Using evolutionary changes to achieve species-specific inhibition of enzyme action — studies with triosephosphate isomerase

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Background: Many studies that attempt to design species-specific drugs focus on differences in the three-dimensional structures of homologous enzymes. The structures of homologous enzymes are generally well conserved especially at the active site, but the amino-acid sequences are often very different. We reasoned that if a non-conserved amino acid is fundamental to the function or stability of an enzyme from one particular species, one should be able to inhibit only the enzyme from that species by using an inhibitor targeted to that residue. We set out to test this hypothesis in a model system.

Results: We first identified a non-conserved amino acid (Cys14) whose integrity is important for catalysis in triosephosphate isomerase (TIM) from *Trypanosoma brucei*. The equivalent residues in rabbit and yeast TIM are Met and Leu, respectively. A Cys14Leu mutant of trypanosomal TIM had a tendency to aggregate, reduced stability and altered kinetics. To model the effects of a molecule targeted to Cys14, we used methyl methanethiosulfonate (MMTS) to derivatize Cys14 to a methyl sulfide. This treatment dramatically inhibited TIMs with a Cys residue at a position equivalent to Cys14, but not rabbit TIM (20 % inhibition) or yeast TIM (negligible inhibition), which lack this residue.

Conclusions: Cys14 of trypanosomal TIM is a non-conserved amino acid whose alteration leads to loss of enzyme structure and function. TIMs that have a cysteine residue at position 14 could be selectively inhibited by MMTS. This approach may offer an alternative route to species-specific enzyme inhibition.

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Introduction

Enzymes from different species that have identical catalytic activities are usually very similar in their amino-acid sequences and three-dimensional structures [1]. This is particularly true at the catalytic site, where the amino acids that form the active site and participate in catalysis are highly conserved. The similarities between homologous enzymes have hampered the design of species-specific inhibitors, in particular those directed toward the active center [2]. To circumvent this problem, attention has been given to regions that are close to the catalytic site but show individual structural features. This strategy has been used to design selective inhibitors of trypanosomal glyceraldehyde-3-phosphate dehydrogenase, but these agents still have inhibitory activity toward the homologous enzyme from other species [3]. Attempts have also been made to produce inhibitors aimed at regions that are critical for enzyme stability [4].

Part of the problem in the design of species-specific enzyme inhibitors is the fact that it is difficult to predict *a priori* whether the chosen non-conserved region is important either for the catalytic activity or for the stability of the enzyme. In this work we present an approach to the localization of non-conserved residues whose perturbation leads to loss of catalytic activity or stability. We focused on amino acids that do not form part of the catalytic center. We show that it is possible to achieve species-specific enzyme inhibition by identifying a non-conserved amino acid that is important for the function of the enzyme from a particular species. An agent that modifies such a residue can inhibit the function of the enzyme from this species but has little effect on the activity of the same enzymes from other species that have a different residue at this position.

We have used triosephosphate isomerase (TIM) in this model study. It is a homodimeric house-keeping enzyme, that exists in all organisms, and the kinetic [5,6], degradation [7] and reactivation [8,9] properties of this enzyme have been thoroughly studied. In addition, the three-dimensional structures of TIM from five species [10–14] are known in detail and the amino-acid sequences from 28 species are also available ([15–39], and accession numbers U02949 (GenBank), Z26875 (EMBL)). Of particular relevance for this study is that TIMs from various

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species have strikingly similar three-dimensional structures [14,40] which makes them ideal model enzymes for investigations of whether non-conserved amino acids can be used as targets for species-specific inhibition.

Results

Analysis of the amino-acid sequences from 28 different species showed that there are only 37 strictly conserved amino acids out of at least 247. The enzymes from rabbit, yeast and *Trypanosoma brucei* are identical in 96 residues out of 248, 248 and 250 amino acids, respectively. We set out initially to ascertain if one of the non-conserved residues or its integrity is centrally important for the stability and/or function of TIM from one of these three species.

We previously observed that rabbit TIM is more sensitive to chloramine T [41], which mainly oxidizes cysteine and methionine residues [42,43], than yeast TIM. There are two cysteines in yeast TIM that are at equivalent positions to two of cysteines in rabbit TIM (see Table 1). Rabbit TIM has three additional cysteines and two methionines that are not present in yeast TIM, and we therefore hypothesized that the higher sensitivity of rabbit TIM to chloramine T was due to the oxidation of one or all of the cysteine and methionine residues that do not have equivalents in yeast TIM. The content of cysteines and methionines in trypanosomal TIM differs from that in both rabbit and yeast TIM (Table 1). We thus compared the inhibitory activity of chloramine T on the trypanosomal enzyme to its effect on rabbit and yeast TIM (Fig. 1).

