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Dissection of the gene of the bifunctional PGK–TIM fusion protein from the hyperthermophilic bacterium *Thermotoga maritima*: Design and characterization of the separate triosephosphate isomerase

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Abstract

Triosephosphate isomerase (TIM), from the hyperthermophilic bacterium *Thermotoga maritima*, has been shown to be covalently linked to phosphoglycerate kinase (PGK) forming a bifunctional fusion protein with TIM as the C-terminal portion of the subunits of the tetrameric protein (Schurig et al., *EMBO J 14*:442–451, 1995). To study the effect of the anomalous state of association on the structure, stability, and function of *Thermotoga* TIM, the isolated enzyme was cloned and expressed in *Escherichia coli*, and compared with its wild-type structure in the PGK–TIM fusion protein. After introducing a start codon at the beginning of the tpi open reading frame, the gene was expressed in E.c.BL21(DE3)/pNBTIM. The nucleotide sequence was confirmed and the protein purified as a functional dimer of 56.5 kDa molecular mass. Spectral analysis, using absorption, fluorescence emission, near- and far-UV circular dichroism spectroscopy were used to compare the separated *Thermotoga* enzyme with its homologs from mesophiles. The catalytic properties of the enzyme at ~80 °C are similar to those of its mesophilic counterparts at their respective physiological temperatures, in accordance with the idea that under in vivo conditions enzymes occupy corresponding states. As taken from chaotropic and thermal denaturation transitions, the separated enzyme exhibits high intrinsic stability, with a half-concentration of guanidinium-chloride at 3.8 M, and a denaturation half-time at 80 °C of 2 h. Comparing the properties of the TIM portion of the PGK–TIM fusion protein with those of the isolated recombinant TIM, it is found that the fusion of the two enzymes not only enhances the intrinsic stability of TIM but also its catalytic efficiency.

Keywords: bifunctional enzyme; hyperthermophiles; PGK–TIM fusion protein; phosphoglycerate kinase; stability; *Thermotoga maritima*; TIM; triosephosphate isomerase

The catalytic role of triosephosphate isomerase (TIM, EC.2.7.23) in glycolysis is to channel the six carbon atoms of fructose 1,6-bisphosphate into substrate phosphorylation by converting dihydroxyacetone phosphate to glyceraldehyde-3-phosphate. All TIMs isolated so far are homodimers with a subunit molecular mass of 26–27 kDa. As shown by denaturation/renaturation experiments, as well as designed monomeric forms of the enzyme, the dimeric quaternary structure is a necessary requirement for full catalytic function (Zabori et al., 1980; Wierenga et al., 1992). Artificial monomers constructed by carefully eliminating quaternary interactions exhibit at most 0.1% of the activity of the native dimer (Borchert et al., 1993, 1994). Altogether, TIM has been shown to be a paradigm for an evolutionarily perfected enzyme (Knowles, 1991). High-resolution structures of homologs from chicken (Banner et al., 1975), yeast (Lolis et al., 1990), trypanosoma (Wierenga et al., 1991; Noble et al., 1993a), *B. stearothermophilus* (Delboni et al., 1995), and *E. coli* (Noble et al., 1993b) have the same topological pattern, a one-domain dimeric $(\alpha/\beta)_8$ barrel, in common. This holds in spite of wide variations observed in the 30 primary structures presently known; identities range from 28 to 100% (Adler, 1994). Sequence alignments allow three regions of maximum conservation to be correlated with the catalytic mechanism and the active site of the enzyme.

In the case of the hyperthermophilic bacterium *Thermotoga maritima*, no distinct TIM can be found. Instead, the enzyme is covalently linked to phosphoglycerate kinase (PGK), forming a bifunctional, tetrameric 654 amino acid fusion protein (Schurig et al., 1995a; Beaucamp et al., 1995; Yu & Noll, 1995). The amino acid sequence of the PGK–TIM fusion protein is coded by two overlapping open reading frames allowing both monofunctional

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PGK and bifunctional PGK–TIM fusion protein to be translated from one and the same gene locus. The ratio of the two enzymes is determined by the efficiency of a (-1)-frameshift event at a C-terminal poly-A stretch on the pgk gene (Schurig et al., 1995a; Schurig, 1995; Wuhrer, 1995).

In this work, we use the *Thermotoga tpi* gene and clone and express non-fused recombinant hyperthermophilic TIM in a mesophilic host, *Escherichia coli*. The stability and activity of the isolated and fused *tpi*-gene products are compared. The results indicate that the fusion to PGK enhances both the intrinsic stability and the catalytic efficiency.

