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Different catalytic properties of two highly homologous triosephosphate isomerase monomers

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ABSTRACT

It is assumed that amino acid sequence differences in highly homologous enzymes would be found at the peripheral level, subtle changes that would not necessarily affect catalysis. Here, we demonstrate that, using the same set of mutations at the level of the interface loop 3, the activity of a triosephosphate isomerase monomeric enzyme is ten times higher than that of a homologous enzyme with 74% identity and 86% similarity, whereas the activity of the native, dimeric enzymes is essentially the same. This is an example of how the dimeric biological unit evolved to compensate for the intrinsic differences found at the monomeric species level. Biophysical techniques of size exclusion chromatography, dynamic light scattering, X-ray crystallography, fluorescence and circular dichroism, as well as denaturation/renaturation assays with guanidinium hydrochloride and ANS binding, allowed us to fully characterize the properties of the new monomer.

Introduction

Sequence differences, both at DNA and protein level, are part of the basis of evolutionary change. Even in naturally occurring highly preserved proteins belonging to closely related species, there are different regions that normally do not influence on major structural or functional change. The highly preserved homodimeric glycolytic enzyme triosephosphate isomerase (TIM) has been well characterized over the last 50 years. Especially TIM's from *Trypanosoma brucei* (TbTIM) and *Trypanosoma cruzi* (TcTIM), which share a similar amino acid sequence identity of 74% and 86%. However, it has been reported that, despite this, differences exist at the level of sensibility to thiol reagents [1,2] and subtilisin [3], equilibrium unfolding [4,5], as well as in the ability to form active dimers from unfolded monomers [6].

This paper demonstrates that variations in the sequence can also be related to significant catalytic differences of the TIM system. For this, a monomeric variant of TcTIM (monoTcTIM) was characterized. The monomeric nature of monoTcTIM was demonstrated using size exclusion chromatography, dynamic light scattering, by the fact that the activity is not dependent on the concentration and because the observed monophasic behavior in the denaturation/renaturation experiments. Although the stability of the monomeric enzyme was about 15% of that of the native dimer and an increase in ANS binding was observed, the overall three dimensional structure was preserved as shown using circular dichroism (CD), intrinsic fluorescence (IF) and X-ray crystallography experiments. It was found that the catalytic turnover is ten times higher than that for monoTbTIM [7], the equivalent monomeric mutant from *T. brucei*. Our results are discussed in light of the current information available for single mutants.

Materials and methods

Bacterial strains and plasmids. Overexpression of monoTcTIM was achieved with the *Escherichia coli* strain BL21 (DE3) [8].

Enzymes and biochemicals. Restriction enzymes, DNA polymerase, T4 DNA ligase and Gdn-HCl were purchased from Boehringer GmbH, Mannheim. All other reagents were obtained from Sigma Chemical Co.

Mutagenesis and purification. Mutagenesis was performed on plasmid pET3a [9]; amino acid insertion was carried out according

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to [10]; and overexpression and purification of monoTcTIM according to [11].

Enzyme characterization. Absorbance measurements were obtained with a Beckman DU7500 spectrophotometer with the cell compartment thermoregulated at 25 °C. Protein concentrations were determined using a BCA assay. TIM activity was followed by changes in the absorbance at 340 nm [12]. Conditions for the reaction that produces dihydroxyacetone phosphate were: 100 mM triethanolamine, 10 mM EDTA, 1 mM dithiotreitol (TED) (pH 7.4), 1.5–3.0 mM D-glyceraldehyde-3-phosphate (GAP), 5–10 μg mL^{-1} α-glycerophosphate dehydrogenase, and 0.2 mM NADH.

Size-exclusion chromatography (*SEC*). Purified TcTIM and monoTcTIM were loaded into a Sephacryl S-200 column, previously calibrated with 20 mM Tris–HCl and 1.0 mM EDTA and pH 7.4. The column was calibrated using proteins of known molecular weight according to [5].

Dynamic light scattering (*DLS*). We used a Zetasizer DLS instrument (Nano Series, Malvern Instruments, USA). To obtain data, 50 μL samples of the protein being studied (900 μg mL^{-1}) were loaded into a Hellma cuvette (Precision Cells, Quartz Suprasil); the particle size analysis tool of the device was used.