The inhibitory activity of chloramine T on rabbit and trypanosomal TIM was almost identical, and was several-fold higher than that on yeast TIM. These results suggested that trypanosomal and rabbit TIM, but not yeast TIM, possess at least one common residue that is modified by chloramine T, inhibiting catalysis. As shown in Table 1 there is only one such residue, corresponding to position 14, which is cysteine in trypanosomal TIM and methionine in the rabbit enzyme. In yeast TIM this position is occupied by a leucine residue. These data suggested that the integrity of the residue at position 14 is essential for the catalytic activity of rabbit and trypanosomal TIM. This hypothesis was investigated by constructing a mutant of trypanosomal TIM, in which Cys14 was replaced by leucine (Cys14Leu TIM), and determining whether the substitution altered the sensitivity of the enzyme to chloramine T, or whether the mutation itself altered the kinetics and/or stability of the enzyme.

Table 1. Positions of cysteines and methionines in rabbit, trypanosome and yeast TIM.

Cysteines			Methionines		
Rabbit	Trypanosome	Yeast	Rabbit	Trypanosome	Yeast
	14		14		
41	39	42			
				50	
66					
			82		
86					
				122	
126	126	126			
217					

The numbers denote the positions of cysteines and methionines in the indicated enzymes. In the alignment of the amino acid sequences and in the three-dimensional structure of the yeast [51] and trypanosome enzymes [40], residue 13 of yeast TIM (leucine), corresponds to cysteine 14 and methionine 14 of trypanosomal and rabbit TIM, respectively.

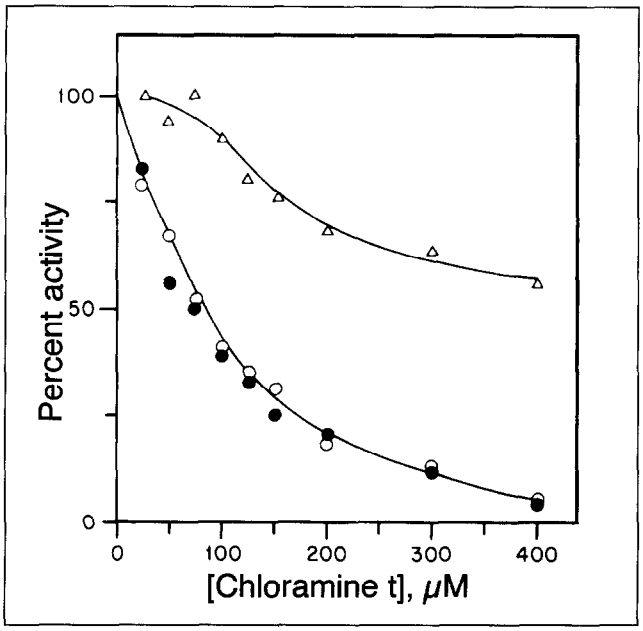

Fig. 1. Chloramine T inhibits the activity of TIM from rabbit muscle (○) and *Trypanosoma brucei* (●) to a greater extent than that of yeast TIM (Δ). The enzymes were preincubated at a concentration of 10 μg ml^{-1} in 100 mM TEA/ 10 mM EDTA, pH 7.4 that contained the indicated concentrations of chloramine T for 24 h at 24 °C. At this time activity was recorded. The activities of control TIM did not change in the preincubation time. The activities for control rabbit, yeast and trypanosome TIMs were 5400, 5800, and 4700 μmol min^{-1} mg^{-1}, respectively; these values are 100 % in the figure.

Properties of the Cys14Leu mutant

Sodium dodecyl sulfate (SDS) gel electrophoresis of extracts from *Escherichia coli* cells expressing the wild-type or the Cys14Leu-mutant enzyme, showed that the enzymes were expressed at equal levels (data not shown). The purification of the mutant enzyme proved difficult, however, because it aggregated when the pH or the salt concentration of the buffers was changed. The tendency of Cys14Leu TIM to aggregate suggested that its stability was lower than that of the wild type, and, in fact, the mutant enzyme lost activity about five times faster than the wild-type enzyme at 46 °C (Fig. 2).

Despite the tendency of Cys14Leu TIM to aggregate, we were able to develop a procedure that permitted a

purification of about 50 % (see Materials and methods), in which the mutant enzyme was obtained from the cellular insoluble fraction that had been extensively washed. This eliminated the possibility that TIM from *E. coli* had been isolated together with the mutant enzyme. The last purification step of Cys14Leu TIM involved a molecular weight filtration through an ACA 34 column. Since Cys14Leu and wild-type TIM had the same elution profile we concluded that Cys14Leu TIM is a dimer.

The mutant TIM had altered kinetics of catalysis. Lineweaver–Burk plots of the activities of wild-type TIM and the Cys14Leu mutant at 25 °C were linear in a concentration range of 0.08 to 3.0 mM of glyceraldehyde-3-phosphate. There were drastic differences in their V_{max}, however (10 and 5500 μmol min^{-1} mg^{-1}, for the Cys14Leu and the wild-type TIM, respectively). The fact that Cys14Leu was partially pure (~50 %) cannot account for the 500-fold difference in V_{max}. The possibility exists, however, that the enzyme in the preparation was largely inactive due to aggregation. Nevertheless the K_M of the Cys14Leu TIM for glyceraldehyde-3-phosphate was different from that of the wild type (1.7 mM and 0.3 mM, respectively). The data obtained for the wild-type TIM were similar to those reported for trypanosomal TIM and for TIMs from other species [44,45]. Substitution of Cys14 with a leucine residue thus caused alterations in the kinetic properties of the enzyme, even though it does not lie at the catalytic site ([46] and see Discussion).

