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'Super-perfect' enzymes: Structural stabilities and activities of recombinant triose phosphate isomerases from *Pyrococcus furiosus* and *Thermococcus onnurineus* produced in *Escherichia coli*

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ABSTRACT

Triose phosphate isomerases (TIMs) are considered to be 'kinetically perfect' enzymes, limited in their activity only by the rates of diffusion of substrate and product molecules. Most studies conducted thus far have been on mesophile-derived TIMs. Here, we report studies of two extremophile-derived TIMs produced in *Escherichia coli*: (i) TonTIM, sourced from the genome of the thermophile archaeon, *Thermococcus onnurineus*, and (ii) PfuTIM, sourced from the genome of the hyperthermophile archaeon, *Pyrococcus furiosus* (PfuTIM). Although these enzymes are presumed to have evolved to function optimally at temperatures close to the boiling point of water, we find that TonTIM and PfuTIM display second-order rate-constants of activity (k_{cat}/K_m values) comparable to mesophile-derived TIMs, at 25 °C. At 90 °C, TonTIM and PfuTIM reach maximum velocities of reaction of ~10^6–10^7 μmol/s/mg, and display k_{cat}/K_m values in the range of ~10^{10}–10^{11} M^{-1} s^{-1}, which are three orders of magnitude higher than those reported for mesophile TIMs. Further, the two enzymes display no signs of having undergone any structural unfolding at 90 °C. Such enzymes could thus probably be called 'super-perfect' enzymes.

1. Introduction

Triose phosphate isomerase (TIM), a glycolytic pathway enzyme which catalyses the reversible isomerization reaction between dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (G3P), is considered to be a kinetically perfect (or catalytically perfect) enzyme. The key characteristic of such an enzyme is that its rate of reaction is only limited by diffusion. The specificity constant of such an enzyme (also described as its catalytic efficiency, or second-order rate constant, k_{cat}/K_m) is generally of the order of 10^8–10^9 M^{-1} s^{-1} [1–3]. A few examples of kinetically-perfect enzymes are triose-phosphate isomerase (TIM), carbonic anhydrase, acetylcholinesterase, and fumarase [4].

TIM is a representative of the prototypical $(\beta/\alpha)_8$ or TIM-barrel fold seen in many enzymes. Its structure consists of eight pairs of alternating beta strands and alpha helices that are connected through short and long loops to form two concentrically arranged cylinders; a barrel-like inner cylinder made up of beta strands forming the hydrophobic core, and an outer cylinder consisting of eight alpha helices, each arranged at an angle to its partner strand located in the inner barrel, and facing the solvent. The eight long loops connecting each of the eight strands to the partner helices that follow them in the sequence come together at the top of the TIM barrel to form the 'mouth' that harbors the catalytic residues [5]. The active sites of all triose phosphate isomerases generally include three important catalytic residues, these being a glutamate (generally present on loop 6) which acts as a nucleophile and deprotonates the substrate, a histidine (whose position is conserved in all TIMs and generally present at position 95/96 on loop 4) which acts as an electrophile, and a lysine (generally present on loop 1) [6–8]. The substrate is held by the active site present at the mouth of the barrel, which takes the form of a 'cavity' covered by a 'lid' that closes to trap the substrate and protect the enediol intermediate (formed during the reaction) from reacting with water present outside the active site. The catalytic groups of atoms contributed by these three residues, which come together in space through the folding of the TIM barrel (and mutual assembly of the eight longer loops connecting the strands to the helices), tend to be perfectly positioned to perform the necessary acid-base catalysis reaction(s) and also to stabilize the enediol intermediate

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which is formed. The entire system is so perfectly designed that a conservative 'Glu-to-Asp' mutation, which conserves the catalytically-important carboxyl group, however, results in a 1000-fold reduction in activity despite there being no other significant change in the structure and arrangement of atoms in the enzyme, because the carboxyl group moves by about 0.1 nm away from the substrate [9].