Equilibrium time determination for denaturation and renaturation of monoTcTIM. IF was used as a probe and changes at native λ_{max} (327 nm) after excitation at 280 nm were obtained. In the denaturation experiments, 150 μg mL^{-1} of monoTcTIM were incubated with increasing concentrations of Gdn-HCl. For the renaturation experiments, monoTcTIM (300 μg mL^{-1}) was first denaturated in 6.0 M Gdn-HCl for 1 h. The renaturation process was initiated by diluting the samples to a final protein concentration of 5 μg mL^{-1} and the final denaturant concentrations were adjusted to the same concentration used in the denaturation experiments. The effect of the added (unfolding) or diluted (refolding) denaturant was kinetically monitored after excitation at 280 nm at the native λ_{max} for 2 min. Data was periodically collected from 0 to 72 h. No significant differences in the IF values were found after 24 h of incubation for both denaturation and renaturation reactions at each denaturant concentration. The changes in IF were monitored using a DM45 OLIS spectrofluorometer with the cell compartment at 25 °C.

Reversibility assays. MonoTcTIM (600 μg mL^{-1}) was incubated at 25 °C for 24 h in 6.0 M Gdn-HCl to complete denaturation. Thereafter, simultaneous dilution of protein and denaturant was performed by adding buffer to reach 10 μg mL^{-1} and 0.1 M final concentrations of monoTcTIM and Gdn-HCl, respectively. The samples were then incubated for 24 h to achieve refolding; following this, IF was determined. No differences were found in the emission spectra of the native and renaturated monoTcTIM using 280 or 295 nm excitation wavelengths in the range from 300–315 to 500–515 nm, respectively (data not shown), indicating complete reversibility of the unfolding and refolding reactions.

Circular dichroism analysis. Spectra were obtained by CD in the far-UV region (180–260 nm). All CD data was collected in a thermoregulated Jasco-720 spectrophotopolarimeter. Molar ellipticity (deg cm^2 $dmol^{-1}$) was calculated as reported in [4]. The data was analyzed using the CDNN program in the 205–260 nm range.

Guanidinium hydrochloride unfolding experiments monitored by changes in IF and CD. In denaturation experiments, monoTcTIM (10, 50, and 100 μg mL^{-1}) was incubated at equilibrium (24 h) in TED 20/1/1 (pH 7.4) at 25 °C. For renaturation assays, samples (600 μg mL^{-1}) were first denaturated; following this, refolding started with the dilution of samples to 10 μg mL^{-1} (final protein concentration) in a range between 0.0 and 6.0 M Gdn-HCl. Protein IF was measured with excitation wavelengths of 280 and 295 nm (4.0 nm bandwidth); the emissions was collected from 300–315 to 510–515 nm, respectively (4.0 nm bandwidth). Only the experiments after excitation wavelength at 280 nm are shown, although similar results were obtained after excitation at 295 nm. The spectral center of mass (SCM) was calculated, as in [5]. The far-UV CD changes (180–260 nm) at 25 °C were monitored at 222 nm for monoTcTIM samples (200 μg mL^{-1} in TED 20/1/1, pH 7.4) using cells with a 0.1/cm path length. All the spectroscopic measurements reported here were obtained after eliminating the reference samples without protein.

ANS fluorescence intensity. Saturating ANS (100 μM final concentration) was added to each sample with or without protein. The excitation wavelength was 360 nm and the emission range was 400–560 nm. Reference spectra with ANS and without protein were subtracted for all conditions.

Crystal structure determination. MonoTcTIM was crystallized by the sitting drop method. One microliter of protein at 25 mg mL^{-1} was mixed with one microliter of the reservoir. Crystals were grown after one week at 9 °C and obtained with a reservoir solution of 100 mM HEPES, pH 7.5, 10% PEG 6000, and 5% MPD. The crystals were cryoprotected by adding PEG 400 (30%) to the reservoir and frozen in liquid nitrogen. Diffraction data was collected on the SER-CAT beamline at APS, Argonne, USA. The data were processed using MOSFLM [13] and reduced using SCALA [14]. The structure was solved using the molecular replacement method using the PHASER program [15] using the coordinates of TcTIM (PDB code 1TCD) as the search model. Refinement and model building were done using the Refmac5 and COOT programs [16,17]. Data collection and refinement statistics are found in Supplementary Table 2. Fig. 2B was generated with PyMOL (http://www.pymol.org). The structure has been deposited in the Protein Data Bank (code 2v5b).

