Structural Differences in Triosephosphate Isomerase from Different Species and Discovery of a Multitrypanosomatid Inhibitor†

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ABSTRACT: We examined the interfaces of homodimeric triosephosphate isomerase (TIM) from eight different species. The crystal structures of the enzymes showed that a portion of the interface is markedly similar in TIMs from *Trypanosoma cruzi* (TcTIM), *Trypanosoma brucei*, and *Leishmania mexicana* and significantly different from that of TIMs from human, yeast, chicken, *Plasmodium falciparum*, and *Entamoeba histolytica*. Since this interfacial region is central in the stability of TcTIM, we hypothesized that it would be possible to find agents that selectively affect the stability of TIMs from the three trypanosomatids. We found that 6,6′-bisbenzothiazole-2,2′ diamine in the low micromolar range causes a desirable irreversible inactivation of the enzymes from the three trypanosomatids and has no effect on the other five TIMs. Thus, the data indicate that it is possible to find compounds that induce selective inactivation of the enzymes from three different trypanosomatids.

Studies of the changes that have occurred in oligomeric proteins during evolution indicate that their tertiary and quaternary structures have been largely conserved; however, many changes have taken place at the level of their primary structure. A statistical analysis of the changes in different portions of oligomeric enzymes showed that amino acid conservation is significantly lower at the interface than at the catalytic site (*1*, *2*). These observations suggest that there may be significant differences in the interfaces of orthologous enzymes from parasites and human. The data could also imply that differences between enzymes from closely related species would be smaller than between enzymes from organisms that are distant in the evolutionary scale. In this context, homodimeric triosephosphate isomerase (TIM) is particular amenable to study the differences and similarities of the interface of oligomeric enzymes from different species, particularly because the crystal structures of TIM from 13 different species are available. This ubiquitous enzyme catalyzes the interconversion between glyceraldehyde 3-phosphate and dihydroxyacetone phosphate by well-known mechanisms (*3*). In most of the species so far described, TIM is a homodimer in which the interface has an area of about 1400 Å^2.

We have previously observed (*4*) that, in its crystal form, TIM from *Trypanosoma cruzi* (TcTIM)[1] has the capacity to bind three hexane molecules; two of these molecules bind to the periphery of the dimer interface. Mattos and Ringe (*5*, *6*) hypothesized that the binding sites for organic molecules could represent potential targets for the design of molecules that are detrimental for enzyme action. Indeed, we observed that a benzothiazole derivative that inactivates TcTIM binds to the same region of the interface in which the two hexane molecules were detected (*7*).

We examined this region of the interface of TIM from three different trypanosomatids, namely, *T. cruzi* (*7*), *Trypanosoma brucei* (*8*), and *Leishmania mexicana* (*9*), and in TIM from five other species: yeast (*10*), chicken (*11*), human (*12*), *Plasmodium falciparum* (*13*), and *Entamoeba histolytica* (*14*). The data showed that the structure of this portion of the interface is markedly similar in the enzymes from the trypanosomatids and significantly different from its counterpart in TIMs from other species. In view of these structural differences and the knowledge that a benzothiazole binds in the interface pocket of TcTIM (*7*), we hypothesized that it would be possible to find agents that are specific for

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[1] Abbreviations: ChTIM, EhTIM, HTIM, LmTIM, PfTIM, TbTIM, TcTIM, and YTIM, triosephosphate isomerase from chicken, *Entamoeba histolytica*, *Homo sapiens*, *Leishmania mexicana*, *Plasmodium falciparum*, *Trypanosoma brucei*, *Trypanosoma cruzi*, and *Saccharomyces cerevisiae*, respectively; **V7**, 6,6′-bisbenzothiazole-2,2′-diamine; EDTA, ethylenediaminetetraacetic acid; NADH, nicotinamide adenine dinucleotide, reduced form; MALDI TOF, matrix-assisted laser desorption ionization time-of-flight.