Among other pursuits, our lab is interested in the structures, stabilities, folding and engineering of TIMs and other TIM-barrel enzymes, and we have previously cloned and expressed a hyperthermophilic TIM, PfuTIM, sourced from the genome of the hyperthermophile archaeon, *Pyrococcus furiosus.* Considerable work from our lab has appeared in the literature about the structural stability of this protein [10–12], and a related psychrophilic TIM homolog from *Methanococcoides burtonii* [13]. However, earlier our focus was not on enzyme activity. Recently, we cloned a TIM sourced from the genome of a thermophile archaeon, *Thermococcus onnurineus*, (TonTIM). In this paper, for the first time, we describe the activities of these two enzymes.

Generally, thermophilic and hyperthermophilic enzymes are expected to be less catalytically-active than their mesophilic counterparts at lower temperatures, because every enzyme is thought to have evolved to function optimally at the optimal temperature of growth of the organism within whose genome it evolved [14]. Again, generally it is also believed that in order to cope with the extreme ambient environment, thermostable enzymes have evolved to have a more rigid and compact tertiary structure, characterized by lower conformational flexibility at lower temperature, resulting in lower catalytic activity. It has further been hypothesized that complementary thermophilic and mesophilic enzyme-pairs could function through very different evolutionarily-optimized catalytic mechanisms [15], facilitated by the fact that thermophilic enzymes display better residue-packing, more salt bridges, increased hydrophobicity and shorter loops in their structures [16].

In this paper we have compared PfuTIM and TonTIM. We show that unlike other thermostable enzymes that show rigidity of structure and lower activity at mesophilic temperatures than their mesophile counterparts, these two enzymes, i.e., PfuTIM and TonTIM show activities, and efficiencies, comparable to their mesophilic counterparts at room temperature, and also that the efficiencies of these enzymes is further increased by approximately 1000-fold when the temperature is raised to 90 °C, with k_{cat}/K_m values reaching an unprecedented ~10^{11} M^{-1} s^{-1}.

2. Materials and methods

2.1. Cloning, expression and purification

The gene for TonTIM was amplified using *T. onnurineus* genomic DNA as template in PCR reactions employing the oligonucleotide with the sequence 5′- AAAGTTAAGGATCCATGACGAAGCTGAAGG-3′ as forward primer and the oligonucleotide 5′- AAACAA-TAACCCGGGTCAAACAATCAGCGATAC -3′ as reverse primer, with BamHI and SmaI sites (underlined) inserted into the primer sequence to facilitate digestion and cloning in frame for expression using the pQE30 vector, with an N-terminal 6xHis tag located upstream of the BamH1 site. The plasmid with the inserted gene was then transformed into XL-1 Blue cells for both expression and purification. Similarly, the PfuTIM gene was cloned between BamHI and HindIII sites using the oligonucleotide 5′-GCAATCGGATC-CATGGCTAAACTCAAGG-3′ as forward primer and the oligonucleotide 5′- GCAATCAAGCTTCTACTCCTTAATTATTCC -3′ as reverse primer for insertion and cloning into the pQE30 vector. The vector with the insert was similarly transformed into XL-1 Blue *E.coli* cells. Subsequently, the vector was also transformed into M15[pREP4] *Escherichia coli* cells for expression. This PfuTIM-encoding clone was generated afresh from *P. furiosus* genomic DNA, instead of using previously-generated clones which are already described encoding the same protein, using similar strategies [12], with the only difference being the presence of a methionine at the junction of the affinity tag and PfuTIM which was not present in the earlier clone. The proteins were produced by inducing the secondary cultures (induced at OD_{600} of 0.6 with 1 mM IPTG in M15[pREP4]) grown by adding appropriate antibiotics, tetracycline (12.5 μg/ml) and ampicillin (100 μg/ml) for TonTIM/pQE30/Xl-1 Blue and kanamycin (50 μg/ml) and ampicillin for PfuTIM/pQE30/M15[pREP4]) for 5–6 h at 37 °C with shaking at 220 rpm. Following this, cells were harvested by centrifuging the culture at 6000 rpm for 10 min. The cell pellet was resuspended in native lysis buffer containing 10 mM Tris, 50 mM NaH_2PO_4 and 300 mM NaCl, pH 8.0. The cells were lysed by sonication and the lysate was separated by centrifuging lysed cells at 16,000 rpm for 1 h. Proteins were purified by Ni-NTA affinity purification as per standard protocols (Qiagen), and bound protein was eluted with buffer containing 250 mM imidazole. The protein, so obtained was subjected to dialysis against 20 mM Tris, pH 8.0.