Thermodynamic data analysis. Spectroscopic data was analyzed according to a two-state equilibrium model involving native (N) and unfolded (U) states: N ⇆ U. The unfolded fraction, F_U, was calculated with $F_U = (y_N - y_{obs})/(y_N - y_U)$; where: y_{obs} is the experimentally determined parameter (IF or CD) and y_N and y_U are the characteristic parameter values of the folded and unfolded conformations, respectively. The values for y_N and y_U in the transition region were obtained by extrapolation of the linear portions of the pre- and post-transitional zones of the respective denaturation or renaturation curve [18]. The data were analyzed using the ORIGIN program (MA, USA). The ΔG between N and U conformations was obtained using: $\Delta G = -RT\ln K_{eq}$; where R is the universal gas constant, T is the absolute temperature and K_{eq} is the equilibrium constant. A least square analysis was used in order to adjust data to the equation: $\Delta G = \Delta G_{(H2O)} - m(\text{Gdn-HCl})$; where $\Delta G_{(H2O)}$ is the ΔG value in absence of denaturant and m reflects the variation of ΔG with respect to the denaturant concentration.

Results and discussion

The design and properties of a monomeric TIM from T. cruzi (monoTcTIM)

In order to be able to compare the equivalent mutation effects in a highly homologous enzyme, the TcTIM gene was mutated at the interfacial loop 3 region (Supplementary Fig. 1); we designed mutations in TcTIM equivalent to those in TbTIM [7] in order to produce a monomeric mutant and analyze the differences in the properties of isolated subunits.

Given that several monomeric TIM mutants in solution exist in equilibrium between monomers and dimers [19,20], the monomeric nature of monoTcTIM was confirmed. SEC experiments were performed for TcTIM and monoTcTIM (Fig. 1A), and the calculation of the Stokes radii of monoTcTIM was obtained (Supplementary Table 1), confirming its monomeric nature.

The following observations indicate that the transition between folded and unfolded monoTcTIM is a unimolecular process: (1) coincidence in the unfolding patterns followed by changes in ter-

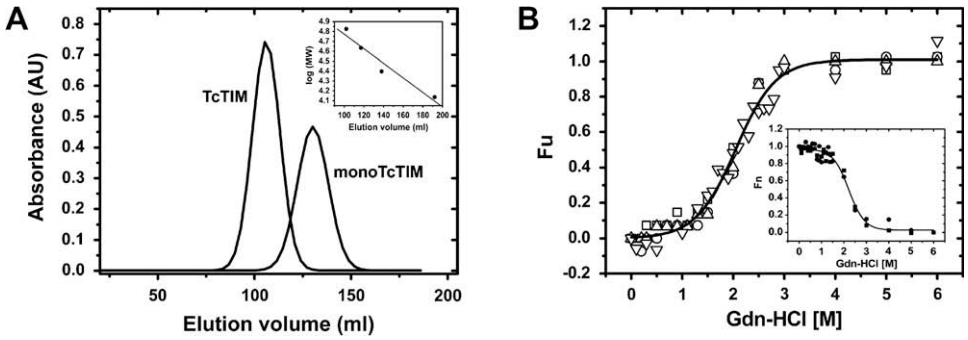

Fig. 1. The monomeric nature of monoTcTIM. (A) Elution profiles. In separate experiments, TcTIM and monoTcTIM (200 μL at 1.2 mg mL^{-1}) were loaded onto a Sephacryl S-200 column. The inset shows the retention volume of the MW standards. (B) Changes in tertiary and secondary structure. Maximum wavelength (λ_{max}) at increasing concentrations: 10(□), 50(○), and 100(Δ) μg mL^{-1} and the mean residue ellipticity values at 222 nm (▽, 200 μg mL^{-1}) were obtained. Inset: unfolding (■) and refolding (●) IF patterns after equilibrium (10 μg mL^{-1}). The lines correspond to the best fit of the data available for the model described in the Materials and methods section.