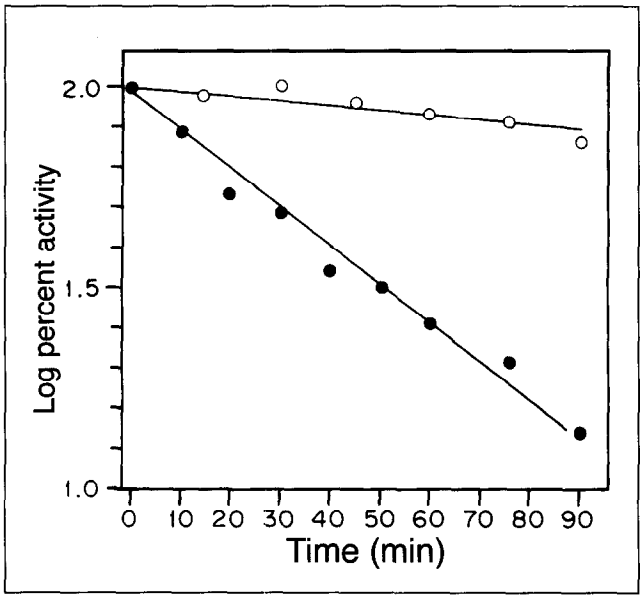

Fig. 2. Cys14Leu TIM has reduced stability compared to the wild-type enzyme. Wild-type (○) and Cys14Leu (●) TIM were preincubated at 46 °C at a concentration of 60 and 130 μg ml^{-1}, respectively, in 100mM MES, 1 mM EDTA and 200 mM NaCl, pH 6.5. At the indicated times aliquots were withdrawn, cooled on ice and activity was measured. The activities of wild-type and Cys14Leu enzymes that were not preincubated were 5100 and 5 μmol min^{-1} mg^{-1} respectively; these values are 100 % in the figure.

Specific inhibition of TIMs that have a cysteine at a position equivalent to Cys14 of trypanosomal TIM

The data on the sensitivity of TIMs from three species to chloramine T, together with the information obtained with the Cys14Leu mutant, indicated that alterations or substitutions of Cys14 of trypanosomal TIM produces loss of stability and function. It was thus possible that an agent that modifies cysteine residues could specifically inhibit TIMs containing cysteine at this position.

The effect of cysteine-reactive chemicals on the activity of TIM from various species has been described previously and, in some cases, species specificity has been noted [45,47,48]. For our purposes a sulfhydryl reagent with access to Cys14 was required. Crystallographic data of trypanosomal TIM [40,46] show that, in each of the two monomers that comprise the TIM dimer, Cys14 is not exposed to the solvent, although it is relatively close to the surface of the protein (~4.3 Å). The other two cysteines of trypanosomal TIM are deeply buried within the structure. We chose methylmethanethiosulfonate (MMTS), a relatively small polar molecule that reacts with accessible cysteines forming a methyl disulfide [49], to study whether selective inhibition of TIMs having a cysteine in position 14 could be achieved.

The exposure of trypanosomal TIM to MMTS resulted in strong inhibition of its activity. The inhibition was concentration and time dependent (Fig. 3). For the inhibition experiments, the enzyme was preincubated with MMTS; aliquots were withdrawn at various times and diluted before determining activity. The activity traces were linear with time, indicating that the effect of MMTS was not reversed by dilution and that the inhibition was probably due to disulfide formation in the preincubation period (see below).

The effect of MMTS on rabbit and yeast TIM was assayed under identical conditions. The reagent at 25 μM inhibited the activity of the rabbit enzyme ~20 %; higher concentrations of MMTS or longer preincubation times, than those shown in Figure 4a, did not increase the extent of inhibition. This could be due to a derivatization of one or more of the cysteines of rabbit TIM. Yeast TIM was completely insensitive to the reagent (Fig. 4b).