2.2. Spectroscopic and chromatographic characterization

2.2.1. Size exclusion chromatography

The dialysed proteins was subjected to analytical gel filtration chromatography by allowing the protein to be resolved on a Superdex 200 10/300 GL column on an AKTA Purifier 10 chromatographic system (GE Healthcare), after equilibration in 20 mM Tris, pH 8.0 buffer. A constant flow rate of 0.5 ml/min was applied throughout the run.

2.2.2. Circular dichroism (CD) spectroscopy

CD spectroscopy was carried out using a Chirascan spectropolarimeter (Applied Photophysics) fitted with a peltier for temperature control. Either 0.1 or 0.2 cm path-length quartz cuvettes were used for collecting far UV CD spectra (198–250 nm) and thermal denaturation data through monitoring of changes in ellipticity with temperature. The thermal denaturation and subsequent renaturation was monitored at 222 nm by applying a constant temperature ramp rate of 1 °C/min as the temperature was increased from 25 to 95 °C (heating) and then decreased from 95 to 25 °C (cooling). The mean residual activity, [θ], was calculated by normalizing data with protein concentration through use of the following equation:

$$[\boldsymbol{\theta}] = \frac{\boldsymbol{\theta obs(in\ \ mdeg)\, x\ \ 100 x\ \ MRW}}{\boldsymbol{1000 x\ \ concentration\ \ (mg/ml) x\ \ path\ \ length(cm)}}$$

where, MRW represents mean residue weight (total molecular weight of the protein/total number of amino acids), [θ] represents mean residue ellipticity and θ_{obs} represents raw ellipticity.

2.3. Dynamic light scattering

Dynamic light scattering data was collected on Zetasizer Nano ZS90 model instrument (Malvern Instruments) The gel filtration-derived sample was further filtered through a 0.22 μm filter, before measurements. A total of 100 readings were averaged to generate the intensity *versus* size plots.

2.4. Activity assay

The triose phosphate isomerase activity was assessed by employing a previously-described non enzyme-coupled assay

using CD spectroscopy, exploiting the principle that if in a racemic mixture only one form of a substrate is utilized by the enzyme as a substrate, the relative concentrations of the two forms (and hence the effective differential absorption) would change with the progress of the reaction [17]. The assay was done with D,L-glyceraldehyde-3-phospahte substrate (DL-G3P), wherein D-G3P is utilized for conversion to dihydroxyacetone phosphate (DHAP), leaving L-G3P as un-reacted residue with change in ellipticity being used to calculate the specific activity by the following equation:

$$\frac{\mu moles\ \ D-G3P}{min-mg\ \ TIM} = 290X\frac{\Delta\theta^{\circ}}{min}X\frac{1}{l(cm)}X\ \ \frac{V(ml)}{mg(TIM)}$$

