Derivatization of the Interface Cysteine of Triosephosphate Isomerase from *Trypanosoma brucei* and *Trypanosoma cruzi* as Probe of the Interrelationship between the Catalytic Sites and the Dimer Interface†

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Received October 12, 1998; Revised Manuscript Received January 25, 1999

ABSTRACT: In the interface of homodimeric triosephosphate isomerase from *Trypanosoma brucei* (TbTIM) and *Trypanosoma cruzi* (TcTIM), one cysteine of each monomer forms part of the intersubunit contacts. The relatively slow derivatization of these cysteines by sulfhydryl reagents induces progressive structural alterations and abolition of catalysis [Garza-Ramos et al. (1998) *Eur. J. Biochem. 253,* 684−691]. Derivatization of the interface cysteine by 5,5-dithiobis(2-nitrobenzoate) (DTNB) and methylmethane thiosulfonate (MMTS) was used to probe if events at the catalytic site are transmitted to the dimer interface. It was found that enzymes in the active catalytic state are significantly less sensitive to the thiol reagents than in the resting state. Maximal protection against derivatization of the interface cysteine by thiol reagents was obtained at near-saturating substrate concentrations. Continuous recording of derivatization by DTNB showed that catalysis hinders the reaction of sulfhydryl reagents with the interface cysteine. Therefore, in addition to intrinsic structural barriers, catalysis imposes additional impediments to the action of thiol reagents on the interface cysteine. In TcTIM, the substrate analogue phosphoglycolate protected strongly against DTNB action, and to a lesser extent against MMTS action; in TbTIM, phosphoglycolate protected against the effect of DTNB, but not against the action of MMTS. This indicates that barriers of different magnitude to the reaction of thiol reagents with the interface cysteine are induced by the events at the catalytic site. Studies with a Cys14Ser mutant of TbTIM confirmed that all the described effects of sulfhydryl reagents on the trypanosomal enzymes are a consequence of derivatization of the interface cysteine.

Triosephosphate isomerase (TIM)[1] has been the subject of numerous structural and mechanistic studies. The kinetics and the energetics of the TIM-catalyzed interconversion between glyceraldehyde 3-phosphate and dihydroxyacetone phosphate have been well established (*1*−*4*). The structures of TIMs from several species have also been unveiled by X-ray crystallography to a high level of resolution (*5*−*13*). The crystallographic data indicate that TIMs that range from thermophilic bacteria to human are dimers formed by two identical subunits. The monomers are formed by eight central β-strands and eight α-helices (numbered 1−8) joined by the corresponding loops, making TIM a member of the family of proteins with $\alpha-\beta$-barrel structures.

An intriguing characteristic of TIM is that the monomer is inactive; only the dimer is catalytically active (*14*−*16*). However, it has been shown that a dimer formed by an intact monomer and a monomer in which catalysis has been blocked by an inhibitor covalently bound to the catalytic site exhibits half of the activity of the intact dimer (*17*). It is also relevant that TIM monomers that have been engineered to have low association constants exhibit catalytic activities that are many times lower than those of the native dimers (*18*−*21*). These observations suggest either that monomer association induces the formation of monomers with high catalytic activity or that intersubunit contacts participate in catalysis. In principle, the latter alternative can be approached with a probe that monitors if during catalysis some of the intersubunit contacts exhibit conformational changes, or if one or more of the interface residues exhibits a property that differs from that in the resting state. We thought that the interface cysteine at position 14 of TIM from *Trypanosoma brucei* (TbTIM) and *Trypanosoma cruzi* (TcTIM) might be particularly useful to examine this possibility. In this work, the residues are numbered according to the aligned amino acid sequence of TbTIM; it is noted, however, that TcTIM has one residue more, that at position 2 (*22*).

† This work was supported in part by Dirección de Personal Académico de la Universidad Nacional Autónoma de México Grants IN206297 (to R.P.-M.) and IN208097 (to A.G.-P.).

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[1] Abbreviations: DTNB, 5,5-dithiobis(2-nitrobenzoate); MMTS, methylmethane thiosulfonate; PCR, polymerase chain reaction; TIM, triosephosphate isomerase; TbTIM and TcTIM, triosephosphate isomerase from *Trypanosoma brucei* and *Trypanosoma cruzi*, respectively.

10.1021/bi982425s CCC: $18.00
Published on Web 03/06/1999

The residue at position 14 in TbTIM is a nonconserved amino acid, but in all TIMs, it forms part of loop 1, which joins the first β-strand and first α-helix. X-ray structures of the TIMs that have been studied show that the side chain of this residue is submerged into loop 3 of the other subunit. The latter is also known as the interdigitating loop, since it fits into a crevice formed by loops 1 and 4 of the other subunit. The importance of residue 14 and loop 3 in dimer stability is well evidenced by data that show that the substitution by site-directed mutagenesis of cysteine 14 of TbTIM for a leucine (*23*), or methionine 14 of human TIM for an asparagine (*24*), yields enzymes with low stability. Along the same line, alterations of loop 3 gave monomers with low association constants (*18*, *21*, *25*).