There are cysteine residues that align with that in position 14 of *T. brucei* in the amino-acid sequences of TIMs from 7 species of the 28 reported to date: the plants *Arabidopsis thaliana* (accession number U02949 (GenBank)), *Coptis japonica* [28], maize [25], rye (accession number Z26875 (EMBL)), and rice [39], and the parasites *Plasmodium falciparum* [31], *Giardia lamblia* [27] and *Leishmania mexicana* [21]. To explore the importance of this residue for TIM activity, we investigated the effect of MMTS on the activity of TIM from maize (Fig. 4c) and from *L. mexicana* (Fig. 4d). The activities of both TIMs were inhibited by MMTS at concentrations and times similar to those observed with trypanosomal TIM. These results indicate that regardless of the overall composition of cysteines in TIM from various

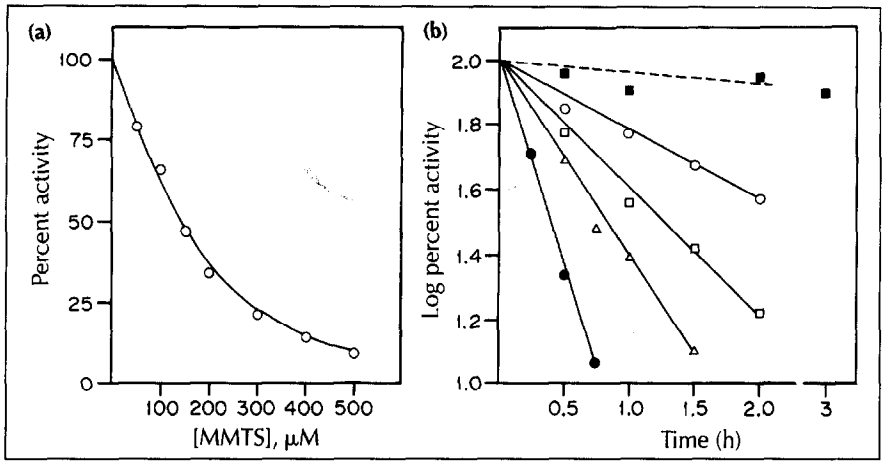

Fig. 3. Cys14Leu TIM has significantly reduced susceptibility to inhibition by MMTS. **(a)** Trypanosomal TIM was preincubated for one hour as in Figure 1 with the indicated concentrations of MMTS. At this time activity was measured. **(b)** Preincubation of trypanosomal and Cys14Leu TIM was as in (a). Cys14Leu TIM (■) was incubated in 900 μM MMTS; wild-type TIM was incubated in 75 (○), 150 (□), 225 (△) or 400 (●) μM MMTS. At the times shown, aliquots were withdrawn for assay of activity. The activities of control wild-type and Cys14Leu TIM were 4800 and 6 μmol min^{-1} mg^{-1} respectively; these values are 100 % in the figure.

species (see cysteine composition in the legend to Fig. 4), it is the modification of the cysteine in position 14 or in an equivalent position that renders the enzyme susceptible to inhibition by MMTS. The fact that the Cys14Leu mutant was inhibited only 5 % by concentrations of MMTS that produced complete inhibition of the wild-type enzyme (Fig. 3a) is consistent with this conclusion.

Inhibition of wild-type trypanosomal TIM by MMTS

MMTS also inhibits other enzymes with accessible cysteines that are essential for function [49,50]. The inhibition of these enzymes by MMTS can be reversed by reducing agents [49,50]. Treatment of trypanosomal TIM that had been inhibited by MMTS with a 100-fold excess of dithiothreitol (over MMTS), however, produced a reversal of inhibition of only 10 %. This suggested that in trypanosomal TIM, MMTS produced largely irreversible structural alterations. To investigate the effect of MMTS on the structure of trypanosomal TIM, the intrinsic fluorescence of this enzyme, incubated with a concentration of MMTS that produced almost complete inhibition of activity, was followed