The factor 290 was taken from work by Fahey and Fischer, 1974 [17], in which a description of the work leading to the determination, and use, of this value in numerous instances of previous work has been described. The enzyme assay was carried out at two different temperatures, 25 °C and 90 °C, falling in the mesophilic and hyperthermophilic ranges of optimal growth temperatures of organisms (and corresponding enzyme activity temperature optima). The reaction was carried out in a buffer containing 10 mM each of triethanolamine, acetic acid and imidazole, of pH 8.0. Amounts of 5 ng/ml and 50 ng/ml of enzyme(s) were used for assays at 25 °C and 90 °C respectively. The experiment was done on a Chirascan (Applied Photophysics) instrument, fitted with a peltier attachment. For all measurements, a cuvette of 1 cm path length was used. First, the enzyme was incubated at a particular temperature for about 10 min and then substrate was added and the change in CD signal was monitored for different substrate concentrations. The substrate stock concentration was kept high, and therefore, the volume of substrate used was low enough to not alter the temperature of the enzyme solution. The linear change of ellipticity was monitored after the mixed solution had attained thermal equilibrium. The change in ellipticity was recorded as a function of time (per second) for a duration of 400 s for assay at 25 °C, and for 100 s at 90 °C. Since, the substrate was prone to degradation or self-hydrolysis at higher temperature, initial velocities for first 30 s was taken for calculation of enzyme's kinetic parameters.

3. Results

3.1. The two TIMs are similar

PfuTIM and TonTIM have molecular weights of around 25 kDa. Both of them are of comparable lengths also with the former being 228 residues-long, and the latter being 226 residues-long. When the sequences of PfuTIM and TonTIM were aligned using Clustal W, they displayed an identity of 92 percent (Fig. 1A) indicating that two enzymes could potentially behave in similar fashion. When the two proteins were subjected to SDS PAGE analysis, both displayed single bands corresponding to ~25 kDa (Fig. 1B).

3.2. Both PfuTIM and TonTIM are tetrameric in nature

Mostly, TIMs tend to be dimeric in nature. To check the oligomeric status of the two TIMs, one derived from a thermophile and the other derived from a hyperthermophile, analytical gel filtration chromatography was performed. In both cases, the proteins eluted at the same elution volume of ~12.4 ml (Fig. 2A) corresponding to a molecular weight suggestive of tetrameric quaternary structural status. The tetrameric status was further confirmed by analysing dynamic light scattering data. Intensity versus size curves are shown in which both the proteins are shown to be mono-dispersed with a single peak showing an average diameter of around 8.6 nm (Fig. 2B).

3.3. A comparison of secondary structure content and structural stability

The far-UV CD spectra of the two proteins suggests that the folding and secondary structural contents are similar in PfuTIM and TonTIM, signifying structural similarity (Fig. 3A). When analysed in respect of thermal stability, by subjecting the proteins to attempts at thermal denaturation through increasing of temperature from 25 °C to 95 °C, and then decreasing it back down to 25 °C, the two TIMs were found to be highly thermostable, showing only a negligible (and insignificant) change in ellipticity upon heating which was reversible upon cooling. In both the proteins, we observed extreme thermal stability, which is not observed in mesophilic proteins (Fig. 3B).

3.4. Comparison of the kinetics of activity

TIMs are considered to be kinetically perfect enzymes, with the specificity constant, or second-order rate constant (kcat/Km) falling in the range of ~10^7–10^8 M^{-1} s^{-1} (or about 10^8–10^9 M^{-1} min^{-1}). The specificity constant ranges from 10^5 M^{-1} s^{-1} in a psychrophile-derived TIM [18] to ~10^8–10^9 M^{-1} min^{-1} in case of most mesophile-derived TIMs [19–25]. These kinetic parameters for mesophilic and psychrophilic TIMs are presented in Table 1, as reported in the literature, using units of 'M^{-1} min^{-1}' (note that we have reported our own experimental values using units of M^{-1} sec^{-1} according to STRENDA guidelines advocated by this journal). Most of the available data on kinetic characterization has been gathered using mesophile-derived TIMs, but characterization of kinetic parameters at higher temperatures is a little difficult because the other enzyme in a coupled enzyme assay denatures upon exposure to higher temperatures even where mesophilic or hyperthermophilic TIMs are reasonably structurally stable themselves, and display some thermal stability. Therefore, only a few studies could be done using a coupled enzyme method. However, kinetics with one hyperthermophilic TIM from *Thermoproteax tenax* has been determined using a coupled enzyme assay at 70 °C [26].

