K  T  V  V  W  N  G  P  M  G  V  F  E  I  D  D  F  A  E     330

GGCACGAAGCAGGTCGCACTTGCGATCGCAGCGCTCACGGAAAAGGGAGCGATCACCGTTGTGGGTGGAGGAGACAGCGCCGCGGCGGTG  1080
 G  T  K  Q  V  A  L  A  I  A  A  L  T  E  K  G  A  I  T  V  V  G  G  G  D  S  A  A  A  V     360

AACAAGTTCGGTCTGGAAGACAAATTCTCCCACGTTTCAACGGGCGGAGGGGCTTCTCTCGAATTCCTTGAAGGAAAAGAACTTCCTGGT  1170
 N  K  F  G  L  E  D  K  F  S  H  V  S  T  G  G  G  A  S  L  E  F  L  E  G  K  E  L  P  G     390

ATTGCCAGCATGCGCATAAAAAAAAAAATAACTCGTAAACTGATCCTCGCTGGGAACTGGAAGATGCATAAAACGATCTCGGAAGCGAAA  1259
 I  A  S  M  R  I  K  K  K  * stop codon pgk
                           I  T  R  K  L  I  L  A  G  N  W  K  M  H  K  T  I  S  E  A  K     420

AAGTTTGTGTCGCTGCTCGTGAACGAACTTCACGACGTGAAAGAGTTCGAAATAGTGGTCTGTCCTCCGTTCACAGCTCTATCTGAAGTG  1349
 K  F  V  S  L  L  V  N  E  L  H  D  V  K  E  F  E  I  V  V  C  P  P  F  T  A  L  S  E  V     450

GGGGAAATACTCTCTGGTAGAAACATCAAATTGGGAGCTCAAAACGTTTTCTACGAAGACCAGGGAGCGTTCACCGGGGAGATTTCTCCT  1439
 G  E  I  L  S  G  R  N  I  K  L  G  A  Q  N  V  F  Y  E  D  Q  G  A  F  T  G  E  I  S  P     480

CTCATGCTGCAAGAGATCGGCGTTGAATACGTGATCGTGGGACATTCCGAGAGAAGGCGTATTTTCAAAGAAGACGACGAGTTCATAAAC  1529
 L  M  L  Q  E  I  G  V  E  Y  V  I  V  G  H  S  E  R  R  R  I  F  K  E  D  D  E  F  I  N     510

AGGAAAGTGAAAGCGGTGCTTGAAAAAGGTATGACTCCTATTCTCTGCGTTGGAGAAACACTCGAGGAAAGAGAGAAAGGGCTCACTTTC  1619
 R  K  V  K  A  V  L  E  K  G  M  T  P  I  L  C  V  G  E  T  L  E  E  R  E  K  G  L  T  F     540

TGCGTTGTGGAAAAACAGGTGAGAGAAGGTTTCTACGGTCTCGACAAAGAGGAAGCAAAGAGAGTGGTAATAGCTTACGAGCCAGTCTGG  1709
 C  V  V  E  K  Q  V  R  E  G  F  Y  G  L  D  K  E  E  A  K  R  V  V  I  A  Y  E  P  V  W     570

GCAATCGGGACAGGAAGGGTGGCGACACCACAGCAGGCACAGGAAGTACACGCGTTCATAAGAAAGCTGCTCTCAGAGATGTACGACGAG  1799
 A  I  G  T  G  R  V  A  T  P  Q  Q  A  Q  E  V  H  A  F  I  R  K  L  L  S  E  M  Y  D  E     600

GAAACAGCGGGATCGATAAGGATTCTCTACGGTGGAAGCATAAAGCCGGACAATTTCCTCGGTCTCATCGTTCAGAGGGATATAGATGGT  1889
 E  T  A  G  S  I  R  I  L  Y  G  G  S  I  K  P  D  N  F  L  G  L  I  V  Q  R  D  I  D  G     630

GGTCTCGTTGGAGGAGCGAGTCTCAAACAGTCTTTCATAGAACTTGCACGAATAATGAGAGGTGTGATTTCCTGA                  1964
 G  L  V  G  G  A  S  L  K  Q  S  F  I  E  L  A  R  I  M  R  G  V  I  S  * stop codon fus   654
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Fig. 4. Complete nucleotide sequence of the *pgk/tpi* gene (*fus* gene) of *T.maritima* and the deduced amino acid sequence. The 30 amino acid N-terminal sequence underlined was obtained by Edman degradation of PGK and the PGK−TIM fusion protein. *fus* gene: nucleotides 1–1964 (654 amino acids plus stop codon, assuming that a −1 frameshift is in effect; see Results); *pgk* orf: nucleotides 1–1200 (399 amino acids plus stop codon); *tpi* orf: nucleotides 1197–1964 (255 amino acids plus stop codon).

reaction rates at pH 5.4 and 7.0 are equal. For the interpretation of the pH profiles at a constant ionic strength of 0.1 M, this has to be kept in mind.

The pH profile for the TIM activity of the fusion protein has an optimum between pH 7 and 9 (Figure 5), in accordance with TIMs from other sources (Plaut and Knowles, 1972; Lambeir *et al.*, 1987). Sulfate, phosphate and arsenate ions inhibit the enzyme (data not shown).