Results and discussion

Cloning and sequencing of the tpi gene

The *tpi* gene of *Thermotoga maritima* was cut out of the plasmid pJEA24. To express the TIM part of the PGK–TIM fusion protein, an ATG start codon was introduced at the 5′ end of the *tpi* gene. The DNA fragment was XbaI/BamHI digested and cloned into the expression plasmid pASK40. Subsequently, *E. coli* JM83 was transformed, leading to the *E. coli* strain *E. coli* JM83/pNBTIM (Fig. 1). The complete DNA sequence of the cloned *tpi* gene was determined and found to be authentic to the *tpi* gene of the fusion protein (Beaucamp et al., 1995; Schurig et al., 1995a).

Expression and purification

The recombinant TIM was expressed as soluble protein from *E. coli* JM83/pNBTIM. In purifying the enzyme, we took advantage of the anomalous thermostability of the *Thermotoga* enzyme, using a heat step to precipitate bulk of the host proteins at 80 °C, after disrupting the cells. Complete removal of nucleic acids was achieved by adding protamine sulfate. As shown by SDS-PAGE (Fig. 2), the recombinant enzyme consists of 28 kDa monomers, in agreement with the calculated subunit molecular mass of 28.53 kDa. Separation of TIM from residual *E. coli* proteins made use of Q-Sepharose. After a final gel filtration, and after concentrating the protein solution, the enzyme was stored at 4 °C. At low salt, *Thermotoga* TIM shows a strong tendency to aggregate at protein concentrations beyond 1 mg/mL; therefore, 400 mM NaCl was added to the storage solutions which, under this condition, allow concentrating the enzyme to concentrations beyond 10 mg/mL.

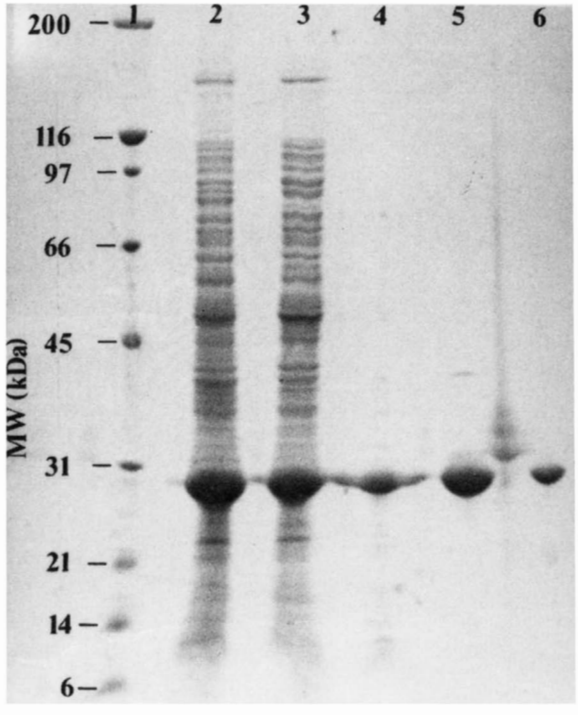

Fig. 2. Purification scheme of recombinant TIM from *E. coli* BL21(DE3)/pNBTIM. 12.5% SDS-PAGE, Coomassie stained. Lane 1, broad-range molecular mass standard; lane 2, crude extract; lane 3, fractions after DNA precipitation; lanes 4–6, combined fractions after heat denaturation, anion exchange chromatography (Q-Sepharose HP) and gel filtration (Superdex 75 pg), respectively.

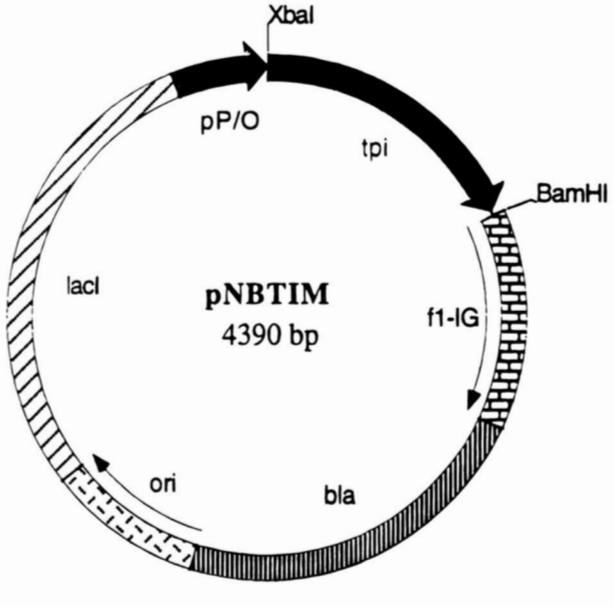

Fig. 1. Plasmid map of pNBTIM. tpi, 806 bp DNA of *Thermotoga maritima tpi* gene inserted by XbaI/BamHI restriction site; f1-IG: intergenic region of phage f1; bla, gene of β-lactamase; ori, replication origin of *E. coli* DNA polymerase; lacI, Lac repressor gene; pP/O, Lac promoter.