In this paper, we have characterized the kinetic parameters of two TIMs, one thermophilic (TonTIM) and the other hyperthermophilic (PfuTIM) at 90 °C for the first time by a non enzyme coupled assay as described in the materials and methods section [17]. The kinetic parameters at 90 °C were compared with those done at 25 °C. The specific activities (calculated from initial velocities at different substrate concentrations) were plotted against substrate (D-G3P) concentration and then the data points were fitted into the Michaelis–Menton hyperbola equation using Origin software, and V_{max} and K_m values were calculated. The Michaelis-Menton plots for PfuTIM and TonTIM at 25 °C and 90 °C can be seen in Fig. 4A and B. It was observed that TonTIM showed specific activity of ~5.7 × 10^4 U/mg at 25 °C and 1.0 × 10^6 U/mg at 90 °C while PfuTIM showed specific activity of 6.194 × 10^4 U/mg at 25 °C and 2.3 × 10^6 U/mg at 90 °C (Table 2). PfuTIM showed almost double specific activity than TonTIM at 90 °C as can be seen in Fig. 4B. Mesophile-derived TIMs generally show K_m in the range of about 0.2–1.45 mM while psychrophilic TIM shows K_m in the range of 1–2 mM (Table 1). TonTIM and PfuTIM showed the K_m values of 0.2076 ± 0.018 mM and 0.6774 ± 0.068 mM respectively at 90 °C. The K_m values for TonTIM and PfuTIM at 25 °C are 1.668 ± 0.194 mM and 1.2194 ± 0.1384 mM respectively (Table 2). The specificity constant, k_{cat}/K_m, is of the order of 10^8 M^{-1} s^{-1} at 25 °C which is 1000 times lower than that obtained at 90 °C, which shows a highly efficient reaction with the value of specificity constant in the range

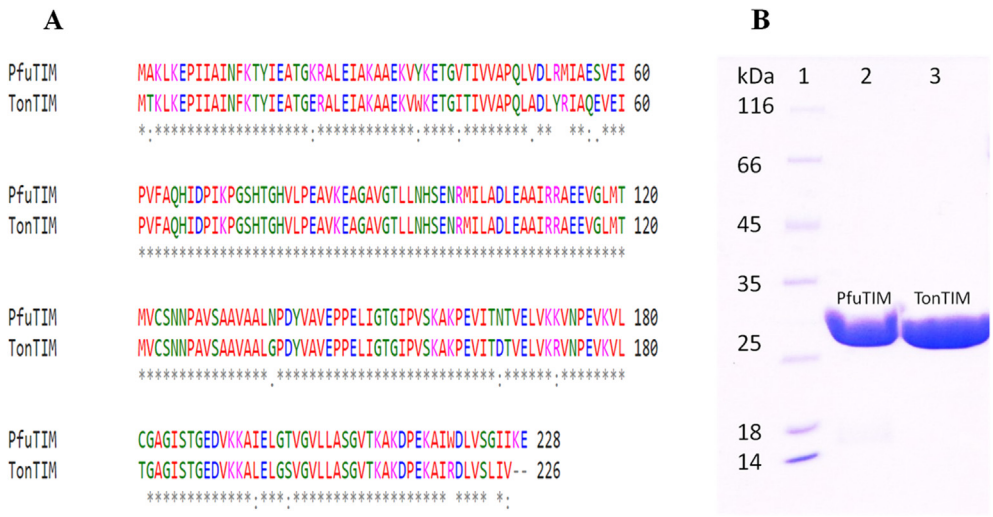

Fig. 1. (A) Sequence alignment of PfuTIM and TonTIM using ClustalW. (B): SDS-PAGE electrophoresis of purified recombinant enzymes, with lane 1 showing molecular weight markers, lane 2 showing purified PfuTIM and lane 3 showing purified TonTIM.