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Table 1. Residues in TcTIM That Contact the Hexane Molecules or Compound **8**[a]

TcTIM	TbTIM	LmTIM	HTIM	YTIM	ChTIM	EhTIM	PfTIM
Ile 69 A	Ile	Ile	Tyr	Tyr	Tyr	Trp	Ser
Arg 71 A	Lys	Lys	Val	Lys	Val	Lys	Phe
Phe 75 A	Phe	Phe	Phe	Phe	Phe	Tyr	Tyr
Tyr 102 B	Tyr	Tyr	Val	Tyr	Val	Ile	Tyr
Tyr 103 A,B	Tyr	Tyr	Phe	Phe	Phe	Phe	Phe
Gly 104 A	Gly	Gly	Gly	His	Gly	His	His
Ile 109 A	Ile	Ile	Leu	Phe	Leu	Gln	Asp
Lys 113 A	Lys	Lys	Lys	Lys	Lys	Lys	Lys
% identity in the pocket[b]	88	88	50	37	50	12	25
% total identity[c]	72	68	52	51	47	46	44

[a] The residues of TcTIM that are less than 4 Å from the hexane or compound **8** molecules are shown in the first column. The equivalent residues in the indicated TIMs are shown. [b] Indicates the percent identity, in which 100% correspond to the residues of TcTIM. [c] Shows the overall identity of the indicated TIMs, in which TcTIM is 100%.

enzymes that have similar interfacial structures. Therefore, we examined several different benzothiazoles and found one (termed **V7**) that inactivates the enzymes from the three trypanosomatids at concentrations that are in the low micromolar range; this agent is without effect on the other five TIMs. These results illustrate that it is possible to discover agents that selectively affect the enzymes from several types of parasites.

MATERIALS AND METHODS

TcTIM (*15*) and TIMs from *T. brucei* (*16*), *L. mexicana* (*17*), human (*12*), yeast (*18*), *Plasmodium falciparum* (*13*), and *Entamoeba histolytica* (*14*) were expressed in *Escherichia coli* and purified as in the indicated references. Chicken TIM was purified as described in ref *19*. After purification, the enzymes dissolved in 100 mM triethanolamine, 10 mM EDTA, and 1 mM dithiothreitol (pH 8) were precipitated with ammonium sulfate (75% saturation) and stored at 4 °C. Before use, the enzymes were extensively dialyzed against 100 mM triethanolamine/10 mM EDTA (pH 7.4). Protein was determined from their absorbance at 280 nm. The extinction coefficient ϵ (M^{-1} cm^{-1}) was 36 440 for TcTIM, LmTIM, TbTIM, and EhTIM, 33 460 for HTIM, 26 664 for YTIM, 25 710 for PfTIM, and 27 960 for ChTIM calculated according to Pace et al. (*20*).

Synthesis of 6,6′-Bisbenzothiazole-2,2′-diamine (**V7**). The synthesis of this compound has been described (*21*). **V7** was dissolved in dimethyl sulfoxide (DMSO). In all experiments, with or without **V7**, the concentration of DMSO was 10% (v/v). In 10% DMSO, **V7** was soluble up to a concentration of 50 μM.

Assay of the Action of **V7** *on the Various TIMs.* Each TIM was incubated at a concentration of 5 μg/mL in a solution of 100 mM triethanolamine, 10 mM EDTA, 10% dimethyl sulfoxide (v/v), and **V7** at the concentrations indicated under Results for 2 h at 36 °C. At this time, an aliquot was withdrawn for assay of activity.

Activity. Activity was determined in the direction of glyceraldehyde 3-phosphate to dihydroxyacetone phosphate (*15*). The decrease in absorbance at 340 nm was followed in a multicell Hewlett-Packard spectrophotometer at 25 °C. The reaction mixture (1 mL) contained 100 mM triethanolamine, 10 mM EDTA, 0.2 mM NADH, 1 mM glyceraldehyde 3-phosphate, and 0.9 units of α-glycerol phosphate dehydrogenase (pH 7.4). The reaction was started by the addition of TIM, usually 5 ng.

Mass Spectrometry. The molecular mass of TcTIM incubated with and without 40 μM **V7** was determined with an Omniflex MALDI TOF mass spectrophotometer from Bruker. After incubation, the samples were dialyzed against water, concentrated in Amicon filters, and their mass spectra determined.

Intrinsic Fluorescence. The emission fluorescence spectra of the enzymes in 100 mM triethanolamine/10 mM EDTA/10% of dimethyl sulfoxide (pH 7.4) were recorded in a RF5000U Shimadzu spectrofluorometer in 4 mm quartz cuvettes at room temperature. The excitation wavelength was 280 nm. To assess the effect of **V7**, the enzymes were incubated with **V7** at 36 °C, and at various times, their fluorescence spectra were recorded. Blanks without protein were also recorded; these were subtracted from the experimental spectra.

RESULTS

The structure of TcTIM in crystals soaked in hexane showed the presence of three hexane molecules; two of the molecules dock in a hydrophobic pocket that is on the periphery of the dimer interface (*4*). This portion of the interface is also capable of binding 3-(2-benzothiazolylthio)-1-propanesulfonate (compound **8**) as evidenced by the crystal structure of the TcTIM−compound **8** complex (*7*). Collectively, these data indicate that the interface of TcTIM has a hydrophobic pocket that is capable of binding hydrophophobic molecules. This portion of the interface was compared to the equivalent regions of other TIMs.