All purification steps were performed at room temperature under aerobic conditions. The homogeneity of the protein preparation was shown by SDS-PAGE and crystallization (see below). The total yield of TIM was 60 mg/L cell culture. For the purification scheme, see Figure 2.

Characterization

Molecular properties

The molecular mass and the quaternary structure of the purified enzyme were determined by SDS-PAGE, analytical ultra-centrifugation, and gel-permeation chromatography (Table 1). The results, 28 ± 2 kDa for the subunit molecular mass, and 56.5 ± 2.5 kDa for the native enzyme, confirm a dimeric quaternary structure, as has been reported for the enzyme from a wide variety of sources, including mesophiles and thermophiles (Wierenga et al., 1992). In the case of the native hyperthermophilic *Thermotoga* PGK–TIM fusion protein, monomeric PGK and dimeric TIM are combined to form a 286 kDa tetramer. For both constituent parts, the high

Table 1. *Comparison of the physicochemical and catalytic properties of triosephosphate isomerase from* Thermotoga maritima *as separate enzyme and integrated in the PGK–TIM fusion protein*

	Separated TIM	TIM_{fus}[a]
Molecular mass[b]		
Monomer (calculated) (kDa)	28.5	71.6
Monomer (SDS-PAGE) (kDa)	28 ± 3	71 ± 3
Native (GPC,AUC) (kDa)	56 ± 4	286 ± 10
Spectroscopic properties		
Absorbance A_{280nm}(0.1%, 1 cm)[c]	0.61 ± 0.03	0.55 ± 0.05
Molar extinction ϵ_M ($M^{-1}cm^{-1}$)	17,375	39,670
Fluorescence: λ_{max} (nm, in 0 M GdmCl)	329	323
Fluorescence: λ_{max} (nm, in 7 M GdmCl)	345	346
Ellipticity [θ_{222nm}] (deg cm^2 $dmol^{-1}$)	−8,030	−14,000
Catalytic properties		
Specific activity (40 °C) (U/mg)	450	400 (1,000)[d]
(80 °C)	1,250	2,840 (7,100)[d]
pH optimum of activity	8.0	7.5–9.0
Activation energy (kJ/mol)	49	80
$K_{M,DHAP}$ (80 °C) (mM)	2.6 ± 0.3	2.7 ± 0.3
Stability		
Thermal transition (°C)	82	90
$(c_{GdmCl})_{1/2}$ (activity at 40°) (M)	3.4	3.8
(fluorescence at 40 °C) (M)	3.6	3.9

[a]For details, cf. Schurig et al. (1995a) and Beaucamp (1996).
[b]SDS-PAGE, SDS-polyacrylamide gel-electrophoresis; GPC, gel permeation chromatography; AUC, analytical ultracentrifugation (sedimentation equilibria).
[c]Determined according to Gill and von Hippel (1989).
[d]Calculated based on the molecular mass of the PGK–TIM fusion protein, and (in brackets) based on the molecular mass of TIM in the fusion protein.

sequence similarity with known mesophilic homologs predicts similar three-dimensional structures. For the natural monomeric PGK, this has recently been confirmed by the high-resolution X-ray determination of the PGK-ATP-3-PG ternary complex (Auerbach et al., 1997). For isolated TIM, preliminary data at 2.6 Å resolution also point to a close similarity of the *Thermotoga* enzyme with the well-known dimeric $\alpha\beta$ barrel topology of mesophilic TIMs (D. Maes, L. Wyns, R.K. Wierenga, N. Beaucamp, R. Jaenicke, unpubl. obs.).

Knowing the tertiary interactions between the domains and subunits in the PGK–TIM fusion protein would be highly desirable. Docking procedures, using the X-ray coordinates of the constituent enzymes, gave ambiguous results (G. Auerbach, R.K. Wierenga, pers. comm.). Crystallization (in 0.1 M HEPES buffer pH 7.5, 0.1 M NaCl, 1.6 M $(NH_4)_2SO_4$, at 12 mg/mL and 20 °C) has been successful (Beaucamp, 1996; Grättinger, 1997); data collection and structure determination are in progress (G. Auerbach, R. Huber, pers. comm.).

Spectral properties

Spectral data, using UV-absorption, fluorescence emission, near-UV and far-UV dichroic absorption of the native and denatured recombinant enzyme is summarized in Table 1. The absorbance maximum and the A_{280}/A_{260} ratio are 278 nm and 1.60, respectively. The molar absorption coefficient and the corresponding specific absorption coefficient of the native protein were calculated using the amino acid composition and the UV absorption spectra of the protein in its native and denatured states (Pace et al., 1995).