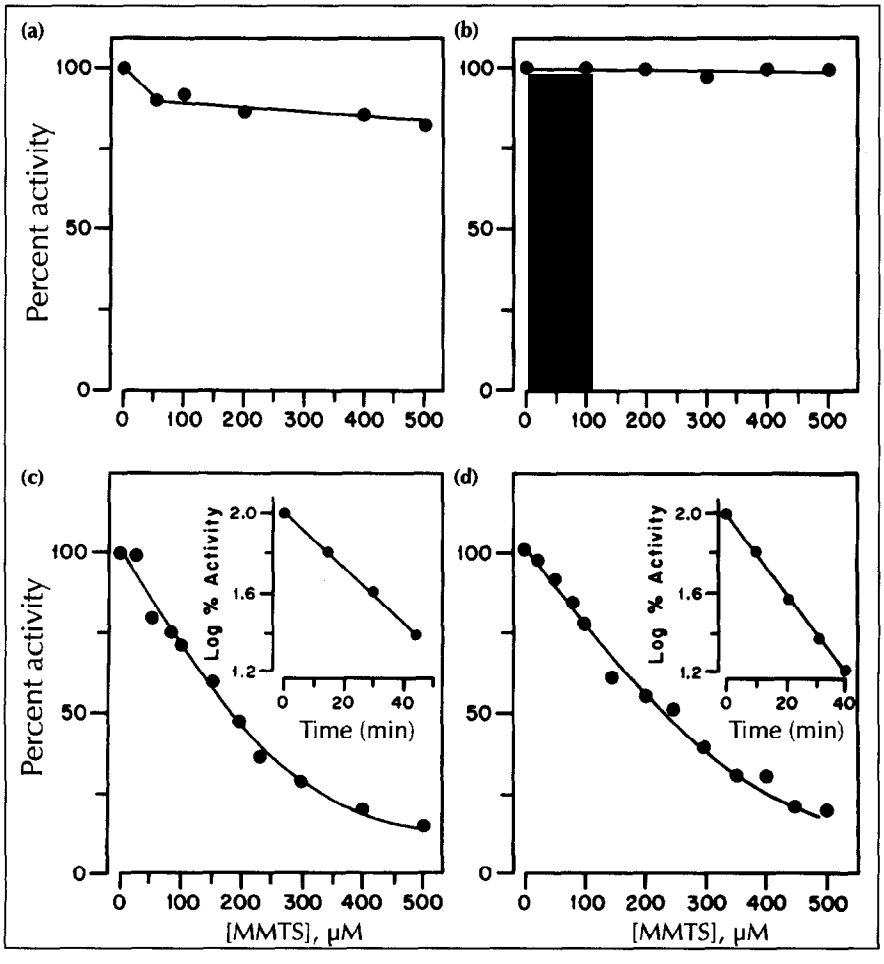

Fig. 4. MMTS strongly inhibits the activity of TIMs with a cysteine residue at a position equivalent to Cys14 of trypanosomal TIM. Effect of MMTS on the actvities of **(a)** rabbit, **(b)** yeast, **(c)** *Leishmania mexicana* and **(d)** maize TIM are shown. The protocol for the assay of the effect of MMTS on rabbit and yeast TIM was as in Fig. 3a. In (c) and (d), the same protocol was followed except that extracts of *Leishmania* and maize were used. For the case of *Leishmania*, the concentration of protein in the preincubation period was 0.068 mg ml^{-1} and that for maize was 0.2 mg ml^{-1}. The activities of the extracts were 6.6 and 1.5 μmol min^{-1} mg^{-1} for *Leishmania* and maize, respectively. The insets in (c) and (d) show the time curve of inhibition with 500 μM MMTS. The content and position of cysteines in rabbit and yeast TIM are shown in Table 1. *Leishmania* TIM has the following cysteines: 15, 40, 118, 127. Maize TIM has cysteines at positions 13, 67, 127, and 218.

over time (Fig. 5). MMTS produced a progressive decrease in fluorescence intensity that was accompanied by a red shift in the peak of maximal emission. Thus, in the presence of MMTS, one or more of the aromatic residues of trypanosomal TIM were exposed to a more polar environment.

The effect of MMTS on the kinetics of catalysis by trypanosomal TIM and on the cysteine content of the enzyme was also determined. The enzyme was incubated with MMTS and the reaction was stopped by centrifugation through Sephadex columns at times in which different degrees of inhibition were assumed to have taken place. Protein concentration, cysteine content and enzymatic activity (at various concentrations of glyceraldehyde-3-phosphate) were determined in the eluates (Table 2). Lineweaver–Burk plots of the activity data were linear; the K_M value showed only slight changes relative to the control. In contrast, the V_{max} progressively decreased with the time of incubation. Thus, MMTS behaved as a non-competitive inhibitor. It is noteworthy that the total cysteine content of the preparations varied in proportion to the V_{max} (Table 2); thus, the formation of inactive enzyme paralleled the derivatization of all the cysteine residues.

Trypanosomal TIM has cysteine residues at positions 39 and 126 in addition to Cys14. A comparison of the structures of yeast and trypanosomal TIMs [40,46,51,52] indicates that the yeast enzyme has cysteines in equivalent positions (42 and 126) to these two residues (Table 1). When yeast TIM was treated with MMTS under conditions identical to those described in Table 2, neither the activity nor the cysteine content showed significant variations (the cysteine content for the control and the treated enzymes were 2.1 and 1.9 per monomer, respectively). We thus inferred that the effects of MMTS on the trypanosomal enzyme result from derivatization of Cys14, which triggers structural alterations that lead to derivatization of the otherwise hidden cysteines. These structural changes also alter the environment of aromatic residues, and abolish catalytic activity.

Table 2. Cysteine content and activity of trypanosomal TIM treated with MMTS.

TIM	Cysteine/monomer	K_M (mM)	V_{max} (μmol min^{-1} mg^{-1})
Control	2.7 (100 %)	0.30	5500 (100 %)
MMTS treated (2 h)	1.3 (48 %)	0.43	2941 (53 %)
MMTS treated (4 h)	0.9 (33 %)	0.42	1666 (30 %)

TIM (1 mg) was incubated with 1 mM MMTS in a final volume of 1 ml TEA/10 mM EDTA, pH 7.4. At the times shown, the mixture was filtered through centrifuge columns. Protein and cysteine content and enzymatic activity were determined with various concentrations of glyceraldehyde-3-phosphate (0.08 to 3.0 mM) in the filtrate. K_M and V_{max} were calculated from linear Lineweaver-Burk plots. The numbers in parenthesis show the percentage decrease of the V_{max} and cysteine content.