There are two main reasons for carrying out these studies in TIMs from *Trypanosoma brucei* (TbTIM) and *Trypanosoma cruzi* (TcTIM). One is that cysteine 14 of each of the two monomers is the only cysteine that forms part of the dimer interface. The other is that incubation of the trypanosomal enzymes with sulfhydryl reagents brings about large structural changes and abolition of catalysis, which result from the derivatization of cysteine 14 by the sulfhydryl reagents (*26*). Therefore, we studied if catalysis and other events that occur at the catalytic site modify the susceptibility of the interface cysteine of the trypanosomal TIMs to thiol reagents. Affirmative data would be indicative of a transmission of structural changes from the catalytic site to the portion of the interface formed by cysteine 14 of one monomer and loop 3 of the other subunit. The results indicate that catalysis or the occupancy of the catalytic site by a substrate analogue does affect the derivatization of the cysteine by two sulfhydryl reagents, 5,5-dithiobis(2-nitrobenzoate) (DTNB) and methylmethane thiosulfonate (MMTS).

MATERIALS AND METHODS

Enzymes. Recombinant TIMs from *T. brucei* and *T. cruzi* were prepared as described elsewhere (*26*, *27*), and maintained as a suspension at 4 °C in 100 mM triethanolamine/10 mM EDTA/1 mM dithiothreitol/1 mM azide, pH 8.0, and 70% ammonium sulfate. For the experiments, the enzymes were dialyzed against 100 mM triethanolamine/10 mM EDTA, pH 7.4.

The Cys14Ser mutant of TbTIM was prepared by polymerase chain reaction (PCR) using the "Expand High Fidelity PCR System" (Boehringer). The mutagenic oligonucleotides were 5′-AACTGGAAGNNNAACGGCTCC-3′ and 3′-TTGACCTTCNNNTTGCCGAGG-5′ (where N = A/C/G/T). The PCR products were ligated to the pCR 2.1 vector (Invitrogen) and sequenced. Once the gene with the appropriate mutation was identified, it was sequenced completely, subcloned into pET3a, and introduced by transformation into BL21(DE3) pLys cells (Novagen). For expression of the Cys14Ser protein, cells were grown as described by Borchert et al. (*27*). The purification of the mutant enzyme was carried out following the methodology described for wild-type TbTIM (*27*).

Substrates. Glyceraldehyde 3-phosphate was prepared by adding 100 mg of glyceraldehyde 3-phosphate diethylacetal and 5 mL of water to 1.5 g of Dowex 50WX2-100 that had been washed with water. The mixture was placed in boiling water and stirred constantly with a spatula for 3 min. The mixture was cooled and filtered through a Millipore filter. The filtrate was lyophilized and the residue dissolved in 5 mL of 100 mM triethanolamine/10 mM EDTA, pH 7.4. The concentration of glyceraldehyde 3-phosphate was around 20 mM. Dihydroxyacetone phosphate was prepared from 25 mg of dihydroxyacetone phosphate dimethylketal dissolved in 2 mL of water. To this solution was added Dowex 50WX2-100 (0.5 g), and after stirring for about 1 min, the mixture was filtered through Millipore filters. The filtrate was incubated at 37 °C for 4 h and then lyophilized. The residue was dissolved in 2 mL of 100 mM triethanolamine/2 mM EDTA, pH 7.4. Before the experiments, the pHs of the two substrates were adjusted with NaOH to the pH of the buffer used in the experiments (100 mM triethanolamine/10 mM EDTA, pH 7.4). Through this procedure, acidification of incubation of mixtures that contained high concentrations of substrates (>1 mM) was prevented.

Effect of Sulfhydryl Reagents and Assay of Activity. The standard conditions for measuring the effect of sulfhydryl reagents on TbTIM and TcTIM were as follows. The enzymes were incubated at a concentration of 5 μg/mL in 0.5 or 0.25 mL of 100 mM triethanolamine/10 mM EDTA, pH 7.4, with the indicated concentrations of thiol reagents, substrate, or phosphoglycolate for 2 h (unless otherwise indicated) at 25 °C. At this time an aliquot was withdrawn and diluted with buffer in order to measure activity. Rate constants were evaluated by fitting the pseudo-first-order kinetic equation to the inactivation of TIMs by thiol reagents. In the absence of sulfhydryl reagents, the activity of the enzymes incubated with or without substrate or phosphoglycolate did not change. The experiments have been carried out at least 3 times.