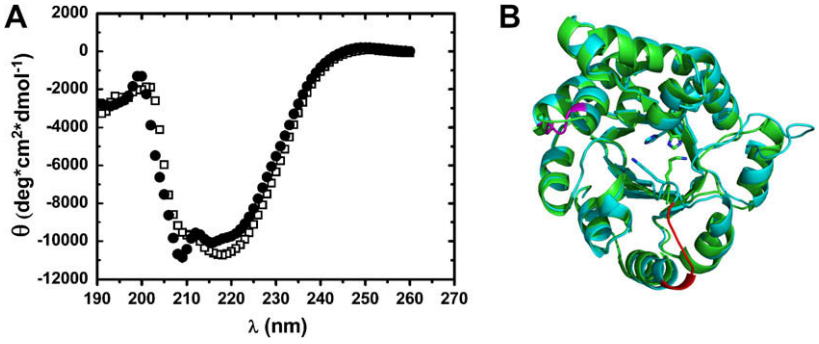

Fig. 2. MonoTcTIM has a native-like structure. (A) CD spectra of TcTIM (□) and monoTcTIM (●), 400 μg mL^{-1} of each protein was analyzed. (B) Model superposition of the crystal structure of monoTcTIM (green, PDB code 2v5b) with the apo structure of a monomer of TcTIM (cyan, PDB code 1tcd). The catalytic residues Lys 13 and His 96 are shown in sticks. Loop 6 and loop 1 of TcTIM are shown in magenta and red, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

tiary and secondary structure (Fig. 1B): (2) both denaturation and renaturation experiments show monophasic behavior (inset Fig. 1B); and (3) no shift in $C_{1/2}$ was observed with increasing protein concentration (10–200 μg mL^{-1}) (Fig. 1B and Table 1).

Moreover, the catalytic activity of monoTcTIM remains unaltered (~0.6 μmol min^{-1} mg^{-1}) using a wide range of protein concentrations (0–40 μg mL^{-1}) (Supplementary Fig. 2), indicating that no changes occur during the oligomeric state of monoTcTIM, in contrast with other monomeric TIM mutants [19,20].

Stability of monoTcTIM

The energy of the unfolding/refolding reactions of monoTcTIM was evaluated using the two-state model described in the Materials and methods section. The results are shown in Table 1. The ΔG values for unfolding and refolding of monoTcTIM were almost the same, indicating that equilibrium was reached in both directions (Table 1). The ΔG value obtained for the unfolding of monoTcTIM is 30% lower than that presented by the monomeric intermediate in the TcTIM unfolding pattern [5]; this value is similar to those reported for other monomeric TIM mutants and only represents between 10% and 15% of the total wild-type enzyme value.

Table 1
Stability of monoTcTIM.

Fitted data	Process	$\Delta G°$ (kJ mol^{-1})	$C_{½}$ (M)
FI_{max} 280 nm/CD_{222}	Unfolding	13.45 (6.4)	2.1
FI_{max} 280 nm	Refolding	13.11 (5.9)	2.2
Mean		13.28 ± 0.2	2.15 ± 0.01

Fittting of experimental data to the model described in the Materials and methods section. For unfolding, the parameters were obtained from the simultaneous fitting of data from Fig. 1B; for refolding, the data correspond to inset Fig. 1B. FI_{max}, maximal fluorescence intensity. *m* Values are in parentheses. $C_{½} = \Delta G°_{H2O}/m$.

One observation from TIM monomerization studies is that some hydrophobic native interactions are lost when the oligomer is disrupted [7,21]. Evidence of this was obtained using ANS. The binding of this dye to predominantly hydrophobic areas of the protein is accompanied by a large increase in its fluorescence quantum yield [22]. TcTIM was used as a control and about a fivefold increase in extrinsic fluorescence for monoTcTIM was obtained (Supplementary Table 1). The significant exposure of the non polar surface could be related to the low stability of monoTcTIM (Table 1).

Structure of monoTcTIM

TcTIM exhibits the characteristic CD spectrum of a $(\beta/\alpha)_8$ protein, with two minima centered at 208 and 218 nm, respectively (□, Fig. 2, see also [6]). At the same protein concentration, the CD spectrum for monoTcTIM is similar to that obtained for TcTIM; however, a shoulder around 210 nm is obtained, indicating some alterations in the secondary structure (●, Fig. 2). Even so, the calculated secondary structure content is very similar for both proteins (Supplementary Table 1), indicating that the main features of the TIM secondary structure are preserved after the mutations. Regarding tertiary structure, the wild-type and the monomeric proteins were analyzed by fluorescence intensity experiments. Minor differences were observed between both proteins in the λ_{max} and the SCM; however, a significant change in the fluorescence

intensity was found for monoTcTIM at both wavelengths of excitation (Supplementary Table 1); one of the reasons for this could due to some alterations in the tertiary structure of the TIM barrel produced after monomerization.