The temperature dependence of the apparent K_m values was determined in the temperature range between 20 and 60°C (Table II). No significant temperature effect was found for the K_m of 3-phosphoglycerate (3-PGA) in the kinase reaction of PGK and the PGK−TIM fusion protein. For both enzymes, biphasic double-reciprocal plots are obtained. Similar negative cooperativity has been found for other PGKs (João and Williams, 1993). The apparent K_m values for 3-PGA of the fusion protein are twice as high as the corresponding values for the monomeric PGK.

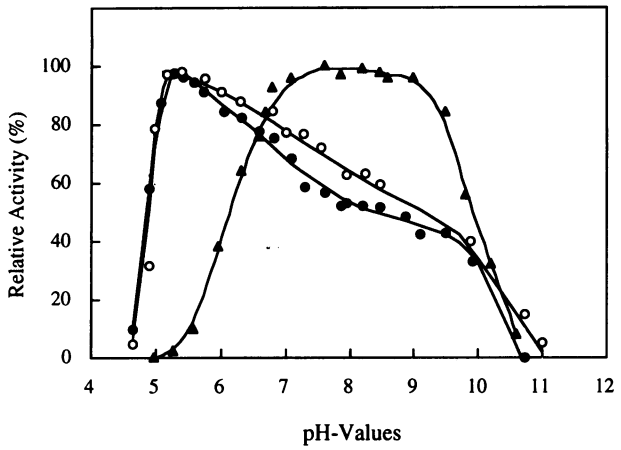

Fig. 5. Effect of pH on the catalytic activity of *T.maritima* PGK (—○—) and the PGK–TIM fusion protein in the PGK reaction (—●—) and in the TIM reaction (—▲—). The following buffers were used: NaAc–HAc, pH 4.6–5.6; MES [2-(*N*-morpholino)ethanesulfonic acid]–NaOH, pH 5.5–6.6; sodium phosphate, pH 5.7–6.8; MOPS [3-(*N*-morpholino)propanesulfonic acid]–NaOH, pH 6.0–7.5; HEPPS [4-(2-hydroxyethyl)piperazine-1-propanesulfonic acid]–NaOH, pH 7.3–8.8; glycine–NaOH, pH 8.6–10.6. All buffers were 50 mM and titrated with NaCl to a ionic strength of 0.1 at the desired pH.

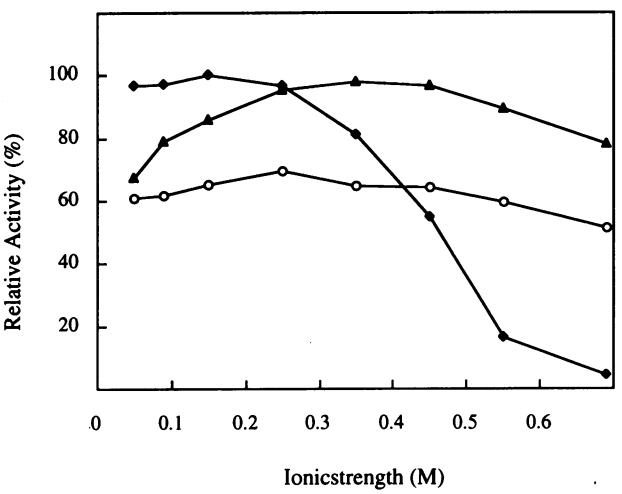

Fig. 6. Effect of ionic strength on the catalytic activity of PGK from *T.maritima*. The following buffers were used: MES–NaOH, pH 5.4 (◆); MOPS–NaOH, pH 7.0 (▲); HEPPS–NaOH, pH 8.6 (○). The increasing ionic strength was titrated with NaCl to the desired value in the reaction mixture.

Table II. Temperature dependence of the apparent K_m values of PGK and the PGK–TIM fusion protein[a]

Enzyme	Substrate	Apparent K_m (mM)		
		20°C	40°C	60°C
PGK	3-PGA	0.4	0.3	0.4
	ATP		0.03	
PGK_{fus}	3-PGA	0.6	0.7	0.9
	ATP	n.d.	0.03	n.d.
TIM_{fus}	DAP	0.3	0.5	2.6

[a]The K_m values for DHAP measured in the TIM reaction are not corrected for arsenate inhibition (see text). For experimental details, see Materials and methods.

In the case of the TIM reaction of the PGK–TIM fusion protein, double-reciprocal plots of the reaction rate versus DHAP concentration between 0.01 and 10 mM were linear. The corresponding K_m values are found to be strongly temperature-dependent, showing a 9-fold increase between 20 and 60°C. This result was complicated by the instability of DHAP at alkaline pH (Michal, 1984). To overcome this problem, initial rates were determined after initiating the reaction with DHAP. An increase in the K_m with increasing temperature has also been observed for a number of thermophilic dehydrogenases, e.g. GAPDH from *Thermoprotheus tenax* (Hensel *et al.*, 1987), *Methanothermus fervidus* (Fabry and Hensel, 1987) and lactate dehydrogenase from *T.maritima* (Hecht *et al.*, 1989). The K_m values reported here are not corrected for inhibition by arsenate, which is present in the coupled assay system (Burton and Waley, 1968).

The temperature dependence of both PGK activity and TIM activity was examined in the respective standard assay systems. Determination of PGK activity beyond 70°C was difficult because of the decomposition of NADH at elevated temperature and acidic pH. In the temperature range between 20 and 70°C, the Arrhenius plots reveal a linear relationship with identical activation energies for the PGK reaction of monomeric PGK and the PGK–TIM fusion protein (38 and 37 kJ/mol). In the case of the TIM assay of the fusion protein, measurements beyond 60°C failed due to the decomposition of DHAP. To compensate for this effect at temperatures ≤60°C, saturation of the enzyme was achieved by increasing the substrate concentration in the assays 6-fold. The corresponding Arrhenius diagram is linear with an activation energy of 80 kJ/mol; this implies that increasing the temperature from 20 to 60°C yields a 40-fold increase in activity.