Table 1 shows the nine residues of either monomer A or B of TcTIM that are less than 4 Å from compound **8** or the hexane molecules. Note that Tyr103 of the two monomers contact the bound molecules, so only one of these residues is considered in what follows. In TIMs from *T. brucei*, and *L. mexicana*, seven of these eight residues are identical to those of TcTM. In the other enzymes, the number of identical residues is lower: four in human and chicken TIMs, three in yeast TIM, two in EhTIM, and only one in PfTIM. The different amino acid composition reflects on the structure of this portion of the interface. As shown in Figure 1A, the overall structure of this region of the interface is markedly similar in the enzymes from the three trypanosomatids and significantly different from human, yeast, chicken, *P. falciparum*, and *E. histolytica* TIMs (Figure 1B). It is noteworthy that there is a strong similarity in the structural arrangements of the latter five TIMs and that, in these TIMs, the region is

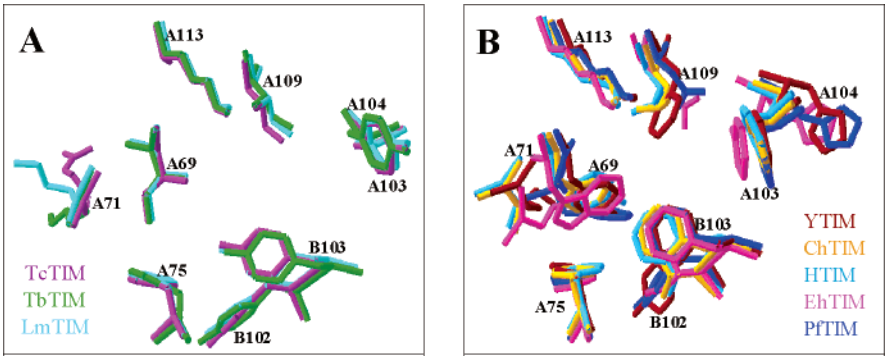


FIGURE 1: Superposition of the structures of the indicated TIMs in the region where binding of two hexane molecules and compound **8** were detected. Panel A shows the structure of TIM from *T. cruzi*, *T. brucei*, and *L. mexicana*; the data are from refs *7*−*9*, respecttively. Panel B shows the equivalent region in TIM from yeast, chicken, human, *P. falciparum*, and *E. histolytica*; the data are from refs *10*−*14*, respectively. The numbers of the indicated residues corresponds to those for TcTIM. The letters A and B at the side of the residues indicate the monomer to which the residue belongs.

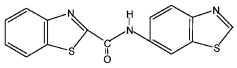
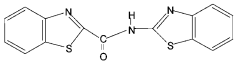
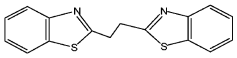
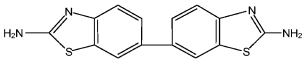
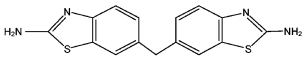
Compound	Structure	Inactivation (%)
V3		NS
V4		NS
V5		40
V6		NS
V7		91
V8		2

FIGURE 2: Effect of the indicated benzothiazoles on TcTIM. The effect of the indicated compounds on TcTIM was assessed at a concentration of 50 μM. NS, not soluble.

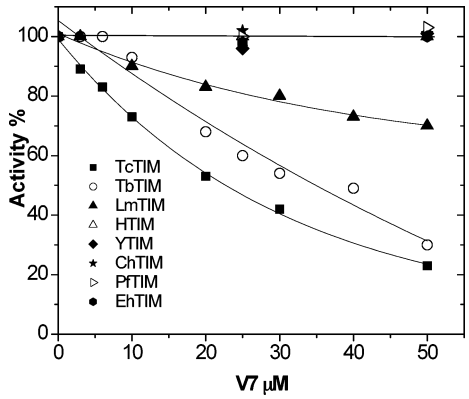


FIGURE 3: Effect of **V7** on the indicated TIMs. The effect of the indicated concentrations of **V7** on the indicated TIMs was assessed as described in the Materials and Methods section.

more tightly packed than in the enzymes from trypanosomatids.