Discussion

A popular approach to achieving selective inhibition of enzyme activity is to design inhibitors targeted to a particular region of the crystal structure of an enzyme. In many cases, the target is at or near the active center. For example, by targeting the adenine binding region of the catalytic site of glyceraldehyde-3-phosphate dehydrogenase, an inhibitor that was 47 times more effective on the trypanosomal than on the mammalian

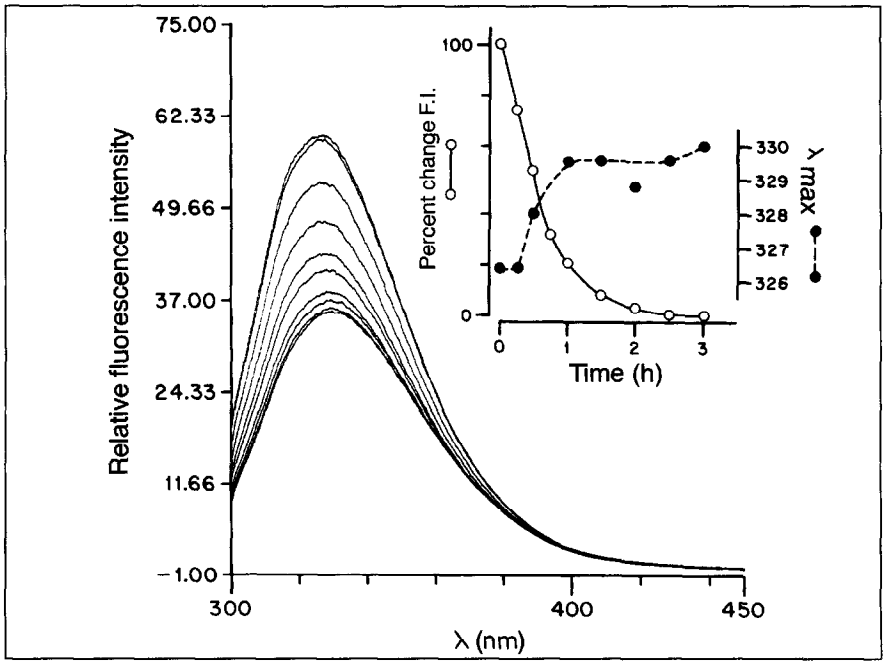

Fig. 5. MMTS produces a progressive decrease in the intrinsic fluorescence of trypanosomal TIM over time. TIM at a concentration of 100 μg ml^{-1} was incubated in 100 mM TEA/10 mM EDTA, pH 7.4 and 500 μM MMTS, and its intrinsic fluorescence spectra recorded at various times (excitation wavelength, 280 nm). The traces obtained were subtracted from blanks that did not contain TIM. From top to bottom the traces are: control (no MMTS added), and after 1, 15, 30, 45, 60, 120, 150 and 180 min incubation with MMTS. The inset shows the decrease in fluorescence intensity (F.I.) with the change between the control and the enzyme treated with MMTS for 180 min taken as 100 %. The inset also shows the wavelength of maximal emission.

enzyme was obtained [53]. The cysteine at the active site of this enzyme has also been targeted for selective inhibition [54]. Other regions of the protein have also been used as targets for design of inhibitors. Peptides directed toward the subunit interface inhibited trypanosomal TIM, and some of the peptides were highly specific for this enzyme [4]. Molecules obtained through other approaches have also been shown to be selective towards enzymes of a given species; suramin and some of its derivatives are selective between mammalian and trypanosomal TIM [55], but the differences in inhibitory activity are not large. To our knowledge, the highest selectivity has been achieved with atovaquone, which is 2000 times more effective in inhibiting ubiquinol–cytochrome *a* reductase activity from *Plasmodium falciparum* than from rat liver [56].

To achieve species-specific inhibition of homologous enzymes, we focused on non-conserved amino acids; if one of these residues or its integrity is essential for the function or stability of the enzyme, it should be possible to find agents that discriminate between homologous enzymes. We have demonstrated the efficacy of this approach using TIM as a model enzyme. We first determined that alterations of non-conserved Cys14 of trypanosomal TIM produced structural perturbations and loss of catalytic activity. Specific inhibition of either purified TIMs, or homogenates containing TIMs, that have this amino acid was achieved using the sulfhydryl reagent MMTS. This reagent had no effect at all, or only a relatively small effect on TIMs that lack Cys14. We stress that MMTS is not specific for TIM; a wide variety of enzymes are inhibited by MMTS [49,50], thus precluding its use *in vivo*. Nevertheless, the data illustrate the validity of the hypothesis.