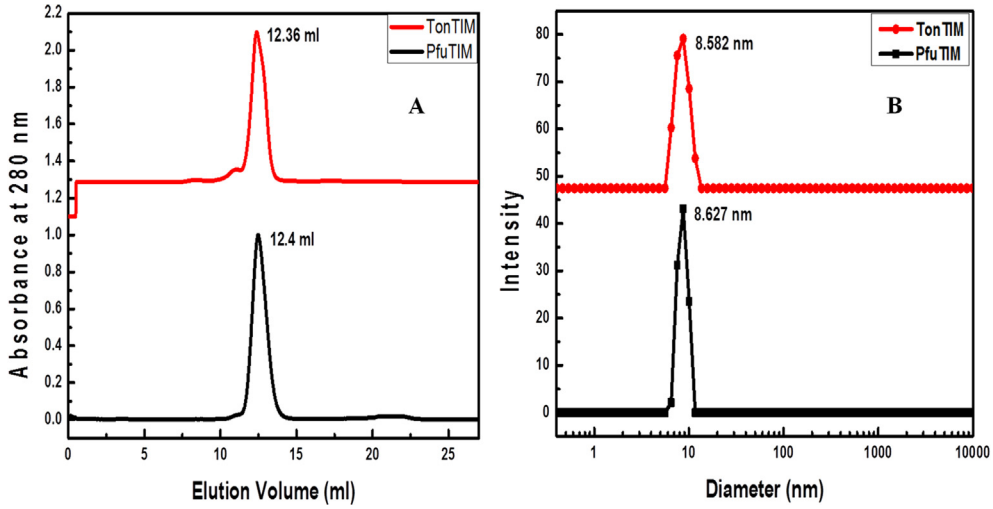

Fig. 2. (A) Gel filtration chromatogram of PfuTIM (in black) and TonTIM (in red) on a Superdex-200 column. (B) Scattering intensity versus size data (diameter in nm) for PfuTIM (in black) and TonTIM (in red) obtained through dynamic light scattering. (For interpretation of the references to colour in this figure caption, the reader is referred to the web version of this article.)

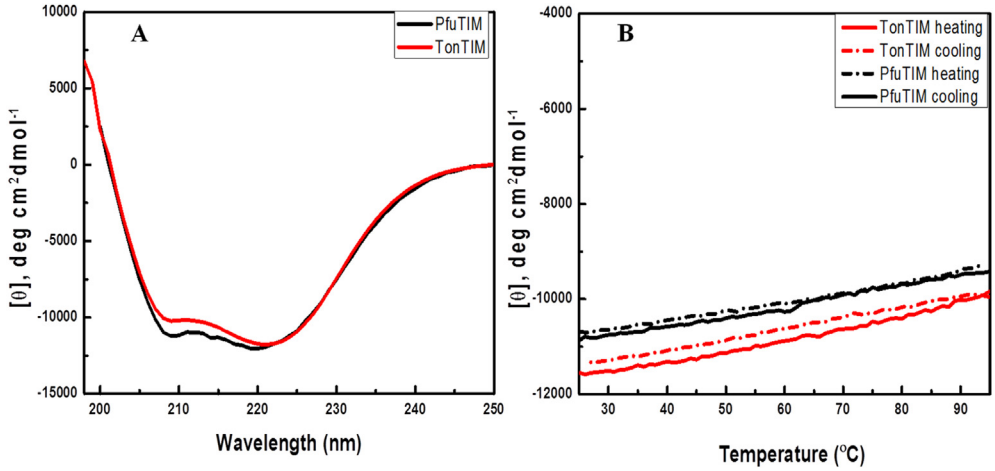

Fig. 3. (A) Far-UV CD spectrum of PfuTIM (in black) and TonTIM (in red). (B) CD ellipticity data collected during heating (continuous lines) and cooling (dotted lines) of PfuTIM (in black) and TonTIM (in red). Heating was done from 25 to 95 °C and cooling from 95 to 25 °C. (For interpretation of the references to colour in this figure caption, the reader is referred to the web version of this article.)