The fluorescence properties of *Thermotoga* TIM reflect the high tryptophan content of the enzyme. Upon denaturation in 7 M GdmCl, the emission maximum exhibits a drastic blue-shift; at the same time, the fluorescence intensity is decreased by a factor of 4.8. With increasing temperature (from 20 to 80 °C), the decrease in fluorescence intensity is of the order of 30%, without a significant redshift, indicating that in the given temperature range the enzyme is not altered in its tertiary and quaternary interactions.

The far-UV circular dichroism of the native enzyme yields a calculated α-helix content of 45% (Chen et al., 1972), in accordance with the results reported for other TIMs (Wierenga et al., 1992; Rentier-Delrue et al., 1993). The near-UV CD provides the characteristics for specific tertiary interactions. Upon denaturation, exposure of the aromatic residues into the chaotropic solvent commonly eliminates the fine structure in the range between 250 and 300 nm. As in the case of the *Thermotoga* PGK–TIM fusion protein (Beaucamp et al., 1997), even in 7 M GdmCl, part of the bands at wavelengths between 265 and 290 nm still show significant amplitude, indicating that the hyperthermophilic enzyme still retains residual structure under strongly denaturing conditions.

Catalytic properties

As depicted in Figure 3, the temperature optimum of the catalytic activity of recombinant TIM coincides with the optimum growth temperature of *Thermotoga maritima*. The linearization of the temperature dependence yields 49 kJ/mol for the activation energy of the isolated enzyme, in contrast to 80 kJ/mol observed for the TIM activity in the PGK–TIM fusion protein (Schurig et al.,

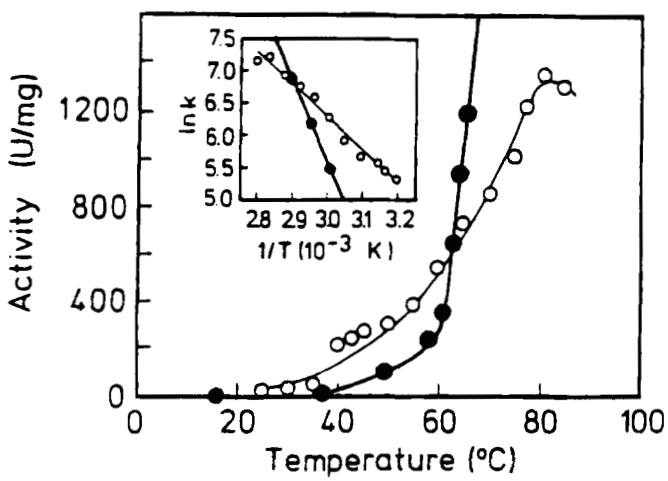

Fig. 3. Temperature dependence of the catalytic activity of TIM in PGK–TIM fusion protein (filled circles) and recombinant separate TIM (open circles) from *Thermotoga maritima*. Activity determined by standard assay: 5 mM DHAP, 1 mM NAD^+, 6 mM NaH_2AsO_4, 20 μg/mL GAPDH in 50 mM HEPPS/NaOH buffer pH 8.0 plus 1 mM EDTA, λ = 366 nm. Because of the marginal activity of *Thermotoga* GAPDH, at 20–60 °C assays were performed with GAPDH from rabbit muscle, at 40–60 °C, with both rabbit and *Thermotoga* GAPDH, beyond 60 °C with GAPDH from *Thermotoga*. Insert: Arrhenius plot of the kinetic data.

1995a; Jaenicke et al., 1996). The difference in the catalytic efficiencies of the two forms of the enzyme is striking. It becomes even more significant, considering the physiological temperature, and the molecular weight of the TIM portion within the PGK–TIM fusion protein. Table 2 summarizes the corresponding activity data at 20, 40, 60, and 80 °C. The k_{cat} of 1,010 min^{-1} at 80 °C is similar to the specific activities of TIMs from mesophilic and thermophilic species at their respective optimum growth temperatures (Gracy, 1975; Krietsch, 1975; Snyder & Lee, 1975; Rentier-Delrue et al., 1993; Kohlhoff et al., 1996). The temperature dependence of the Michaelis constant, K_M(DHAP), of the PGK–TIM fusion protein and isolated recombinant TIM does not show significant differences: above 60 °C, the K_Ms are identical, with no significant changes at higher temperature.

Regarding the pH optima of the catalytic activity, the isolated recombinant TIM construct shows a distinct pH optimum at pH 8, whereas PGK–TIM is active over a wider pH range, between pH 7 and 9 (Table 1). Considering the neutral internal pH within the *Thermotoga* cell, both the higher efficiency and the broader pH optimum may be taken as additional evidence for the observation that TIM represents an "evolutionarily perfected enzyme" (Knowles, 1991).

Stability

TIM integrated into the PGK–TIM fusion protein has been shown to be intrinsically stable at temperatures up to 100 °C, i.e., far beyond the optimum growth temperature of *Thermotoga maritima* (T_{opt} = 80 °C) (Schurig et al., 1995a). To establish the stabilizing or destabilizing effect of the fusion of the genes, deactivation and denaturation transitions of the recombinant isolated TIM were measured, using pH, temperature, and GdmCl to destabilize the enzyme.