Activity in the direction of glyceraldehyde 3-phosphate to dihydroxyacetone phosphate was measured at 25 °C as described elsewhere (*28*) with 1 mM substrate, α-glycerophosphate dehydrogenase, and NADH to trap dihydroxyacetone formed, and 5 or 7.5 ng of enzyme/mL of reaction mixture. In the direction of dihydroxyacetone phosphate to glyceraldehyde 3-phosphate, the K_m and V_{max} were determined at 25 °C in a mixture of 1 mL that contained 100 mM triethanolamine/10 mM EDTA, pH 7.4, 1 mM NAD, 4 mM arsenate, 120 μM dithiothreitol, 1 unit of glyceraldehyde-3-phosphate dehydrogenase, various concentrations of dihydroxyacetone phosphate, and 50 ng of TbTIM or TcTIM. Activity was calculated from the increase in absorbance at 340 nm as a function of time. It is pointed out that the enzymes for trapping the products of the reactions were in large excess, doubling the concentration of the trapping enzymes did not modify the activities observed.

Protein concentration of the enzymes was determined from their absorbance at 280 nm using the molecular extinction coefficients (ϵ_{280}) of 34 950 M^{-1} cm^{-1} for TbTIM and Cys14Ser TbTIM and 33 460 M^{-1} cm^{-1} for TcTIM (*22*).

RESULTS

Increasing concentrations of sulfhydryl reagents, such as DTNB and MMTS, induce a time- and concentration-dependent irreversible inhibition of the activities of TbTIM and TcTIM that results from derivatization of their interface cysteines (*26*). The inhibiting effect of DTNB and MMTS on TbTIM is shown in Figure 1 (A and B, respectively); the

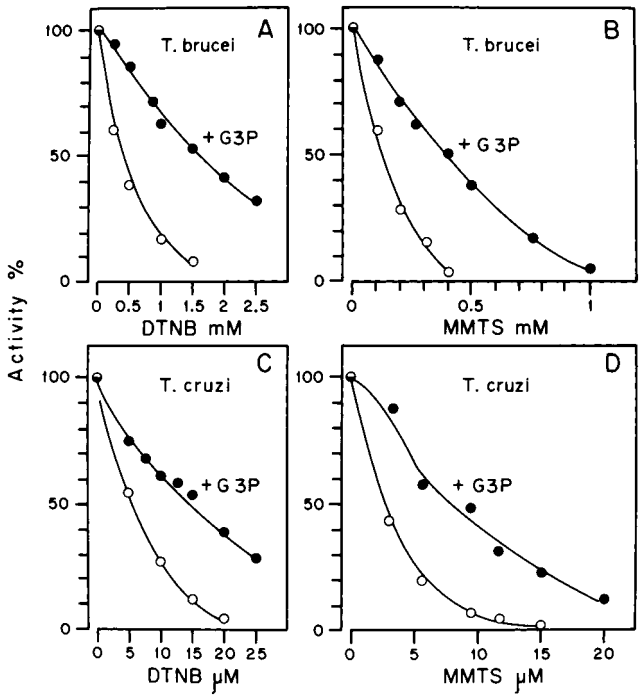

FIGURE 1: Effect of DTNB and MMTS on the activity of TbTIM and TcTIM incubated with and without glyceraldehyde 3-phosphate. TbTIM (A and B) and TcTIM (C and D) were incubated at a concentration of 5 μg/mL in a mixture of 100 mM triethanolamine and 10 mM EDTA, pH 7.4; the mixtures also included the indicated concentrations of DTNB (A and C) or MMTS (B and D), and 5 mM glyceraldehyde 3-phosphate where shown. After 2 h of incubation, an aliquot was withdrawn and diluted in order to measure activity; the latter was measured at a concentration of 7.5 ng/mL (see Materials and Methods). The results are expressed as percent of the activity in which 100% was 2900 and 3200 μmol min^{-1} mg^{-1} for TbTIM and TcTIM, respectively.

data also show that the inclusion of glyceraldehyde 3-phosphate in the reaction mixture induced partial protection against the inhibiting action of the two sulfhydryl reagents.

The activity of TcTIM is also inhibited by sulfhydryl reagents, but this enzyme is nearly 100 times more sensitive than TbTIM; previous data indicated that this is due to a higher accessibility of the interface cysteine of TcTIM to sulfhydryl reagents (*26*). Therefore, we explored if these two enzymes exhibit differences in the protective effect of substrate against thiol reagents. In the experiments with TcTIM, much lower concentrations of DTNB and MMTS (Figure 1C,D, respectively) were used in order to obtain an inhibition comparable to that observed in TbTIM. Notwithstanding the relatively high susceptibility of TcTIM to the thiol reagents, substrate also protected against their detrimental effect. It is noted that the extent of protection was similar to that observed in TbTIM.