Table 1
Comparative table representing the reported, or measured, kinetic parameters of psychrophilic, mesophilic and and hyperthermophilic TIMs.

Protein (source)	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (M^{-1} min^{-1})	Reference
Vibrio marinus, *v*TIM (psychrophilic)	1.9 ± 0.2	4.2 ± 0.2 × 10^5	2.2 ± 0.2 × 10^5	[18]
Trypanosome brucei brucei (mesophilic)	0.25 ± 0.05	3.7 × 10^5		[19]
Rabbit muscle (mesophilic)	0.32 ± 0.05	5.1 × 10^5		[20]
Chicken muscle (mesophilic)	0.39	2.60 × 10^5		[21]
	0.47	2.56 × 10^5		[22]
Yeast, yTIM (mesophilic)	1.22 ± 0.05	7.9 × 10^5		[19]
	1.27	1.0 × 10^6		[20]
E. coli, *e*TIM (mesophilic)	1.03 ± 0.1	5.4 ± 0.1 × 10^5	5.2 ± 0.6 × 10^5	[18]
Schistosoma mansoni (mesophilic)	1.1	6.9 sec^{-1}		[23]
Helicobacter pylori, HpTIM (mesophilic)	3.46 ± 0.23	8.8 × 10^4		[24]
Human skeletal muscle (mesophilic)	0.34			[25]
	Sp. act. = 7200 U/mg			
Thermoproteus tenax (hyperthermophilic)	0.2			[26]
	Sp.act. = 6200 U/mg			

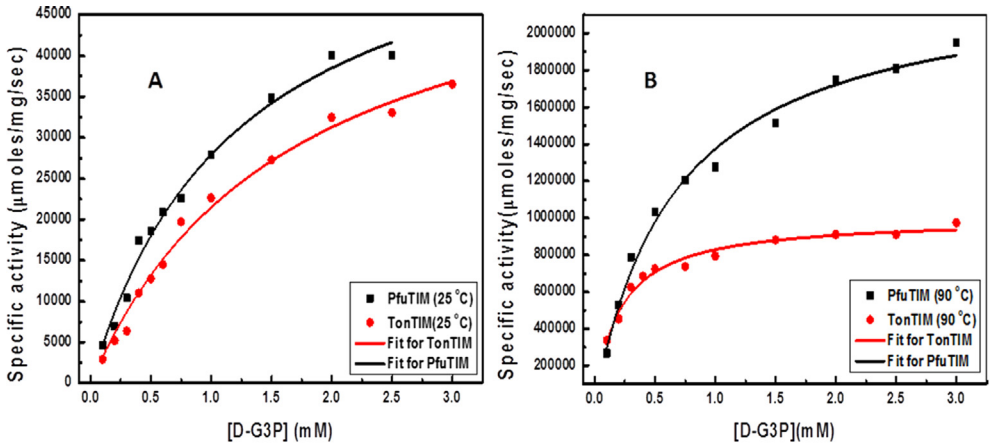

Fig. 4. Michaelis–Menton plots for triose phosphate isomerase activity in PfuTIM (in black) and TonTIM (in red), for observations at (A) 25 °C and (B) 90 °C. The data points represent the specific velocities at different substrate concentrations and the continuous hyperbolae represent the best fits. (For interpretation of the references to colour in this figure caption, the reader is referred to the web version of this article.)

of 10^{11} M^{-1} s^{-1}. This rate is extremely high and exceeds the rate of diffusion as well. However, the rates of diffusion of substrate are elevated at elevated temperatures explaining the rationale behind the increased enzyme activity at higher temperature (90 °C).