We reasoned that by targeting this portion of the interface, it would be feasible to find molecules that affect TIMs from the three parasites but not TIMs from the other species. Therefore, we searched for such compounds. In a previous work (*22*), we found that among several dozens of molecules that were tested, the only molecules that affect TcTIM in the micromolar range contain a benzothiazole group. We also observed that inactivation of TcTIM by molecules with a single benzothiazole group, such as compound **8**, required the binding of two molecules and that a single molecule sufficed to induce strong inactivation of TcTIM if it contained two covalently bound benzothiazole groups (compound **10** in ref *22*). However, compound **10** was a weak inhibitor of TbTIM and LmTIM. Thus, we synthesized six molecules that contain two benzothiazole groups searching for a multitrypanosomatid inhibitor. Three were insoluble even in media that contained 10% DMSO (Figure 2). Among the remaining three, we found that compound **V7** at a concentration of 50 μM inactivated TcTIM by 80−90%. In this connection, it is noted that **V8**, which is formed by two benzothiazole groups with the same orientation as in **V7** but separated by a methylene group, hardly affected TcTIM (Figure 2). This suggests that inactivation by a given benzothiazole involves very precise interactions between the compound and the enzyme; in addition, it would appear that the rigidity of **V7** is an important factor for inactivation.

Effect of **V7** *on TIMs from Different Species.* The effect of different concentrations of **V7** was examined in TcTIM and in the TIMs that are shown in Figure 1. As shown in Figure 3, we found that **V7** induced half-maximal inactivation of TcTIM and TbTIM at concentrations of 21 and 35 μM, respectively. We calculated that half-maximal inactivation of LmTIM was attained with about 100 μM (Figure 3). Remarkably, at the maximal concentration that could be studied, **V7** had no effect at all on the other five TIMs.

It is important to note that, in our experimental protocol, we incubate the enzyme with the substance to be tested, and after 2 h an aliquot of the mixture (generally 1 μL) is withdrawn to measure the remaining activity by adding it to 1 mL of reaction medium. This 1000-fold dilution of the compound did not reverse the action of the compound. In some experiments, we have followed activity for more than 20 min; in all cases, the activity traces of the inhibited enzyme were linear. Thus, the action of **V7** on the TIMs from the trypanosomatids is irreversible, a characteristic that has been considered as advantageous in the development on antiparasitic drugs (*23*). The irreversible action of **V7** distinguishes it from inhibitors that act when they are in the aggregated state (*24*).

In regard to its mechanism of action, it is noted that **V7** is a bidentate molecule of considerable basicity that in all likelihood is protonated at pH 7.4, thus yielding two possible positively charged tautomers that could establish hydrogen bonds with some enzyme residues (*25*). Otherwise **V7** is a rather chemically inert molecule that under the conditions of the experiments would not form covalent bonds with amino acids. In fact, we found by mass spectrometry that the mass of TcTIM treated with **V7** and the control were the same. In both cases, the mass was 27 049.75 Da, which corresponds to that of the monomers.

Effect of **V7** *on the Intrinsic Fluorescence of the Various TIMs.* The irreversibility of the action of **V7** suggested that the inactivation of TIMs from the trypanosomatids is accompanied by structural alterations. To explore this possibility, we recorded the emission spectra of the intrinsic fluorescence of the three TIMs from trypanosomatids as well as that of TIMs from human, yeast, and chicken during their incubation with 40 μM **V7**. It is noted, however, that at the wavelength at which the fluorophores of TIM are excited (280 nm) **V7** exhibited a strong fluorescence with a maximum at 376 nm. However, at 330 nm, the wavelength of maximum emission of TIM, the fluorescence of **V7** was almost nil (Figure 4A). Therefore, to assess whether **V7** induces structural alterations, the intensity of fluorescence of TIM at 330 nm was recorded.

The incubation of TIM from the three trypanosomatids with **V7** induced a progressive decrease of their intrinsic fluorescence (Figure 4B). The structural changes paralleled the loss of activity. When the same experiment was carried out with human, yeast, and chicken TIMs, no effect on activity or fluorescence was observed. These experiments illustrate that the decrease of activity induced by **V7** is due to alterations of enzyme structure.