Effect of Substrate on the Sensitivity of TbTIM and TcTIM to Sulfhydryl Reagents. The effect of different concentrations of added glyceraldehyde 3-phosphate on the inhibiting effect of MMTS and DTNB was determined in TbTIM and TcTIM (Table 1). As the concentration of added glyceraldehyde 3-phosphate was increased, the inhibition by the sulfhydryl reagents diminished. However, it is noted that in the presence of TIM, glyceraldehyde 3-phosphate rapidly reaches an equilibrium with dihydroxyacetone phosphate, the ratio of the two species in the media being about 1:20 in favor of the latter (*1*, *3*). In our experimental conditions, the equilibrium between glyceraldehyde 3-phosphate and dihydroxyacetone phosphate in the media was reached in seconds. Accordingly, the results reflect the effect of the two substrates at a ratio of 20 dihydroxyacetone phosphate per 1 glyceraldehyde 3-phosphate. In TbTIM and TcTIM, the respective K_ms for glyceraldehyde 3-phosphate are 0.6 and 0.8 mM, whereas the K_m for dihydroxyacetone phosphate in the two enzymes is 2 mM. Thus, the data of Table 1 suggest that maximal protection is induced when the enzymes are close to saturation with dihydroxyacetone phosphate.

Table 1: Effect of Different Concentrations of Added Glyceraldehyde 3-Phosphate on the Inhibition Produced by DTNB on TbTIM and TcTIM[a]

	% activity			
	T. brucei		*T. cruzi*	
added G3P (mM)	DTNB	MMTS	DTNB	MMTS
–	4	10	3	4
0.5	12	14	5	7
1	18	20	8	8
3	33	37	18	17
5	67	59	32	28
10	89	85	36	53

[a] The enzymes were incubated in the conditions of Figure 1. TbTIM was incubated with 1.5 mM DTNB or 0.4 mM MMTS, and TcTIM with 15 μM DTNB or 12 μM MMTS; these mixtures were supplemented with the indicated concentrations of glyceraldehyde 3-phosphate. At the end of 2 h, activity was determined. The results are expressed as percent of the activity of the controls in which 100% was the activity of TIMs incubated without the sulfhydryl reagents. Note that under the conditions of the experiment, glyceraldehyde 3-phosphate rapidly reached an equilibrium with dihydroxyacetone phosphate at a ratio of 20 dihydroxyacetone phosphates per 1 glyceraldehyde 3-phosphate (*1*, *3*). Therefore, the incubation media contained a mixture of the two substrates.

Studies with a Cys14Ser Mutant of TbTIM. Substrate protects against the action of sulfhydryl reagents in the trypanosomal enzymes (Figure 1). Thus, it may be asked if sulfhydryl reagents affect catalysis independently of their effect on cysteine 14. Previous studies on the inhibition of TbTIM and TcTIM by thiol reagents had been carried out in the resting enzymes (*26*); thus, there was the possibility that in the presence of substrate, thiol reagents derivatized other cysteines that exist in the trypanosomal enzymes (for amino acid sequence and cysteine composition of the two enzymes, see ref *22*). Therefore, we carried out studies with a mutant enzyme of TbTIM in which its cysteine 14 was changed to a serine by site-directed mutagenesis.

Cys14Ser TbTIM exhibited catalytic properties that were almost identical to those of the wild type; the K_m and V_{max} of the Cys14Ser TbTIM for glyceraldehyde 3-phosphate were 0.6 mM and 3780 μmol min^{-1} mg^{-1}, respectively, whereas in wild-type TbTIM, these values were 0.6 mM and 4170 μmol min^{-1} mg^{-1}. In the standard reaction mixture for measuring TIM activity with 1 mM glyceraldehyde 3-phosphate, 5 mM MMTS did not affect the activity of the mutant enzyme, albeit under the same conditions, wild-type TbTIM was inhibited by about 50% in 10 min. This indicates that (i) that the effect observed with MMTS in wild-type TbTIM in the presence of glyceraldehyde 3-phosphate is indeed due to its effect on its cysteine 14, and (ii) that MMTS does not compete with substrate for the catalytic site, although substrate does protect against the action of MMTS.