The above purification protocols yielded homogenous PGK and PGK–TIM fusion protein with specific activities of 400 and 200 U/mg respectively in the PGK backward reaction. Although accurate determination of the specific catalytic activity in the forward reaction failed because of restrictions of the coupled assay (Bücher, 1955), PGK was shown to exhibit at least three times the activity of the PGK–TIM fusion protein. The specific activity of the fusion protein measured in the TIM assay exceeded 500 U/mg, which extrapolates to 20 000 U/mg at the optimum growth temperature (see above). This high catalytic efficiency is in agreement with the activity reported for TIMs from mesophilic hosts at their respective growth temperatures (Noltmann, 1972; Jaenicke, 1991).

Stability measurements

To quantify the thermal stability of both PGK and the PGK–TIM fusion protein, the irreversible heat inactivation was monitored in the temperature range 70–105°C. Incubation in 50 mM phosphate buffer, pH 7.5, in the presence of 1 mM EDTA for 2 h at varying temperatures yielded a thermal transition of 100°C for both enzymes, measured as loss of PGK activity. Since phosphate is known to inhibit TIM, the corresponding experiment for the isomerase reaction of the fusion protein had to be performed in 50 mM HEPPS [4-(2-hydroxyethyl)piperazine-1-propanesulfonic acid]–NaOH buffer, pH 7.5, in the presence of 1 mM EDTA and 5 mM cysteamine. The

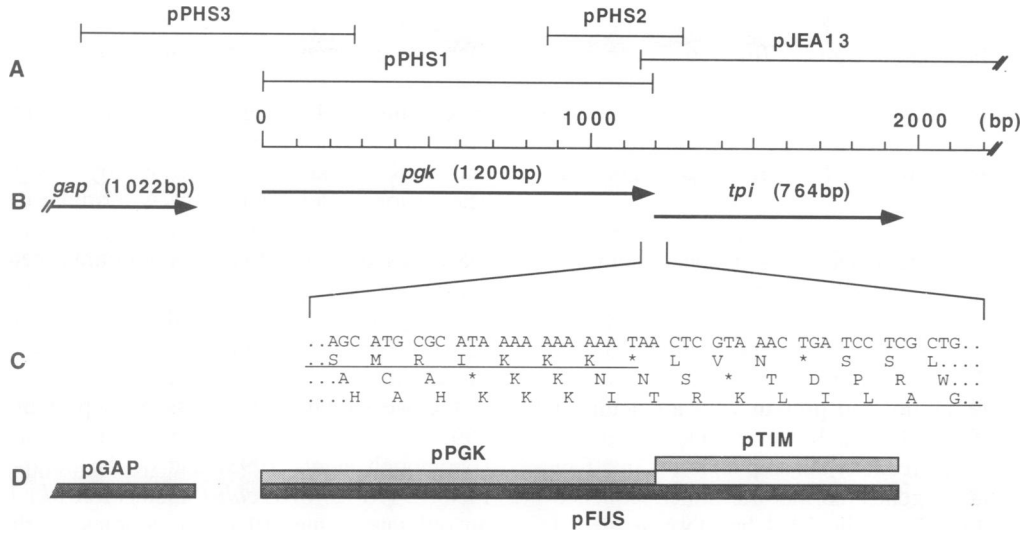

Fig. 7. Schematic diagram of the apparent *gap* operon of *T.maritima*. (**A**) The clones and PCR products which overlap to constitute the gene cluster. (**B**) The open reading frame (ORF) in the coding strand corresponding to the *gap* (Tomschy *et al.*, 1993), *pgk* and *tpi* genes. (**C**) The three phase translation of the frameshift region between the *pgk* and *tpi* genes (nucleotides 1177–1221 of the *fus* gene; Figure 4); the *pgk* and the *tpi* ORF are underlined. (**D**) The plasmid constructs used to over-express the corresponding genes in *E.coli*.

significantly reduced thermal transition of TIM inactivation of 88°C may have resulted from specific ion effects.

Identification and cloning of the T.maritima pgk and tpi genes

The *pgk* gene was amplified by polymerase chain reaction (PCR) using two degenerate primers corresponding to the identical N-terminal 12 amino acids of PGK and the PGK–TIM fusion protein (Figure 4) and to the last seven amino acids of a published *T.maritima* C-terminal PGK sequence. This 65 amino acid C-terminal fragment (clone ESTMT-1011) was found by random sequencing of cDNA and genomic libraries of *T.maritima* (Kim *et al.*, 1993) and showed high homology to the PGK of *T.aquaticus*. Polymerase chain reaction yielded an ~1.2 kb fragment pPHS1 (Figure 7A). Sequencing showed that the amplified sequence and the sequence of Kim *et al.* (1993) were only 80% identical.