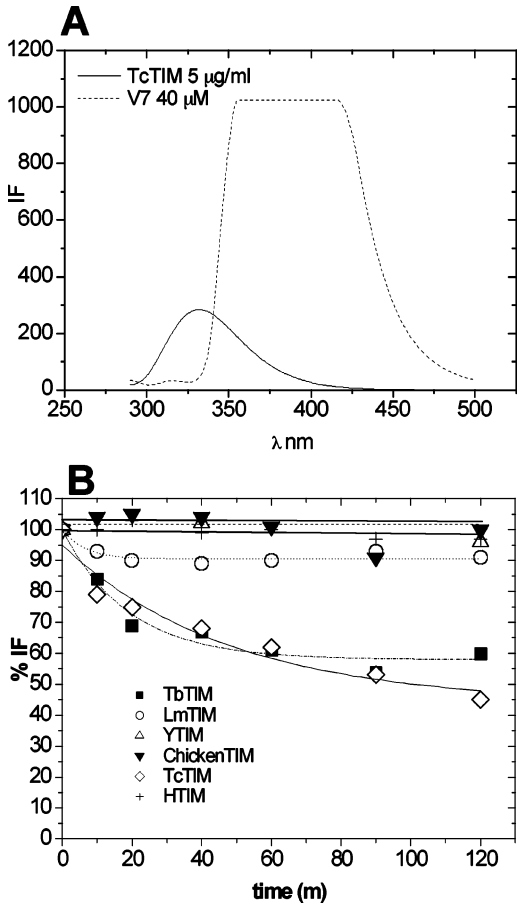


FIGURE 4: (A) Intrinsic fluorescence spectra of TcTIM and fluorescence of **V7**. (B) Effect of **V7** on the intrinsic fluorescence of the indicated TIMs. In panel A, the intrinsic fluorescence spectrum of TcTIM at a concentration of 5 μg/mL (solid line) in 100 mM triethanolamine/10 mM pH 7.4 and the fluorescence of **V7** at a concentration of 40 μM were recorded at an excitation wavelength of 280 nm. In panel B, the indicated TIMs at a concentration of 5 μg/mL were incubated in 100 mM triethanolamine/10 mM EDTA/10 dimethyl sulfoxide (pH 7.4) and 40 μM **V7** at 36 °C. At the indicated times the fluorescence of the samples at 330 nm was recorded.

DISCUSSION

Mattos and Ringe (*5*, *6*) proposed that the analysis of the crystal structure of enzymes exposed to organic solvents could reveal binding sites for organic molecules. They also put forth that these sites could be targeted for the design of molecules that are deleterious for enzyme action. In consonance with the proposal, we found that a region in the periphery of the interface of TcTIM has the capacity to bind two hexane molecules (*4*) as well as a benzothiazole derivative that causes inactivation of the enzyme (*7*). This portion of the interface was examined in TIMs from eight different species. The data showed that this portion of the interface is strikingly similar in the enzymes from the three trypanosomatids that were studied. Of equal importance is that, in the five nontrypanosomatidal that were examined, this region differs markedly from that of the trypanosomatids. In this connection, it is noteworthy that we found a bis(benzothiazole) that inactivates the three trypanosomatidal TIMs and does not affect the other TIMs. Therefore, it would appear that the structural similarities and differences that may exist in the interfaces of oligomeric enzymes may be targeted to find agents that selectively affect the enzymes from different organisms.

We point out that we have tried to obtain crystals of TcTIM in complex with **V7**; however, the attempts have been unsuccessful. Therefore, it would seem that the drastic structural changes induced by **V7** hinder the crystallization of the complex. In this respect, it is relevant to note that **V7** induces strong changes in the intrinsic fluorescence of TcTIM, TbTIM, and to a lower extent in LmTIM (Figure 4). Moreover, when we used relatively high concentrations of TcTIM and prolonged incubations times, it was clearly evident that **V7** induces aggregation of the enzyme. In all likelihood, this effect of **V7** accounts for the irreversibility of its action. On the other hand, as described elsewhere (*7*), we were able to crystallize TcTIM in complex with one molecule of compound **8**. We probably succeeded because inactivation and structural changes of TcTIM induced by **8** requires the binding of two molecules. Thus, the enzyme in complex with one molecule of **8** has the stability that is necessary for crystallization.

In the absence of crystallographic data, it is not possible to define the precise interactions between **V7** and the enzyme. However, we found that three enzymes that have nearly identical hydrophobic pockets are sensitive to **V7** and five enzymes that have a different hydrophobic pocket are insensitive to **V7**. Thus, there is a strong correlation between

the structure of the pocket and the sensitivity of the enzymes to the bis(benzothiazole) **V7**. Along this line, it is important to note that the sensitivities of TcTIM and TbTIM to **V7** are markedly similar, whereas that of LmTIM is lower. This indicates that although **V7** very likely binds to the same region of the three trypanosomatid TIMs, the magnitude of the overall response to this agent is affected by other factors. Nonetheless, the overall data illustrate that enzymes from closely related parasites have common structural features that are not shared by orthologous enzymes from organisms that are far away in the evolutionary scale. We also show that by targeting specific structures on these common species it is possible to find agents that induce selective inactivation of the enzymes from three different trypanosomatids.

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