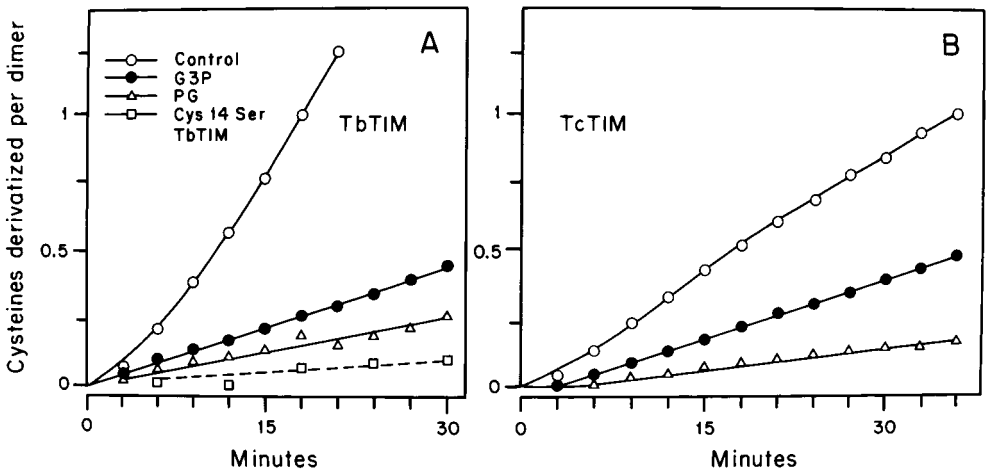

FIGURE 2: Effect of substrate and phosphoglycolate on nitrobenzoate formation during the incubation of DTNB with TbTIM (A), Cys14Ser TbTIM (A, open squares), and TcTIM (B). The enzymes (300 μg) were incubated at 25 °C in 1 mL of a mixture that contained 100 mM triethanolamine and 10 mM EDTA, pH 7.4. The mixtures also contained 1.5 mM DTNB for the experiments with TbTIM and Cys14Ser TbTIM, and 12 μM DTNB for those with TcTIM. In addition, 5 mM glyceraldehyde 3-phosphate or 5 mM phosphoglycolate was included as shown. Recording of the absorbance of the mixture at 412 nm was started immediately after the enzymes were added. The blank (no enzyme) that contained 1.5 mM DTNB exhibited a slight increase in absorbance at 412 nm; this was subtracted from the experimental values.

The effect of DTNB on Cys14Ser TbTIM was studied by incubating the enzyme in the conditions of Figure 1 with a concentration of DTNB (2 mM) that produced almost complete inhibition of the activity of wild-type TbTIM. DTNB diminished the activity of the mutant enzyme by 25%, thus confirming that the deleterious effect of sulfhydryl reagents in the trypanosomal enzymes is a consequence of derivatization of Cys 14. Further evidence for this conclusion is provided below (Figure 2).

Mechanism of Protection by Substrate against Thiol Reagents. The preceding data indicated that substrate protects against the action of sulfhydryl reagents on the interface cysteine of TbTIM and TcTIM. Since derivatization of the interface cysteine of TcTIM and TbTIM triggers irreversible structural alterations that lead to abolition of catalysis (*26*), the protective effect of substrate on the action of the thiol reagents could be consequence of hindrances in the reaction of sulfhydryl reagents with the interface cysteine, or to prevention of the structural alterations that are produced by derivatization.

Derivatization of cysteines with DTNB yields nitrobenzoate which has an absorption maximum at 412 nm (*29*). Therefore, the possibility that substrate hinders the reaction of sulfhydryl reagents with the interface cysteine was explored by recording the formation of nitrobenzoate during the incubation of TcTIM and TbTIM with DTNB with and without glyceraldehyde 3-phosphate. Recording was carried out for 30 min, since at longer times of incubation the derivatized enzymes undergo aggregation (more evident in TbTIM), and thus the increase in absorbance does not necessarily reflect formation of nitrobenzoate. In consonance with previous data (*26*), incubation of TbTIM and TcTIM with DTNB brought about a progressive increase in absorbance at 412 nm (Figure 2, panels A and B, respectively). However, the recordings also showed that with substrate, the rate of formation of nitrobenzoate was significantly diminished. Note that with TcTIM, a 100 times lower concentration of DTNB was used, and, thus, the rates of reduction of DTNB by the two enzymes cannot be compared. That the reduction of DTNB is indeed due to derivatization of the interface cysteine is illustrated by data that show that in Cys14Ser TbTIM hardly any reduction of DTNB took place (Figure 2A, open squares).

The results with DTNB are mechanistically relevant since they illustrate that the effect of substrate is to decrease the reaction between sulfhydryl reagents and the interface cysteine. This could result from a diminution in the influx of the thiol reagents to the interface cysteine, or from a conformational change that leads to a slowing of the reaction between the sulfhydryl reagents and the interface cysteine.

Effect of Phosphoglycolate and Other Compounds on the Action of DTNB and MMTS on TbTIM and TcTIM. Phosphoglycolate is a powerful competitive inhibitor in TbTIM; $K_i = 0.026$ mM (*20*). The crystal structure of TbTIM in complex with 2-phosphoglycolate (*19*, *30*) shows that it occupies the catalytic center of TIM in a form analogous to that of substrate. In confirmation of other reports, we observed that phosphoglycolate inhibited the activity of TbTIM; it also inhibited the activity of TcTIM. With 1 mM glyceraldehyde 3-phosphate as substrate, the inhibition curves in TbTIM and TcTIM by different concentrations of phosphoglycolate were markedly similar (Figure 3A). This is in agreement with the very similar crystallographic structure of the catalytic sites of the two trypanosomal TIMs (13). It was also observed that dilution of incubation mixtures that contained phosphoglycolate yielded enzymes that exhibited the activity of enzymes not treated with the inhibitor (not shown). Hence, in the two enzymes the action of phosphoglycolate is fully reversible.