In a parallel experiment, we used TIM homology PCR to amplify a fragment of the *tpi* gene from *T.maritima*. The genomic DNA was found to be an excellent substrate for PCR between two degenerate oligonucleotide primers, corresponding to highly conserved regions in the TIM sequences known to play an essential role in the mechanism of the enzyme, i.e. around His111 (His495 in Figure 4; Lodi and Knowles, 1991) and in the phosphate binding α-helix (Belasco *et al.*, 1978). The resulting 484 bp fragment was cloned, sequenced and used to probe Southern blots of digested *T.maritima* genomic DNA. This probe identified an *Eco*RI fragment of ~2 Kb, pJEA13 (Figure 7A), which was isolated from a subgenomic library. Clones of this fragment were found to carry the full-length *tpi* sequence. The 5′-terminus of this gene lacks promoter elements and a start codon. Instead, the final 19 codons of an open reading frame (ORF) abut the 5′-terminus of the *tpi* gene are identical to the 3′-terminus sequence found in pPHS1. The complete organization of the gene cluster was confirmed by sequencing additional PCR products (Figure 7A and B): (i) pPHS3 between the *gap* gene coding for glyceraldehyde 3-phosphate dehydrogenase (Tomschy *et al.*, 1993) and the *pgk* gene; and (ii) pPHS2 between the *pgk* gene and the *tpi* gene, confirming the transition between the two genes.

These results show that: (i) the *fus* gene (the connection of the *pgk* and *tpi* genes is called the *fus* gene, indicating that this gene is sufficient to encode the PGK–TIM fusion protein, see below) is part of the apparent *gap* operon of *T.maritima*; (ii) since the *tpi* gene lacks a start codon and promoter elements, it must be expressed together with the preceding *pgk* gene and, therefore, cannot be present as a distinct enzyme in *T.maritima*; (iii) both genes are not an in-frame fusion (Figure 7C). The *pgk* gene ends with a stop codon that overlaps the TIM coding sequence. A mechanism such as a programmed reading frameshift must be in effect upstream of the PGK stop codon. The most common types of frameshifts are −1, but +1 and even higher shifts in either direction (translational hops) have been observed in retroviruses, retrotransposons, bacterial transposons and in yeast (Atkins *et al.*, 1991; Farabough *et al.*, 1993). Depending on signals in the sequence of the mRNA, such translational frameshifts yield efficiencies of 50% and more.

Further evidence for these findings came from expression experiments on the previously mentioned genes in *Escherichia coli* (Figure 7D). (i) The *pgk* gene was over-expressed in *E.coli* as a distinct entity (pPGK), yielding an active, thermostable and monomeric PGK with spectroscopic and physicochemical properties identical to those of the PGK purified from *T.maritima* (Schurig *et al.*, in preparation). (ii) After introduction of an initiation Met codon (such that the engineered N-terminus would start with MITRKà) and subcloning in an expression vector, a modified *T.maritima tpi* gene (pTIM) supported growth of a TIM-deficient *E.coli* strain on minimal medium. Furthermore, a thermostable TIM activity was detected in the crude extract (Adler, 1994). (iii) Cloning of the complete *fus* gene (pFUS), including the frameshift, supported the independent expression of PGK and the PGK–TIM fusion protein in *E.coli*. This finding indicates that the *fus* gene with the two overlapping reading frames

is sufficient to encode both PGK and the fusion protein and that the mechanism of frameshifting in *T.maritima* and in *E.coli* are essentially the same (N.Beaucamp, unpublished result).

Nucleotide sequence of the T.maritima pgk and tpi genes

The nucleotide sequence and the deduced amino acid sequence of PGK and the PGK−TIM fusion protein are shown in Figure 4. The complete primary structure was determined by sequencing the PCR products pPHS1 and pPHS2 and clone pJEA13. As deduced from the DNA sequence, the 1200 bp (including stop codon) of the *pgk* gene encode a 399 amino acid protein with a calculated molecular mass of 43.19 kDa. If we assume that a −1 negative frameshift upstream of the stop codon is in effect to link the *pgk* and *tpi* genes (so that the linker sequence might be ...IKKKITRKL...), the 1964 bp DNA sequence of the *fus* gene (including the stop codon) encodes a 654 amino acid protein with a calculated mass of 71.6 kDa. These predicted masses are in accord with those observed with the native enzymes.

Discussion

The hyperthermophilic bacterium *T.maritima* represents one of the deepest branches in the bacterial domain of the phylogenetic tree. It uses carbohydrates as one of its primary carbon sources and, under normal growth conditions, a number of glycolytic enzymes are expressed at relatively high levels. As shown in the present study, *T.maritima*, instead of producing a separate enzyme with TIM activity, elaborates a bifunctional 'polyprotein' that carries both PGK and TIM activity. On the other hand, a second PGK, distinct from the PGK−TIM fusion protein, could be purified to homogeneity from the crude extract of the bacterium. The present data clearly demonstrate that the TIM part of the fusion protein is covalently linked to the PGK part. Given the molecular mass of the subunits (as determined by gradient SDS−PAGE), the difference between the fusion protein, 70 kDa, and PGK, 43 kDa, corresponds well to the known molecular mass of a TIM subunit from other sources, 27 kDa. It is remarkable that in the bifunctional enzyme, two reactions are combined which do not represent consecutive steps in glycolysis.