The pH-induced denaturation shows the common bell-shaped profile, with transitions at pH 3.0, 5.3, and 9.0 (Fig. 4). Whether the decrease in activity at pH 5.3 is attributable to the anomalous titration of a histidine residue (Lodi & Knowles, 1991) cannot be determined. What is evident from the activity and fluorescence profiles in the alkaline pH range is that deactivation precedes denaturation; in the range of the carboxylate titration, complete protonation causes synchronous loss of the native structure *and* function.

Comparison of recombinant separate TIM with the intrinsic stability of the enzyme in the natural PGK–TIM complex shows that the gene fusion leads to a significant increase in stability of the two gene products: after 2 h of incubation at the given temperatures, the temperatures $T_{1/2}$ at which 50% deactivation are observed are 82 °C (i.e., close to the optimum growth temperature) for the recombinant separate TIM, and 90 °C for TIM within the natural

Table 2. *Catalytic properties of separated recombinant TIM*

Temperature (°C)	$K_{M,DHAP}$ (mM)	v_{max} (mol min^{-1} ml^{-1})	k_{cat} (min^{-1})
20	3.6 ± 0.2	1.2	0.3
40	1.2 ± 0.2	2.1	1.8
60	2.5 ± 0.2	890	360
80	2.6 ± 0.2	2,660	1,010

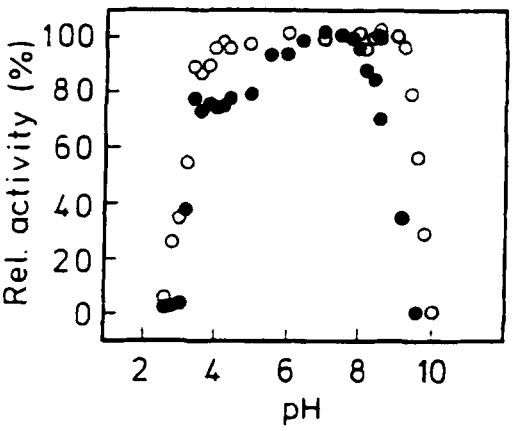

Fig. 4. pH-dependent deactivation and denaturation of recombinant TIM from *Thermotoga maritima*. The catalytic activity and fluorescence emission were determined at 40 °C. Samples (20 μg/mL) were incubated at varying pH for 48 h. Residual activity (filled circles) monitored by standard assay at 40 °C (conditions, cf. Fig. 3); fluorescence emission at 328 nm (λ_{exc} = 280 nm) (open circles).

PGK–TIM fusion protein (Fig. 5A). Deactivation kinetics confirm this result: the half-times of deactivation exceed the above data by one order of magnitude (Hess, 1991). No significant deactivation can be observed at temperatures below 70 °C; however, at optimum and maximum temperature, the half-times of deactivation are 4 h and 1 min, respectively. Linearization of the deactivation kinetics at temperatures beyond 70 °C yields an activation energy of 275 kJ/mol (Fig. 5B).

The GdmCl-induced deactivation/denaturation profiles reflect a complex behavior. At low GdmCl levels, up to concentrations close to the cooperative unfolding transition, significant activation of up to 180% occurs, without any detectable alterations of both fluorescence emission and dichroic absorption (Fig. 6A). This finding may be explained by either the effect of high ionic strength on the catalytic activity or an increase in flexibility of the protein at low denaturant concentration. In the case of *Thermotoga* glyceraldehyde-3-phosphate dehydrogenase (which shows the same effect), the second alternative has been shown to be valid, making use of H-D exchange experiments at varying temperature (Wrba et al., 1990; Jaenicke, 1996a). In the unfolding transition, at $(c_{GdmCl})_{1/2} \sim$ 3.6–3.8 M, again, the loss of catalytic activity and the change in the native fluorescence properties precede the breakdown of the secondary structure, monitored by far-UV CD (Fig. 6A–C).

Determining the GdmCl-induced deactivation/denaturation transitions at varying temperature, the half-concentrations of GdmCl are shifted from 4 M at 20 °C to 1 M at 80 °C; at the same time, the GdmCl-induced activation decreases steadily; at ≥80 °C, i.e., at the temperature at which the enzyme reaches its natural flexibility, it vanishes (Fig. 6B). That denaturants show additive effects has often been observed and is expected for thermodynamic reasons (Tanford, 1968; Privalov, 1979; Ghélis & Yon, 1982; Jaenicke, 1991).