Since TbTIM and TcTIM have similar catalytic properties, an identity of nearly 70% (*22*), and very similar X-ray structures (*13*), it may not be surprising that in the two enzymes substrate protects against thiol reagents by the same mechanism. However, since cysteine 14 is buried in the interface, and DTNB and MMTS have different dimensions and markedly different structure, it seems rather peculiar that the extent of protection by substrate against the two thiol reagents is also in the same range. In this regard, the results

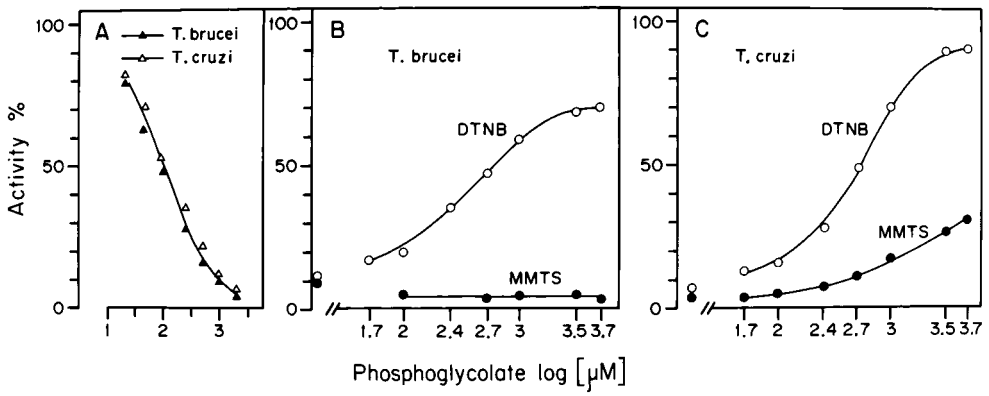

FIGURE 3: Effect of different concentrations of phosphoglycolate on the activity of TbTIM and TcTIM (A) and on the action of DTNB and MMTS on TbTIM (B) and TcTIM (C). In (A), the activities of TbTIM and TcTIM were measured with 1 mM glyceraldehyde 3-phosphate and the indicated concentrations of phosphoglycolate. In (B) and (C), the incubating conditions were as in Figure 1; with TbTIM, the incubation mixture contained 1.5 mM DTNB or 0.4 mM MMTS; with TcTIM, 15 μM DTNB or 12 μM MMTS. The mixtures also contained the indicated concentrations of phosphoglycolate. After 2 h, the activity of the enzymes was determined.

Table 2: Apparent Rate Constants (M^{-1} s^{-1}) for the Reaction of DTNB and MMTS with Cysteine 14 of TbTIM and TcTIM[a]

additions	*T. cruzi*	*T. brucei*
DTNB	24 ± 0.6	0.15 ± 0.01
DTNB + G3P	7.6 ± 0.7	0.03 ± 0.003
DTNB + PG	3.3 ± 0.3	0.03 ± 0.002
MMTS	40 ± 2.3	0.58 ± 0.03
MMTS + G3P	14.3 ± 1	0.15 ± 0.01
MMTS + PG	12.1 ± 1	0.85 ± 0.08

[a] The enzymes were incubated in the conditions of Figure 1 with DTNB or MMTS and 5 mM added glyceraldehyde 3-phosphate or 5 mM phosphoglycolate as indicated. TbTIM was incubated with 0.75, 1.5, and 3 mM DTNB, or 0.2, 0.4, and 0.8 mM MMTS. TcTIM was incubated with 7.5, 10, 15, 20, and 40 μM DTNB, or 5, 7.5, 10, and 15 μM MMTS. At various times, aliquots were withdrawn for measurements of activity. Plots of ln activity versus time were linear. The slopes were then plotted against DTNB or MMTS concentrations, and the apparent rate constants (±, standard error) for the reaction were calculated from these slopes.

of experiments on the effect of phosphoglycolate on the action of DTNB and MMTS on the two enzymes are relevant (Figure 3).

In TbTIM and TcTIM, phosphoglycolate protected against the action of DTNB (Figure 3, panels B and C, respectively). Protection by phosphoglycolate was higher in TcTIM, but in the two enzymes, the protection curves as a function of phosphoglycolate concentration were very similar, reaching a maximum at a concentration of about 1 mM phosphoglycolate. Phosphoglycolate also decreased the rate at which the interface cysteines of TbTIM and TcTIM were derivatized by DTNB (Figure 2A,B, triangles). Thus, phosphoglycolate, similarly to substrate, protected against the action of DTNB by slowing its reaction with the interface cysteine.

The results on the effect of phosphoglycolate on DTNB action differ drastically from those obtained with MMTS. Phosphoglycolate up to a concentration of 5 mM did not protect TbTIM against MMTS action (Figure 3B). In TcTIM, phosphoglycolate protected against MMTS action, but not to the extent observed with DTNB (Figure 3C).