Topologically, three different combinations of the two enzymes would be possible: PGK may be added either to the N- or the C-terminus of TIM or one of the proteins may be inserted into the sequence of the other, forming an internal domain. There are examples in the family of α/β-barrel enzymes for each of these configurations (Brändén and Tooze, 1991). For instance, pyruvate kinase folds into four well-defined domains, a central α/β-barrel plus three additional independent portions: one at the N-terminus (residue 1–42), another looped out from the end of β-strand 3 of the TIM barrel (residue 116–223) and finally an open twisted α/β domain at the C-terminus (residue 388–530) (Muirhead *et al.*, 1986). Our characterization of the *T.maritima fus* gene demonstrates linkage of the N-terminus of the TIM to the C-terminus of the PGK. The *gap* gene (which encodes GAPDH; Tomschy *et al.*, 1993) is followed by the *pgk* and *tpi* genes (encoding PGK and TIM). The observed gene cluster (Figure 7) resembles the operon of several bacteria, such as the *gap* operons of *B.megaterium* (Schläpfer and Zuber, 1992) and *Corynebacterium glutamicum* (Eikmanns, 1992). Our expression experiments suggest that both enzymes, the fusion protein (70 kDa per subunit) and PGK (43 kDa), are encoded by this gene segment. The fusion protein shares the N-terminal domain of 399 amino acids with PGK, followed by ~255 amino acids of the TIM sequence. Obviously, a programmed translational frameshift is required for synthesis of the PGK−TIM fusion protein. Such natural subversion of the triplet decoding upon translation is involved in the expression of several procaryotic and eucaryotic genes. Expression of the bacteriophage λ tail assembly protein (Levin *et al.*, 1993), of the POL3 gene of retrotransposon Ty3 of yeast (Farabough *et al.*, 1993) and of the polypeptide release factor-2 (RF-2) of *E.coli* (Adamski *et al.*, 1993) involve an obligate frameshift event. Studies on the mechanism pointed out some general principles for such natural frameshifts (Gesteland *et al.*, 1992; Farabough *et al.*, 1993). All programmed frameshifts depend on two elements, a 'recoding site' (Gesteland *et al.*, 1992), which allows non-triplet translocation (slippage) of the mRNA, and a stimulator (Atkins *et al.*, 1990), which increases the efficiency of recoding. It was found that secondary structure elements (like pseudoknots) and rare codons stimulate the frameshift events by causing a translational pause. In other cases an adjacent in-frame termination codon and a Shine−Dalgarno interaction between 16S rRNA and a sequence upstream of the shift site increase the frameshift efficiency (Adamski *et al.*, 1993). With the *fus* gene, no secondary structure elements nor a ribosome binding site are detectable, but the poly(A) sequence may act as a slippage site in combination with the in-frame stop codon (Figure 7C). The *E.coli* RNA polymerase was found to slip on 10–11 nucleotide long runs of A or T bases (Wagner *et al.*, 1990).

The amino acid sequences deduced from the nucleotide sequences of both the PGK and the TIM part of the *fus* gene do not appear to differ to a large extent from the sequences of the corresponding enzymes from psychrophilic, mesophilic and thermophilic organisms. The sequence identity of the TIM part of the fusion protein from *T.maritima* to TIMs from other sources ranges from 35% for the enzyme from the psychrophile *Moraxella* to 50% for the moderate thermophile *B.stearothermophilus* (Rentier-Delrue *et al.*, 1993). For the PGK sequence, the identities are even higher, ranging from 35% for *Methanothermus fervidus*, a thermophilic archaeon (Fabry *et al.*, 1990) to 61% for *B.stearothermophilus* (Davies *et al.*, 1991). Neither the amino acid nor the nucleotide composition provide a simple explanation for the thermal stability of *T.maritima* PGK and the PGK−TIM fusion protein (Böhm and Jaenicke, 1994).

From a structural point of view, the proposed link between the C-terminus of PGK and the N-terminus of TIM is conceivable: the crystal structures of both PGK (Banks *et al.*, 1979) and of TIM (Wierenga *et al.*, 1992) reveal that the C- and N-termini of the two enzymes are solvent exposed and sterically accessible to chain extensions. As shown by various independent methods, the monofunctional PGK is a monomeric enzyme with a molecular mass of 43 kDa. This result is in agreement

with all other known PGKs reported so far. In contrast, the PGK–TIM fusion protein appears to be tetrameric, summarizing the evidence gained from ultracentrifugal analysis and light scattering. Generally, TIMs have been reported to be homodimers held together by an interfacial loop located opposite to the N-terminus (Wierenga *et al.*, 1992) and several residues that fold into this loop are found to be conserved in the *T.maritima* TIM primary sequence. Assuming this topology to hold for the enzyme, a fusion of the C-terminus of PGK to the N-terminus of TIM should allow the normal dimerization of the isomerase, with PGK projecting from the back of each of the monomer α/β-barrels. However, experimental evidence from sedimentation analysis, gel permeation chromatography and dynamic light scattering experiments clearly indicates that association of the PGK–TIM fusion protein goes beyond the dimer. Combining the results of sedimentation velocity ($s^0_{20,w}$) and dynamic light scattering ($D_{20,w}$), the calculated molecular mass ($M_{s,D}$ = 286 kDa) corresponds precisely to the tetramer, using the monomer molecular weight (M = 70 kDa). This result is confirmed by high speed sedimentation equilibria which, even under conditions of meniscus depletion (Yphantis, 1964), do not allow significant dissociation of the tetramer to dimers or monomers to be detected. The structural basis of this higher order oligomerization of the apparently well-conserved domains cannot be deduced from examination of the primary sequences.