Reconstitution

The GdmCl-induced denaturation is only partially reversible; the yield of reconstitution (monitored by fluorescence emission) is strongly temperature dependent, and drops from 80% at 20–40 °C

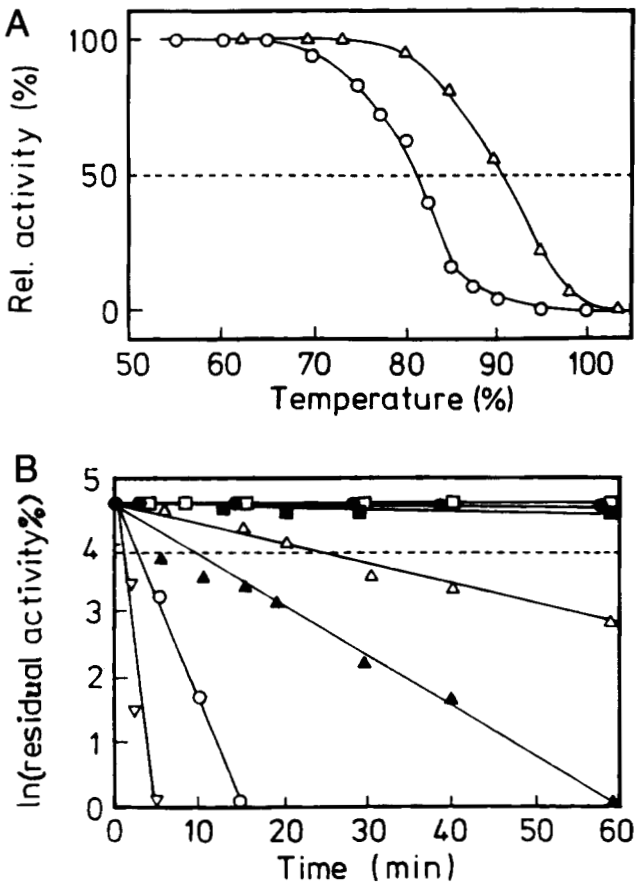

Fig. 5. Thermal denaturation of recombinant TIM from *Thermotoga maritima* (20 μg/mL) in 50 mM sodium phosphate buffer pH 8.0. Residual activity monitored by standard assay at 40 °C. **A:** Residual activity of separate TIM (open circles) and TIM integrated in PGK–TIM fusion protein (open up triangles), after two hours of incubation at given temperatures. Dotted line: temperatures for 50% residual activity. **B:** Kinetics of thermal deactivation. Incubation for given time periods at 70 °C (open squares), 75 °C (filled circles), 80 °C (filled squares), 85 °C (open up triangles), 90 °C (filled up triangles), 95 °C (open circles), 100 °C (open down triangles). Dotted line: half-time for 50% residual activity.