The rate constants for the inactivation of the trypanosomal TIMs by DTNB and MMTS in the presence and absence of phosphoglycolate were determined and compared to those obtained with glyceraldehyde 3-phosphate (Table 2). In all cases, the plots of ln activity of TbTIM and TcTIM versus time with various concentrations of DTNB and MMTS were linear, obeying a pseudo-first-order reaction. The plots of the pseudo-first-order rate constants depended linearly on reagent concentration, indicating second-order kinetics. From the latter data, the apparent second-order rate constants (M^{-1} s^{-1}) were calculated (Table 2). In the two TIMs, substrate and phosphoglycolate decreased the rate constant of the reaction with DTNB. In TcTIM, phosphoglycolate also protected against MMTS action, but in TbTIM, no such effect was observed; in fact, phosphoglycolate induced a slight, but reproducible increase in the rate of reaction of MMTS. These results, together with those in Figure 3, are strongly suggestive that the species that occupies the catalytic site hinders the action of sulfhydryl reagents to different extents, and that these become apparent with thiol reagents of different size or chemical characteristics.

In the conditions of Figure 3, phosphoglycerate, phosphoenol pyruvate, and glucose 6-phosphate at a concentration of 5 mM did not protect against the action of DTNB and MMTS in TbTIM and TcTIM.

DISCUSSION

Many of the studies on the conformational changes that occur in TIM during catalysis have focused on the extensive movement of loop 6. Kinetic (*31*−*34*), crystallographic (*6*, *7*, *19*, *35*−*37*), and site-directed mutagenesis experiments (*38*) have shown that loop 6, which is far from the dimer interface, closes over the catalytic site in order to prevent formation of methylglyoxal from the enediol intermediate of the isomerization reaction. Here, we explored if events at the catalytic site and/or loop 6 are transmitted to the dimer interface by determining the susceptibility of the interface cysteine of TbTIM and TcTIM to thiol reagents.

TcTIM is far more susceptible to thiol reagents than TbTIM (*26*). The crystal structure of TcTIM shows that the separation of the dimer interface in the region between loop 1 of one monomer and helix 3 of the other monomer is larger in TcTIM than in TbTIM (*13* and see Figure 4). Hence, it is probable that the pathway for the influx of thiol reagents to cysteine 14 is through this region of the enzymes. There are, however, unexplained questions regarding the entrance of thiol reagents to the interface cysteine. Loop 3 of one subunit projects between loops 1 and 4 of the other subunit; in this

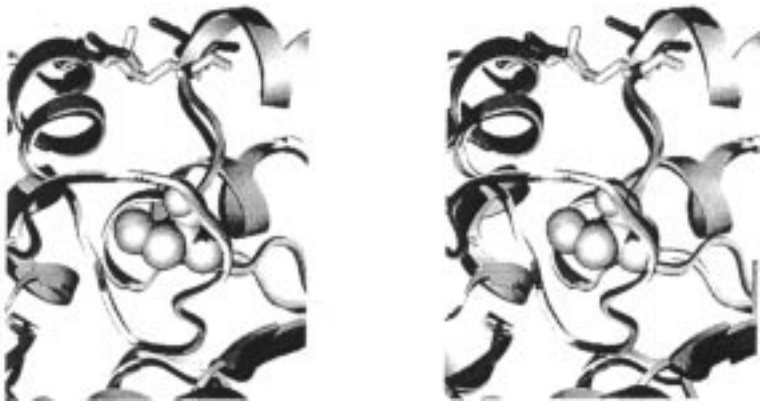

FIGURE 4: Stereoview of TbTIM (gray) and TcTIM (black) in the region of cysteine 14. The side chain of cysteine 14 of one monomer, displayed in CPK format, is enclosed by loop 3 of the other monomer; in this region no significant difference has been observed in the two TIMs. Also shown is the portion of the interface between loop 1 of one monomer (left side) and helix 3 of the other monomer (right side). The side chains of glutamine 19 of TbTIM and glutamic acid 19 of TcTIM after the end of loop 1 and at the beginning of helix 1 are depicted as sticks. Aspartic acids 86 of TbTIM and TcTIM, at the end of helix 3 and in front of residue 19, are also depicted as sticks. Note that in TbTIM the residues of the two monomers face each other, whereas in TcTIM the side chains point in opposite directions. Also note that the separation between monomers is significantly larger in TcTIM than in TbTIM.

Table 3: Atoms within 4 Å from the Sulfur of the Interface Cysteine of *T. Cruzi*

monomer A			monomer B		
atom	residue	distance	atom	residue	distance
Cp	14(A)	3.3	Cp	14(B)	3.3
Op	14(A)	3.1	Op	14(B)	3.2
CD	68(B)	4.0	CD	68(A)	3.9
CA	71(B)	4.0			
Cp	77(B)	3.9	Cp	77(A)	3.7
Op	77(B)	3.4	Op	77(A)	3.4
			Np	78(A)	3.9
CA	78(B)	3.9	CA	78(A)	3.8
C	78(B)	3.9	C	78(A)	3.8
Np	79(B)	3.4	Np	79(A)	3.3
CB	79(B)	3.7	CB	79(A)	3.7
			OG	79(A)	3.9
CD	82(B)	3.9	CD	82(A)	4.0