One difference between the two PGK-active enzymes is the higher catalytic efficiency of the monomeric PGK compared with the fusion protein, measured in both the glycolytic and gluconeogenetic directions. Since domain movements have been suggested as an essential element of the catalytic mechanism, the reduced activity of the PGK–TIM fusion protein would lend support to the notion that this domain movement is hindered in the bifunctional enzyme complex. The difference is also reflected in the K_m values for the substrate 3-PGA. Other catalytic properties, such as the effect of pH, temperature and ionic strength on the catalytic activity and the activation energy of the enzymatic reaction, are similar. The differences in the temperature dependence of the various K_m values (Table II) confirm earlier observations which indicated that under physiological conditions enzymes do not necessarily operate with maximum efficiency. In this context, Wetlaufer (1980) has pointed out that proteins must normally fulfil multiple functions (including folding, targeting, catalysis, degradation as nutrient, etc.) and that it is likely to be impossible to optimize all of them simultaneously. Obviously, in the case of a hyperstable bifunctional enzyme complex, this holds to an even higher degree than for simple thermophilic enzymes, where anomalous temperature effects on K_m have been previously reported (Hecht *et al.*, 1989).

As in other hyperstable enzymes investigated so far, PGK and the PGK–TIM fusion protein require high temperatures to exhibit significant catalytic activity. In the mesophilic temperature region, both enzymes are essentially inactive. This corroborates earlier observations that indicate that homologous enzymes from mesophiles and extremophiles occupy 'corresponding states' with respect to their catalytic properties if they are compared under their respective physiological conditions (Wrba *et al.*, 1990; Jaenicke, 1993).

Considering the optimal growth temperature of *T.maritima* (80°C), both PGK and the PGK–TIM fusion protein are sufficiently stable to carry out their physiological function without requiring extrinsic stabilizing factors. Experimental constraints did not allow determination of the optimum and maximum temperatures of catalysis for both enzymatic reactions. However, considering the half-times of inactivation and the intrinsic stability of other *Thermotoga* enzymes, the temperature limit of thermal denaturation for the two enzymes is likely to exceed the optimal growth temperature of the bacterium (Juszczak *et al.*, 1991; Blamey and Adams, 1994; H.Schurig, unpublished results).

Materials and methods

Bacterial strains and media

A culture of *T.maritima* (MSB 8, DSM 3109) was kindly provided by Drs K.O.Stetter and R.Huber (Lehrstuhl für Mikrobiologie, Universität Regensburg, Regensburg, Germany). Cells were grown at 80°C according to Huber *et al.* (1986). Frozen cells were also obtained from Dr M.W.W.Adams (University of Georgia at Athens, Athens, GA). *Escherichia coli* strains used in cloning experiments were XL1-blue (Stratagene) and BL21DE3 (Studier and Moffatt, 1986). TIM-deficient *E.coli* strain DF502, Δ(*rha*, *pfkA*, *tpi*), *pfk*B1, *his*$^-$, *pyrd*$^-$, *edd*$^-$, *F*$^-$, *str*^{r}, was a construct of Dr D.Fraenkel (Harvard Medical School, Cambridge, MA). *Escherichia coli* strains were grown in Luria Bertani medium (1% tryptone, 0.5% yeast extract, 0.5% sodium chloride) supplemented with agar (1.3%) and antibiotics (12 μg/ml oxytetracyclin or 100 μg/ml ampicillin), when appropriate.

Separation of PGK and PGK–TIM fusion protein

All chromatographic purification steps were performed aerobically at room temperature. Chromatographic materials were purchased from Pharmacia (Uppsala, Sweden).

Crude extract. Frozen cells (60 g wet weight) were thawed, resuspended in 250 ml 50 mM Tris–HCl buffer, pH 7.5, containing 1 mM EDTA (buffer A) and passed through a French press (SLM Aminco Instruments, Urbana, IL) at 15 000–20 000 p.s.i., i.e. (1.0–1.4)×10^8 N/m^2. After the addition of 15 μg/ml DNase I and 5 mM $MgCl_2$, the suspension was incubated at 37°C for 45 min. Insoluble material was removed by centrifugation (2 h at 48 000 *g*). The pellet was resuspended in standard buffer and again centrifuged (1 h at 48 000 *g*); subsequently the two supernatants were pooled.

Ammonium sulfate precipitation. Solid ammonium sulfate was added to the crude extract in portions of 20 g/h under continuous stirring up to 35% saturation. The suspension was further stirred for 1 h at 4°C. Following centrifugation (2 h at 48 000 *g*), ammonium sulfate was added to bring the supernatant to 70% saturation. After spinning down precipitated protein (2 h at 48 000 *g*), the pellet was dissolved in buffer A plus 30% saturated ammonium sulfate.

Hydrophobic chromatography. The 35–70% ammonium sulfate pellet was redissolved and applied to a 160 ml phenyl-Sepharose FF (high sub) column (5.0×8 cm) equilibrated with buffer A plus 30% saturated ammonium sulfate. The column was washed extensively with this buffer until all coloured bands were eluted. Two different peaks with PGK activity were eluted by applying a linear gradient of 30–0% ammonium sulfate in a total volume of 1.6 l (10 column volumes). A flow rate of 5 ml/min was used throughout this procedure. Active fractions of PGK (first PGK-active peak) and the TIM–PGK fusion protein (second PGK-active peak) were pooled separately.

Purification of PGK

Ion exchange chromatography. The pooled fractions were dialyzed against 20 mM Tris–HCl buffer, pH 8.0, plus 1 mM EDTA (buffer B) and applied to a 70 ml Q-Sepharose HP column (2.6×13 cm) equilibrated with this buffer. The column was washed at a flow rate of 8 ml/min until the baseline was reached. The PGK activity could be eluted with a linear gradient of 0–0.5 M NaCl contained in 10 column volumes.