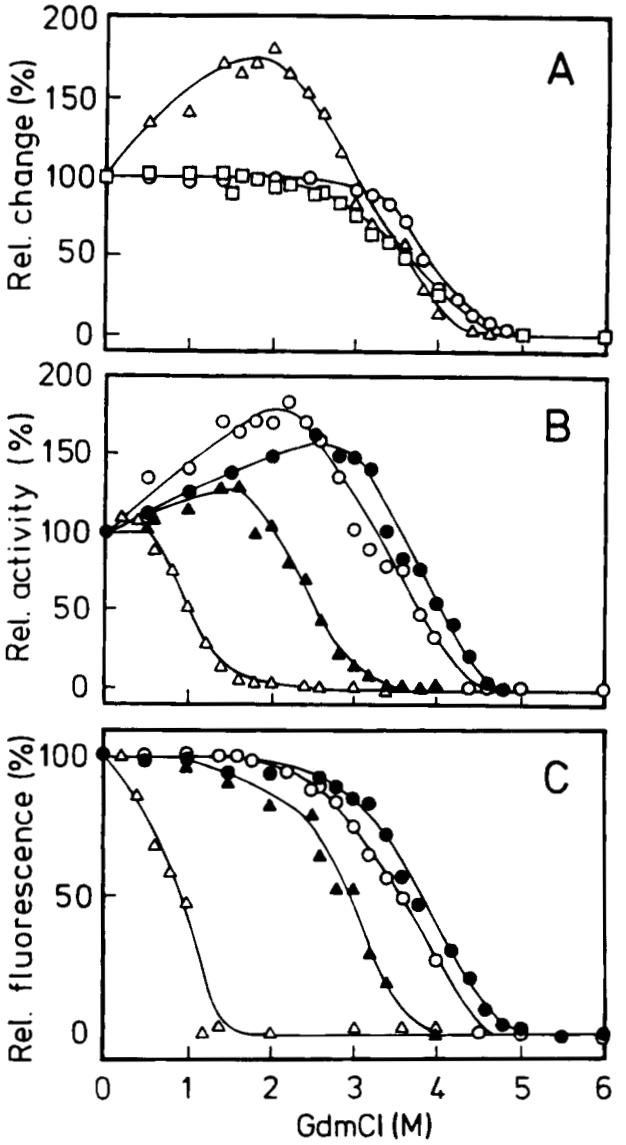

Fig. 6. GdmCl-induced denaturation of recombinant TIM from *Thermotoga maritima* in 50 mM sodium phosphate buffer pH 8.0, incubated for 48 h. **A:** Effect of GdmCl on catalytic activity at 20 μg/mL (open up triangles); fluorescence emission at 328 nm (λ_{exc} = 280 nm) and 20 μg/mL (open squares); dichroic absorption at 222 nm and 50 μg/mL (open circles). **B:** Activation/deactivation at 20 °C (filled circles), 40 °C (open circles), 60 °C (filled up triangles), 80 °C (open up triangles). Standard assay at 40 °C. **C:** Denaturation at 20 °C (filled circles), 40 °C (open circles), 60 °C (filled up triangles), 80 °C (open up triangles), monitored at λ_{em} = 328 nm (λ_{exc} = 280 nm).

to zero at 80 °C (Fig. 7). The complete irreversibility under optimum growth conditions of *Thermotoga* holds also after long-term incubation, in contrast to previous results for glyceraldehyde-3-phosphate dehydrogenase from *Thermotoga maritima*, which was shown to be accessible to high yields of reconstitution over the whole temperature range from 10–80 °C (Rehaber & Jaenicke, 1992; Jaenicke, 1996a; Jaenicke et al., 1996). Comparing the half-concentrations $(c_{GdmCl})_{1/2}$ of denaturation and renaturation, it is obvious that, even in the temperature range in which at least partial regain of fluorescence is observed, the transitions do not coincide; instead, there is a wide range of "hysteresis" between the two profiles

Conclusions

Thermotoga maritima PGK–TIM fusion protein forms a tetramer, in contrast to the monomeric and dimeric states observed for the two separate enzymes. As in the case of certain archaea where dimeric PGKs have been discovered, it is tempting to assume that the occurrence of enhanced quaternary interactions might represent a general mechanism of thermal stabilization (Jaenicke, 1991, 1996b; Hess et al., 1995). The present experiments show that the integration of TIM into the PGK–TIM fusion protein also has a stabilizing effect. However, given the small differences between the free energies of stabilization of thermophilic and mesophilic proteins, any "traffic rules" of stabilization have to be taken with care (cf. Schurig et al., 1995b). This holds also at the level of the highly conserved amino acid sequences and topologies of the two enzymes: so far, neither the amino acid composition nor sequence statistics allowed strategies of stabilization to be defined (Böhm & Jaenicke, 1992, 1994; Hilbert et al., 1993).

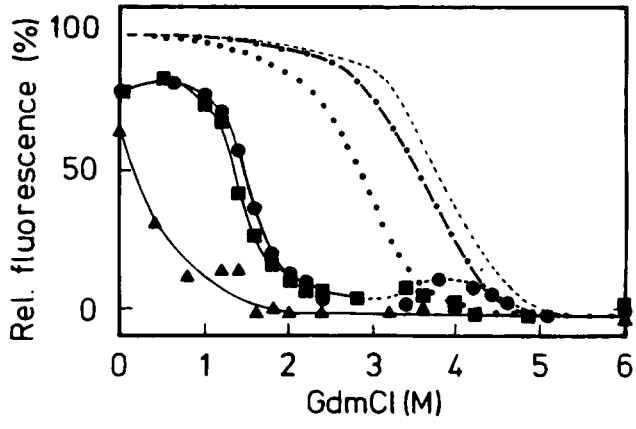

Fig. 7. GdmCl-induced unfolding/refolding of recombinant TIM from *Thermotoga maritima* (20 μg/mL) in 50 mM sodium phosphate buffer pH 8.0. Fluorescence emission at 328 nm (λ_{exc} = 280 nm) was monitored after 48 h incubation at given GdmCl concentrations at 20 °C (filled circles), 40 °C (filled squares), and 60 °C (filled up triangles). For comparison, the corresponding denaturation transitions at 20 °C (- - - - -), 40 °C (—·—) and 60 °C (·····) are included (cf. Fig. 6C).

In comparing the PGK–TIM fusion protein and its constituent parts in detail, deactivation experiments proved the thermal stability of PGK to be closely similar for the separate monomer and the tetrameric fusion protein, indicating that tetramerization within the fusion protein does not involve strong interactions between the PGK subunits (Schurig et al., 1995a). In the case of the isolated TIM (after cloning the separate *tpi* gene, and expression of the dimeric enzyme), the comparison showed that the fusion of PGK to TIM not only enhances the intrinsic stability of TIM but also its catalytic efficiency. Thus, the higher order of quaternary structure of the fusion protein contributes significantly to the intrinsic stability of TIM. It remains to be shown how the difference in stability is related to additional quaternary interactions in the PGK–TIM fusion protein, on the one hand, and improved packing of the single polypeptide chains, on the other. That additional quaternary interactions must be involved is evident from reactivation/renaturation experiments (Beaucamp et al., 1997): the observed low yields are indicative for the kinetic partitioning between folding and aggregation due to wrong domain and/or subunit interactions (Zettlmeissl et al., 1979; Jaenicke, 1996b). The three-dimensional structure of the PGK–TIM fusion protein will provide the definite answer to this question.