To gain further insight into the structural properties of monoTcTIM, its three dimensional structure was determined by means of X-ray crystallography. The low alpha-carbon RMSD value (0.88 Å) indicated no significant changes in the main chain of the monomeric enzyme when compared with TcTIM (PDB code 1TCD, Fig. 2B). Likewise, no differences in the general structure of the main chain were detected between monoTIM from *T. brucei* (PDB code 1TRI) and monoTcTIM (alpha-carbon RMSD 1.3 Å). However, in the monomer present on the asymmetric unit in the crystal (Fig. 2B), residues 16–21, corresponding to loop 1, are not seen on the electron density map and residues 170–177, corresponding to the catalytic loop 6, are less visible on the same map. Also, the catalytic His 96 and Lys 13 residues are in a different, non catalytic position compared to that occupied by the same residues in the wild-type enzyme (Fig. 2B, Supplementary Fig. 3). Thus, although the overall architecture of the protein is well-preserved, the presence of small but important changes indicates that the monomeric enzyme is unable to perform optimal catalysis.

For all the parameters described above, the known properties of monoTbTIM are fairly similar to those reported here for monoTcTIM i.e., the magnitude of the ΔG of unfolding, the Stokes radii and the general properties of the X-ray crystal structure [23]. Overall, our results show that the enzymatic activity presented by monoTcTIM (see below) is only due to a monomeric species of the molecule.

Differential activity of monoTcTIM and monoTbTIM

Although the catalytic activity of TcTIM and TbTIM native dimers is approximately the same, monoTcTIM presents a K_{cat} ten times higher than that observed for monoTIM from TbTIM (Table 2). Regarding substrate affinity (K_m), in monoTcTIM, this parameter is twice the value reported for monoTbTIM; however, the V_{max} of monoTcTIM is 83 times higher than that observed in monoTbTIM, indicating that equivalent mutations produce a more efficient monomeric protein in TcTIM, although with less affinity than the same protein from TbTIM.

It is difficult to predict the precise location of the monomer catalytic differences. A clue could be given by the presence of Pro44, a residue located in the loop 2 of TcTIM (Supplementary Fig. 1). It has been suggested that a mutation of serine for proline at this position, could enhance the catalytic activity after monomerization [21]. The results obtained for monoTcTIM agree with those stated above, because the mutant RMMO-1 [21] that contains the Pro44 residue in the primary sequence shows a significant increase in K_{cat} compared to monoTbTIM, which lacks this residue (Table 2).

Table 2
Catalytic activity of monoTcTIM.

Protein	K_m (mM)	V_{max} (μmol min^{-1})	K_{cat} (s^{-1})	References
TbTIM	0.45[a]	9300[b]	6166.66[a]	a: [23] b: [25]
monoTIM	5.3	0.007	5.2	[7]
	4.1	Nr	5.16	[26]
monoTIM-SS	6.1	Nr	7.83	[27]
RMMO-1	1.19	Nr	58	[21]
RE-TIM	17.2	Nr	0.46	[27]
TcTIM	0.66[c]	9670[c]	4333.33[d]	c: [1]
	0.9[e]	7498[e]		d: [28] e: [11]
monoTcTIM	11.2 ± 2.6	0.58 ± 0.09	52.7	This work

Kinetic parameters for some TIMs and monomeric mutants. nr, not reported.

The differences in the catalytic activity of two highly similar proteins indicate the great interdependency acquired by the biological dimer. Any changes at the sequence level are communicated between the monomers to develop the most efficient possible enzyme. In this sense, it is noteworthy that, for all the studied TIMs from several organisms, their catalytic activity remains the same despite their sequence differences [1,24]. This study shows that the catalytic properties of isolated enzymatic TIM subunits do differ, thus providing another tool for the study of the mechanisms of catalysis, protein stability and dimeric nature of this enzyme.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.03.085.

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