Gel permeation chromatography. The collected active fractions were concentrated to ~10 mg/ml using an Amicon ultrafiltration cell (50 ml; Amicon, Lexington) and a PM-30 membrane. An aliquot of the concentrated solution (1.5 ml) was applied to a 120 ml Superdex 75 pg column (1.6×60 cm). The column was equilibrated with buffer B with 100 mM NaCl at a flow rate of 1 ml/min. The purified enzyme was pooled, dialyzed against buffer B with 0.01% NaN_3 and stored at 4°C.

Purification of TIM–PGK fusion protein

Ion exchange chromatography. The pooled fractions were dialysed against 80 mM Tris–HCl buffer, pH 7.5, in the presence of 1 mM EDTA and 5 mM cysteamine (buffer C); subsequently they were applied to a 70 ml Q-Sepharose HP column (2.6×13 cm) equilibrated with this buffer. The column was washed at a flow rate of 8 ml/min until the baseline was reached. The PGK activity could be eluted with a linear gradient of 0–0.5 M NaCl contained in 10 column volumes.

HiTrap Blue. The collected active fractions were concentrated in an Amicon ultrafiltration cell (50 ml) using a PM-30 membrane. After dialysis against buffer C, the solution containing the TIM–PGK fusion protein was loaded on a HiTrap Blue column (5 ml) in portions of ~5 mg. After washing the column with buffer C at a flow rate of 4 ml/min, the PGK activity was eluted in a sharp peak using a pulse of buffer C with 1 M NaCl.

Gel permeation chromatography. The collected active fractions were concentrated to ~10 mg/ml using a Centricon 30 microconcentrator (Amicon, Lexington). An aliquot of this solution (1.5 ml) was applied to a 120 ml Superdex 200 pg column (1.6×60 cm). The column was equilibrated with buffer C with 100 mM NaCl at a flow rate of 1 ml/min. The purified enzyme was pooled, dialyzed against buffer C with 0.01% NaN_3 and could be stored at 4°C without significant loss of activity over months.

Protein concentrations were determined using the BCA (bicinchoninic acid) protein assay (Pierce, Rockford), following the procedure recommended by the manufacturer, with bovine serum albumin as standard.

Enzyme assays

Unless otherwise noted, substrates, coenzymes and helper enzymes in the enzyme assays were purchased from Boehringer (Mannheim, Germany). The enzyme activity of PGK was assayed using the backward reaction from 3-PGA to 1,3 bisphosphoglycerate, as described by Bücher (1955). The reaction was initiated by the addition of a suitable amount of enzyme to the assay mixture: 7 mM 3-PGA, 5 mM $MgCl_2$, 5 mM cysteamine, 1 mM EDTA, 2.2 mM ATP, 0.26 mM NADH, 20 μg/ml glyceraldehyde phosphate dehydrogenase (GAPDH) in 50 mM test buffer. Unless otherwise noted, the test buffer was 50 mM sodium phosphate buffer, pH 6.0. The forward reaction was used for a qualitative characterization of the enzymes (Bücher, 1955), because it has the disadvantage that the change in concentration of the substrate is not linearly related to the change in optical density.

TIM activity was determined in the direction from DHAP to glyceraldehyde phosphate, measuring the change in ultraviolet absorbance resulting from NADH formation in the coupled assay with GAPDH and arsenate (Plaut and Knowles, 1972). The standard assay mixture contained 5 mM DHAP (Sigma, St Louis), 1 mM NAD^+, 6 mM NaH_2AsO_4, 20 μg/ml GAPDH in 50 mM HEPPS–NaOH buffer, pH 8.0, with 1 mM EDTA. In both cases, changes in absorbance of NADH were measured at 366 nm and 40°C (standard assay) in an Eppendorf Photometer 1101M. The concentrations of the substrates, DHAP and 3-PGA, were determined enzymatically (Michal, 1984). Blank experiments were performed in the absence of the enzyme in order to correct for NADH autoxidation at high temperature and acidic pH. The pH of buffer solutions were corrected at elevated temperatures, making use of published temperature coefficients ΔpK/°C. At temperatures beyond 40°C, the rabbit muscle GAPDH (as auxiliary enzyme) was replaced by the recombinant thermostable GAPDH from *T.maritima* (Tomschy *et al.*, 1993).

Kinetic measurements

The ionic strength of the buffers used for pH-dependent activity measurements was adjusted to 0.1 M using NaCl. The kinetic parameters V_{max} and K_m were obtained from an unweighted least squares analysis of plots of $[S_0]/v_0$ versus v_0 (Hanes plot), with v_0 as initial velocity and $[S_0]$ as initial substrate concentration. Direct linear plots were used to calculate confidence limits of K_m and V_{max} (Henderson, 1992).

Enzyme stability

To investigate the long-term stability of the enzymes at high temperature, 200 μl portions of the enzyme solution were placed in 0.5 ml safe-lock microtubes (Eppendorf, Hamburg, Germany) at protein concentrations of ~10 μg/ml and overlaid with Nujol mineral oil (Perkin Elmer, Überlingen, Germany). After incubation at the desired temperature, samples were cooled rapidly and assayed immediately for residual activity.

Determination of molecular masses

The molecular masses and the subunit composition of the purified enzymes were determined by SDS–PAGE, gel permeation chromatography and sedimentation analysis as described before and in a previous study (Schurig *et al.*, 1995).