Why do perturbations of Cys14 affect enzyme stability and function? This residue is not part of the catalytic site. In trypanosomal TIM, Cys14 lies in a four amino-acid stretch that joins the first β-strand with the first α-helix of the eight-strand β-barrel structure of the enzyme. It is located ~4.3 Å from the nearest molecular surface, and is adjacent to Lys13, which is a conserved amino acid that lies at the catalytic site and serves to bind the substrate [45]. It is particularly important that the side chain of Cys14 contacts the interdigitating loop of the other subunit of the TIM homodimer. Recently it was shown that this loop is essential for dimer stability; modification of the loop gave rise to stable monomers with catalytic activity [57]. Mutagenesis studies have shown that the integrity of residues at the subunit interface is important for enzyme stability. Substitution of Asn78 of yeast TIM [58], or substitution of His47 for an Asn in trypanosomal TIM [59], destabilizes the enzyme. Our data show that alterations of catalysis induced by perturbations of Cys14 occur simultaneously to structural modifications. It is probable that the alterations that TIM undergoes when Cys14 is genetically replaced or chemically modified are due to perturbations of inter-subunit contacts.

Significance

Proteins from different species with identical enzymatic activities have highly conserved three-dimensional structures, even though they have regions of highly diverged amino-acid sequence. The development of compounds that selectively inhibit an essential enzyme of an infectious agent has usually focused on the active site, a region that is often highly conserved. We hypothesized that if we could identify and target a non-conserved amino acid that is important for either the function and/or stability of the enzyme from a specific species, selective inhibition of that enzyme could be achieved.

We tested this hypothesis using the glycolytic enzyme triosephosphate isomerase from different species, and showed that Cys14 is such an amino acid in the enzyme from *Trypanosoma brucei*. Other protozoan parasites and plants also have this residue in equivalent positions, but mammalian enzymes do not. We showed that only the enzymes that have a residue equivalent to Cys14 are inhibited by a reagent targeted to cysteine residues. By using non-conserved residues as targets, it is theoretically possible to have a compound that exhibits 100 % selectivity. This principle could be applied to the rational design of drugs against infectious agents. Although in the present studies TIM was used as a model enzyme, there is no reason *a priori* why our general approach for the identification and targeting of essential, non-conserved amino acids could not be applied to other enzymes and proteins of interest to achieve species-specific inhibition.

Materials and methods

Materials

TIM from rabbit muscle and yeast, MMTS, glyceraldehyde-3-phosphate, NADH, 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB), α-glycerophosphate dehydrogenase and chloramine T were obtained from Sigma. The rabbit enzyme was purchased as a lyophilized powder; this was dissolved in 100 mM triethanolamine (TEA)/10 mM ethylene diamine tetraacetic acid (EDTA). Yeast TIM was an ammonium sulfate suspension. This suspension was centrifuged and the pellet dissolved in the aforementioned buffer. The solution was passed through Sephadex G-100 centrifuge columns equilibrated with the same buffer. The eluted enzyme was used for the experiments.

Expression and purification of TIM from T. brucei

The peTTIM1 plasmid was a generous gift of Dr P.A.M. Michels (International Institute of Cellular and Molecular Pathology, Brussels, Belgium). *E. coli* strain BL21(DE3) [60] was transformed with the plasmid and the enzyme was expressed and purified exactly as described by Borchert *et al.* [59]. The enzyme was precipitated with 65 % ammonium sulfate and stored at 4 °C. Prior to all experiments, the precipitate was dissolved in 100 mM TEA/10 mM EDTA, pH 7.4 and filtered through Sephadex G-50 centrifuge columns equilibrated with the same buffer.

Site-directed mutagenesis of TIM from T. brucei

Substitution of the cysteine at position 14 for leucine was performed on a XbaI–BamHI fragment that had been subcloned in the pTZ19R vector according to Kunkel [61], using the oligonucleotide 5'-AGCCAACTGGAAGCTTAACGGCTCC-CAAC-3'. The mutation was confirmed by DNA sequence determination and the gene was subcloned into pET3a and introduced by transformation into BL21(DE3) cells. For expression of the Cys14Leu protein, cells were grown as described for the wild-type TIM, but induction with isopropyl-β-d-thiogalactopyranoside was performed for 3 h at 20 °C.

Purification of Cys14Leu TIM from T. brucei

E. coli cells (from 1 l culture) expressing Cys14Leu TIM were suspended in 40 ml of 25 mM 2[N-morpholino]-ethanesulfonic acid (MES), 1 mM EDTA, 1 mM dithiothreitol and 0.2 mM phenylmethylsulfonyl fluoride (PMSF) pH 6.5 and disrupted by three passages through a French Press. The mixture was centrifuged at 100 000 x g for 60 min. The pellet was suspended in the same buffer, but containing 100 mM MES and no PMSF, and centrifuged again. The pellet was suspended and stirred at 4 °C for 30 min in the 100 mM MES buffer that in addition contained 200 mM NaCl. After centrifugation, the enzyme was precipitated from the supernatant between 45 and 65 % $(NH_4)_2SO_4$ saturation. It is noted that the $(NH_4)_2SO_4$ precipitate could not be dissolved in buffers at pH 7.4 or 8.0 as it underwent marked aggregation. Better solubilization was achieved with the 100 mM MES, 200 mM NaCl buffer, although some aggregation took place. The aggregate was discarded by centrifugation; the remaining soluble enzyme was applied to an ACA 34 A column (120 x 1.5 cm) and eluted with the same buffer. Again, some aggregation occurred in the column. A portion of the enzyme eluted between 140 and 160 ml. SDS gel electrophoresis [62] of this preparation showed that Cys14Leu TIM was about 50 % pure at this stage. The ease with which the enzyme aggregated precluded further purification. About 1 mg of protein was obtained. In separate experiments, pure wild-type TIM was passed through the ACA 34 A filtration column (no aggregation was observed); it eluted in nearly the same volume as the mutant enzyme. This indicated that Cys14Leu TIM was obtained as a dimer with a molecular mass of ~54 kDa.