Materials and methods

DNA techniques

The *Thermotoga* tpi plasmid pJEA24 was a gift from Dr. Elliot Adler. The *tpi* gene was cloned into the XbaI/BamHI restriction site of the plasmid pASK40 (Skerra et al., 1991). Subsequently, *E. coli* JM83 was transformed, and positive clones were selected by ampicillin resistance.

All cell strains were grown in Luria Bertani medium (1% tryptone, 0.5% yeast extract, 0.5% sodium chloride) with 100 mg/mL ampicillin or 1.3% agar added, when appropriate.

The gene sequence was determined by the didesoxynucleotide chain termination method (Sanger et al., 1977), using the T7-Sequencing™ kit from Pharmacia (Uppsala, Sweden). Oligonucleotides corresponding to the triosephosphate isomerase (tpi) nucleotide sequences (Adler, 1994) were provided by MWG Biotech (Ebersberg, Germany). For further details, see Yanisch-Perron et al. (1985), Dubendorff and Studier (1991), and Schurig et al. (1995a).

Protein expression and purification

To express *Thermotoga* TIM, 10 mL of an overnight culture were added to 1 L of LBamp medium and inoculated at 26 °C. After growing for 16 h at 26 °C, up to OD_{546} nm ≥4, the cells were harvested by centrifugation (Sorvall GS3, 5000 rpm, 15 min, 4 °C) and resuspended in 70 mM Tris buffer pH 8.5, 2 mM EDTA. Subsequently, the cell suspension was passed twice through a french press, and protamine sulfate was added in an amount corresponding to half the mass of nucleic acids estimated from the absorption at 260 nm. After centrifugation (Sorvall SS34, 15,000 rpm, 1 h, 4 °C), the supernatant was diluted fivefold and heated to 80 °C for 45 min, followed by one further centrifugation step (Sorvall SS34, 15000 rpm, 1 L, 4 °C). The supernatant was loaded on a 60 mL Q-Sepharose HP column equilibrated with 70 mM Tris buffer pH 8.0, 2 mM EDTA, 5 mM cysteamin, and eluted with 10 column volumes 70 mM Tris buffer pH 7.5, 2 mM EDTA, 0–1.0 M NaCl, flow rate 4 mL/min. For storage, active fractions were pooled, with 1% NaN_3 added. For crystallization, the protein was loaded on a Superdex 75 pg gel filtration column; active fractions were concentrated by Centricon 30 cutoff filters to a final concentration of 10 mg/mL. To avoid reversible aggregation of the enzyme at concentrations beyond 1.3 mg/mL, 400 mM NaCl was added.

Enzyme assay and protein characterization

SDS PAGE, gel permeation chromatography, and analytical ultracentrifugation were performed as described earlier (Schurig et al., 1995; Hofmann, 1995). For enzyme assays, K_M values and thermal stabilities, see Schurig (1995) and Beaucamp (1996).

Spectroscopic methods

UV absorption spectroscopy made use of a Kontron Uvikon 391 Spectrophotometer. Protein concentration: 0.6 mg/mL. Fluorescence emission spectra (λ_{exc} = 280 nm) were monitored in a Perkin–Elmer MPF-3 fluorimeter. Protein concentration: 10 μg/mL. Solvent: sodium phosphate buffer pH 8.0, 2 mM EDTA. For complete denaturation, 7 M GdmCl was added. Circular dichroism spectra were measured in an Aviv CD spectropolarimeter 62DS. Protein concentration: 0.7 mg/mL in 0.01 cm cuvettes for far-UV CD spectra, and 2.5 mg/mL in 1 cm cuvettes for near-UV spectra, respectively.

GdmCl-induced equilibrium transitions

For denaturation, native TIM at 20 μg/mL (in 50 mM sodium phosphate buffer pH 8.0 plus 2 mM EDTA), was incubated for 48 h at varying GdmCl concentration. The fraction of native protein was detected by enzyme activity, fluorescence or dichroic absorption. Subsequent incubation for further 24 h was applied to make sure that equilibrium was reached. For reconstitution, GdmCl-denatured protein was diluted with the above buffer, again to a final concentration of 20 μg/mL. Solutions were incubated for 48 and 72 h at the respective GdmCl concentrations; reactivation and renaturation were monitored by enzyme activity and fluorescence emission.

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