N-terminal sequencing

Determination of N-terminal sequences were performed by Dr R. Deutzmann (University of Regensburg) using an Applied Biosystems Sequencer 120A/477A.

Recombinant DNA techniques

DNA manipulations made use of standard methods (Sambrook *et al.*, 1989). Oligonucleotides were supplied by MWG-Biotech (München). Enzymes used in recombinant DNA manipulations were obtained from Boehringer (Mannheim), New England Biolabs and Stratagene.

Identification and cloning of the tpi reading frame. Opposing oligonucleotide primers complementary to two highly conserved regions of TIM were used to amplify a *tpi* fragment from *T.maritima* genomic DNA. The sense primer, 5′-GATCAAGCTTATCGATCTGCAG TGG ATH CTN GGN CAY WSN GA-3′ (where N represents all four nucleotides; H represents A, C and T; Y represents C and T; and W represents A and T), was designed to correspond to a region of *tpi* encoding the catalytic histidinyl residue (residues 491–497 in the *T.maritima tpi* sequence given in Figure 4). The antisense primer, 5′-GATCAATTCT-CGAGGATCC YTT NAG NGA NGC NCC NCC NAC NAG RAA NCC RTC NAC RTC-3′ (where R represents A and G), was designed to correspond to a region of *tpi* that comprises a conserved α-helix involved in the binding of the substrate phosphoryl group (residues 626–638 in the *T.maritima tpi* sequence). This strategy is analogous to that used to amplify a *tpi* fragment from *Moraxella* spp. strain TA137 (Rentier-Delrue *et al.*, 1993).

The fragment generated by PCR amplification was inserted in the *Bam*HI–*Pst*I site of pBS(+) and sequenced to eliminate the possibility that contaminated *tpi* sequences had been amplified. This fragment was used to probe Southern blots of digested *T.maritima* genomic DNA, identifying an *Eco*RI fragment of ~2 kb. This *Eco*RI fragment was isolated from a size-selected subgenomic library in pUC18 and was found to contain the complete *tpi* reading frame on sequencing using primers complementary to regions of the PCR-amplified *tpi* fragment. The *tpi* reading frame is located ~60 bp from one end of the fragment, preceded by and overlapping with the last 19 codons of *pgk*. This clone did not complement DF502.

The oligonucleotides 5′-GATCCATATG ATA ACT CGT AAA CTG-3′ and 5′-GGCCCTGCAGGATCC TCA GGA AAT CAC ACC-3′ were used to introduce an N-terminal start codon to the *tpi* reading frame (such that the N-terminus would begin as MITRKà) and 3′-end cloning sites by PCR amplification. The resulting fragment was introduced into the *Nds*I–*Bam*HI site of pET11a and into the *Nde*I–*Pst*I site of pBSx1c-*Nde*I, a modification of the vector used to express chicken TIM (Blacklow and Knowles, 1990) that replaced the start codon *Nco*I site with an *Nde*I site (engineered by Dr S.Pollack). Lysates of BL21 transformants of the pET11a clone were assayed for thermostable TIM activity and DF502 transformants of the pBSx1c-*Nde*I clone were tested for growth on glycerol-supplemented minimal plates.

Identification and cloning of the pgk reading frame. A 3–6 kb subgenomic library of *T.maritima* DNA was constructed as described by Ostendorp *et al.* (1993) and used as template for PCR. Three different reactions were performed to obtain DNA fragments of the *pgk* gene (pPHS1), the region between the *pgk* and *tpi* genes (pPHS2) and between the *gap* and *pgk* genes (pPHS3). A schematic diagram outlining the procedure is shown in Figure 7A. The following oligonucleotides were used as primers: pPHS1, sense primer, 5′-ATG GAG AAG ATG ACK ATC AGR GAT GTK GAT CTK AA-3′ (encoding amino acids 1–12 of the N-terminus of PGK and the fusion protein); antisense primer, 5′-TTA CCA KGG KAG TTC TTT KCC TTC (amino acids 1–7 plus the stop codon of the C-terminus from clone ESTMT 1011, Kim *et al.*, 1993).

pPHS2, sense primer, 5′-TTGAGCTCTTCAAGCAGAAGC-3′ (nucleotides 902–920 of the *pgk* gene); antisense primer, 5′-CGCTTCCGAGATCGTTTTATGCAT-3′ (nucleotides 1233–1256 of the *fus* gene). pPHS3, sense primer, 5′-GGAAAGCTCGTGAAGGTGGCTTCC-3′ (nucleotides 932–955 of the *gap* gene; Tomschy *et al.*, 1993); antisense primer, 5′-ACAGCGGGAACAAACTTCACT-3′ (nucleotides 261–281 of the *pgk* gene). All primers were designed to introduce flanking restriction sites into the amplified fragments for subsequent cloning into the *Xba*I–*Hin*dIII site of pKM1. The pKM1 plasmid used to over-express PGK and the PGK–TIM fusion protein was provided by Klaus Maskos (ETH, Zürich, Switzerland); pKM1 is a derivative of pASK40 (Skerra *et al.*, 1991) with a T7 promoter replacing the *lac* promoter/operator. The PCR was carried out in a volume of 100 µl using 10 ng DNA and 100 pmol primer. The annealing temperature was 55°C.

The sequences were deposited at the EMBL gene bank under the accession numbers X75437 for the *pgk* reading frame and L27492 for the *tpi* reading frame.

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