arrangement, the side chain of cysteine 14 (Figure 4), which is part of loop 1 (residues 14−19), becomes closely packed within the core of loop 3 (residues 66−79). Indeed, in the two monomers of TcTIM, there are 12 and 13 atoms at a distance of less than 4 Å from the sulfur of the interface cysteine 14 (Table 3). Given the structural arrangement of cysteine 14, the question that arises is how thiol reagents get across loop 3 and react with the sulfur of the interface cysteine. Furthermore, this work shows that catalysis and phosphoglycolate impose an additional barrier to the influx of thiol reagents to the interface cysteine. Thus, there is also the question of how catalysis or the occupancy of the catalytic center by phosphoglycolate affects its derivatization.

In this context, the work of Gracy and co-workers (*39*) on the deamidation of asparagines 15 and 71 of human and rabbit TIM is particularly relevant. It is known that asparagines adjacent to glycines may undergo a spontaneous reaction that leads to deamidation of asparagines (*40*). In human and rabbit TIM, asparagines 15 and 71 are followed by glycines, and Gracy and co-workers (*41*−*43*) showed that repeated catalytic cycles, or the occupancy of the catalytic site by a substrate analogue, confer to the enzyme a conformation prone for asparagine deamidation. In the resting state, the enzyme hardly undergoes deamidation. Other works showed that deamidation of the two asparagines does not occur at random; substrate-induced deamidation of asparagine 71 appears before deamidation of asparagine 15 (*41*, *43*).

Asparagine 71 forms part of loop 3; thus, the data of the latter investigators indicate that the occupancy of the catalytic site induces changes in the conformation of loop 3 making asparagine 71 prone to deamidation; indeed, one of their conclusions was that there are connections between the catalytic site and the dimer interface (*44*). Our data show that the occupancy of the catalytic site by substrate or an analogue has a strong influence on the access of thiol reagents to the cysteine that is entrapped in loop 3. Therefore, it is likely that both substrate-induced deamidation of asparagine 71 of loop 3 and resistance to agents that derivatize the interface cysteine, which is enclosed within loop 3, involve structural changes in the region of loop 3 and the enclosed cysteine.

As noted, loop 3 of one subunit docks between loops 1 and 4 of the other subunit. X-ray studies of TIM in which the association constants between monomers have been largely decreased through alteration of interface residues show that in monomeric TIM the orientations of the side chains of the active site lysine 13 and histidine 96 differ from those in wild-type TIM (*21*); the disposition of loop 4 is also different, and loop 1 is very mobile. In monomeric TIM, the binding of phosphoglycolate to the catalytic site induces the catalytic site residues to adopt the conformation of the wild type (*20*) and a tighter packing of loops 1 and 4. As a consequence, it is possible that in dimeric TIM the occupancy of the catalytic site enhances the packing of loops 1 and 4 with the interdigitating loop 3 of the other subunit, thus diminishing the interaction of cysteine 14 with sulfhydryl reagents.

Alternatively, it could be that events at the catalytic center change the geometry of cysteine 14 in relation to the residues in loop 3, and thereby confer a relatively high resistance to thiol reagents. Indeed, there are van der Waals contacts between loop 3 of one subunit and the catalytic lysine 13 of the other subunit. However, we examined the crystal structure of TIM with empty and filled catalytic sites, and failed to detect apparently important differences of intersubunit contacts in the region of loop 3 and the side chain of residue

14. This is in consonance with the data of Lolis and Petsko (*30*) in yeast TIM, who failed to detect significant differences between empty monomers and monomers filled with phosphoglycolate. This may imply that the entrance of thiol reagents to the interface cysteine depends on fluctuations that occur in this region of the enzyme, and that these are modulated by the state of the catalytic site. In fact, it has been reported that phosphoglycolate increases the thermostability of TIM (*20*).

Although at the moment it is not possible to define the precise mechanism through which catalysis or the occupancy of the catalytic site confers resistance to thiol reagents, the present data illustrate that the structural arrangements between loop 3 and the interface cysteine differ in enzymes with empty and filled catalytic sites. Moreover, it is also important that the experiments with phosphoglycolate indicated that there are gradations in the barrier to thiol reagents to the interface cysteine. For example, in TbTIM, phosphoglycolate effectively diminished derivatization by the relatively large and negatively charged DTNB, but it failed to protect against the action of the smaller and neutral MMTS (Figure 3B). Studies with thiol reagents of different chemical characteristics could perhaps yield more information on the structure and properties of the barrier, and also provide data for the design of agents that specifically perturb the interface cysteine of the trypanosomal enzymes.

ACKNOWLEDGMENT

We thank Dr. P. A. M. Michels (Research Unit for Tropical Diseases, ICP-TROP, Brussels, Belgium) for the TbTIM gene, and Dr. Juan P. Pardo for helpful discussions.

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BI982425S