Extracts from Leishmania *and maize*

Leishmania mexicana strain MHOM/MX/85/Solas [63] was grown in RPMI 1640 supplemented with 10 % fetal-calf serum at 28 °C and 5 % CO_2. Extracts were prepared by grinding 200 mg of cells (wet weight) with 300 mg silicon carbide. The ground cells were suspended in 5 ml of 25 mM Tris-HCl, 1 mM EDTA and 250 mM sucrose, pH 7.8 and centrifuged at 500 x g for 3 min. The supernatant was again centrifuged at 15 000 x g for 15 min. The supernatant was used to assay for activity.

Embryos from maize (Montecillo A 6) (a kind gift of M. Gavilanes-Ruiz, Facultad de Química, UNAM) were obtained by dissection. These were washed with sodium hypochlorite and then extensively with distilled water. Embryos were placed over filter papers laid on Petri dishes (3.5 inch diameter) that contained 7 ml water. They were allowed to grow in the dark at 29 °C for 1 h. The material was collected, frozen in liquid nitrogen and ground. The ground material was suspended in 250 mM sucrose, 70 mM Tris-HCl, 2 mM EDTA, pH 8.0 that also contained 10 μg ml^{-1} chymostatin, 50 μg ml^{-1} *n*-tosyl-L-phenylalanine chloromethyl acetone and 100 μg ml^{-1} *n*-α-*p*-tosyl-L-lysine ketone. This mixture was homogenized and filtered through gauze. The filtrate was centrifuged at 1300 g for 10 min. The supernatant was collected and centrifuged at 105 000 g for 60 min. The supernatant was used for assay of TIM activity.

Determination of protein concentration

Protein concentration was determined according to Lowry *et al.* [64] for rabbit TIM or by using $\epsilon_{1\%}$(280 nm) values of 13.1 for yeast TIM. For trypanosomal TIM we used an extinction coefficient of 13.0 estimated from its aromatic residue content [65].

Activity measurements

Enzymatic activity was determined at 25 °C in 100 mM TEA/10 mM EDTA, pH 7.4, with glyceraldehyde-3-phosphate, as substrate, the coupling enzyme α-glycerophosphate dehydrogenase and NADH as described previously [41]. The decrease in absorbance at 340 nm was used to calculate TIM activity. In all cases, including those in which the enzyme was preincubated with inhibitors, activities were linear with time. When the kinetics of the enzyme were studied, the decrease in optical density that took place within the first two minutes of the experiments was used for calculation of activity rates.

Effect of MMTS on TIM activity

TIM from the various sources was preincubated in 100 mM TEA/10 mM EDTA, pH 7.4, with the concentrations of MMTS indicated under in the Results section. At various times, aliquots were withdrawn from the mixtures and diluted. Dilution was at least 1000-fold when pure enzymes were used, and at least 100-fold when homogenates were studied. It was noted that MMTS, at the concentration present in the activity mixture, did not affect the activity of the coupling enzyme, α-glycerophosphate dehydrogenase.

Determination of cysteine content

The cysteine content of TIM was determined with DTNB according to Ellman [66] after exposing the enzymes to 1 % SDS. When the effect of MMTS on the cysteine content of TIM was assayed, the enzyme treated with MMTS was passed twice through Sephadex centrifuge columns. Protein and cysteine content were determined in the eluate.

Intrinsic fluorescence of trypanosomal TIM

The emission-fluorescence spectra of the enzyme was recorded between 340 and 450 nm at an excitation wavelength of 280 nm with a Shimadzu RF5-000U spectrofluorometer. The concentration of enzyme was 100 μg ml^{-1} in 100 mM TEA/10 mM EDTA, pH 7.4. Blanks without enzyme were also recorded and subtracted from the samples that contained enzyme.

Analysis of crystallographic data

Geometric analysis of protein structures were done on Protein Data Bank [67] files 3TIM and 1YPI corresponding to trypanosomal and yeast TIM, respectively. Solvent accessibility was estimated with the program ACCESS (Scott R. Presnell, University of California, San Francisco, CA), an implementation of the Lee and Richards' algorithm [68], using a probe radius of 0.14 nm and a slice width of 0.001 nm. Distance from Cys14 to the molecular surface was measured from its sulfur atom to the nearest solvent exposed atom in the protein using BIOGRAF software (Molecular Simulations, Inc. Waltham, MA).

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