4. Discussion

Enzymes are bio-molecular catalysts designed to significantly accelerate the rates of the reactions they catalyse. The most significant parameter that defines the power of enzyme catalysis is the ratio k_{cat}/K_m, which is also called the specificity constant, or second-order rate constant [4], Reactions with specificity constants of ~10^8- 10^9 M^{-1} s^{-1} are called diffusion-limited reactions as the rate limiting step is the diffusion of substrate. Two models discuss the high efficiencies of such enzymes and the extremely high rates of reaction catalyzed by them. One is the Alberty-Hammes-Eigen model in which the enzyme's active site is considered to be a hemispherical reaction site on a plane with the rest of the protein modelled as a wall [27,28]. In 1972, the reaction catalysed by carbonic anhydrase (which dehydrates H_2CO_3) was monitored and the second order rate constant was discovered to be 1.5 × 10^{10} M^{-1} s^{-1}, i.e., one order of magnitude higher than that postulated to be possible using the Albert-Hammes-Eigen model [29]. This paradox was explained by a model proposed by Chou et al. which considered the active site to be a cavity within the enzyme, and which includes factors called the spatial factor and the force-field factor, in the interplay between enzyme and substrate. This model postulated that the upper limit could reach 10^{10} M^{-1} s^{-1}. Zhou and Zhong (1982) compared the two models and discussed roles played by the protein surface present outside the active site. They pointed out that if the Van der Waals' binding energy is very small, the protein outside the active site could behave as a 'wall' blocking the flow of

Table 2
Kinetic parameters of PfuTIM and TonTIM at 25 °C and 90 °C.

Parameters	PfuTIM		TonTIM	
	25 °C	90 °C	25 °C	90 °C
K_m (mM)	1.2194 ± 0.1384	0.6774 ± 0.068	1.668 ± 0.194	0.2076 ± 0.018
k_{cat} (sec^{-1})	1.548 × 10^6	0.575 × 10^8	1.43 × 10^6	0.25 × 10^8
k_{cat}/K_m (M^{-1} sec^{-1})	1.269 × 10^9	0.8488 × 10^{11}	0.857 × 10^9	1.204 × 10^{11}
Activity (U/mg)	6.194 × 10^4	2.3 × 10^6	5.73 × 10^4	1.0 × 10^6

substrate molecules to the active site, but that if the van der Waals' energy happened to be more than 3 kT (where k the Boltzmann constant and T the absolute temperature), the protein outside the active site could act like an 'accelerator', actually speeding up the diffusion of the substrate to the active site around the enzyme molecule [30]. The limits to which such acceleration may be facilitated by particular enzymes are not described, or understood. The high rates of the reactions catalysed by TIMs have lead many to declare TIM to be 'kinetically perfect'. Analyses of Michaelis–Menten kinetics based on changes in metabolite concentrations associated with mutational changes of kinetic parameters of enzymes have been performed [31]. These suggest that selection pressure for higher reaction rates increases k_{cat}, while K_m may either decrease or increase depending upon an enzyme's stage of evolutionary development. An increase of both k_{cat} and K_m would be required to increase the performance of an enzyme already exhibiting a k_{cat}/K_m ratio close to the diffusion-controlled limit [31]. TIM is classified as a diffusion-controlled enzyme, and extensive work on TIM has been carried out by Knowles and co-workers [1,3,7–9,21,22] who suggest that TIM can't work any faster. However, these authors haven't necessarily reckoned for mechanisms involving 'acceleration' by the enzyme's surface outside the active site. We have shown that both PfuTIM and TonTIM show 100-to-1000 fold enhanced catalytic efficiency at 90 °C. This could owe to minor structural optimization at these temperatures, supported by the tetrameric quaternary structure (unlike dimeric mesophile TIMs) and a globular shape involving four cavities and roles played by surface areas outside the active site.

Conflict of interest

None.

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