Factors That Control the Reactivity of the Interface Cysteine of Triosephosphate Isomerase from *Trypanosoma brucei* and *Trypanosoma cruzi*†

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ABSTRACT: The amino acid sequences and X-ray structures of homodimeric triosephosphate isomerase from the pathogenic parasites *Trypanosoma brucei* (TbTIM) and *Trypanosoma cruzi* (TcTIM) are markedly similar. In the two TIMs, the side chain of the only interface cysteine (Cys14) of one subunit docks into loop 3 of the other subunit. This portion of the interface is also markedly similar in the two enzymes. Nonetheless, Cys14 of TcTIM is nearly 2 orders of magnitude more susceptible to the thiol reagent methylmethane thiosulfonate (MMTS) than Cys14 of TbTIM. The causes of this difference were explored by measuring the second-order rate constant of inactivation by MMTS (k_2) under various conditions. At pH 7.4, k_2 in TcTIM is 70 times higher than in TbTIM. The difference decreases to 30 when the amino acid sequence of loop 3 and adjoining residues of TbTIM are conferred to TcTIM (triple mutant). The pK_a values of the thiol group of the interface cysteine of TcTIM and the triple mutant were 0.7 pH unit lower than in TbTIM. Because this difference could account for the different sensitivity of the enzymes to thiol reagents, we determined the k_2 of inactivation at equal levels of ionization of their interface cysteines. Under these conditions, the difference in k_2 between TcTIM and TbTIM became 8-fold, whereas that of the triple mutant to TbTIM was 1.5 times. The substrate analogue phosphoglycolate did not modify the pK_a of the thiol group of the interface, albeit it diminished the rate of its derivatization by MMTS. In the presence of phosphoglycolate, under conditions in which the interface cysteines of the enzymes had equal levels of protonation, the difference in k_2 of TcTIM and TbTIM became smaller, whereas k_2 of the triple mutant was almost equal to that of TbTIM. Thus, from measurements of the reactivity of the interface cysteine in various conditions, it was possible to obtain information on the factors that control the dynamics of a portion of the dimer interface.

Triosephosphate isomerase (TIM)[1] is a glycolytic enzyme that catalyzes the interconversion between glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. The crystal structures of TIMs from a wide variety of species have been determined (*1*−*10*). Except for TIM from *Thermotoga maritima* which is a tetramer (*10*), all TIMs so far described are formed by two identical monomers which have a central core formed by β-strands surrounded by α-helices. The eight β-strands and α-helices are joined by loops numbered 1−8. The catalytic residues in each monomer belong to the same polypeptide chain; however, only in its dimeric form is TIM catalytically active (*11*−*13*). In this respect, it has been advanced that the association of the two monomers brings about the proper orientation of the residues (*14*−*18*). However, there are reports that suggest that the dimer interface plays a dynamic role in catalysis (*19*).

A large portion of the enzyme is formed by the dimer interface. A conspicuous region of the interface is formed by loop 3. The residues of loop 3 of each of the two subunits submerge into the other subunit. Another relevant feature of loop 3 is that, in each monomer, the residues of loop 3 surround the side chain of residue 14 of the other subunit (the numbering system of TIM from *Trypanosoma brucei* will be used). Site-directed mutagenesis studies of residue 14 or loop 3 (*16*−*18*, *20*) have shown that this portion of the interface is central in dimer stability. Likewise, it has been shown that chemical perturbation of Cys14 of TIMs from trypanosomatides provokes structural alterations and abolition of catalysis (*9*, *20*−*25*).

The portion of the interface formed by loop 3 and residue 14 has an additional interest. TIMs from the pathogenic parasites *Tryanosoma cruzi* (*23*), *Trypanosoma brucei* (*3*), *Leishmania mexicana* (*26*), *Plasmodium falciparum* (*7*), *Giardia lamblia* (*27*), and *Entamoeba histolytica* (*28*) possess a cysteine in an equivalent position. In TIMs from many other species, position 14 is occupied by a different residue; for example, in mammalian TIM, it is a methionine

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[1] Abbreviations: MMTS, methylmethane thiosulfonate; PCR, polymerase chain reaction; TIM, triosephosphate isomerase; IPTG, isopropyl β,D-thiogalactopyranoside; TbTIM and TcTIM, triosephosphate isomerase from *Trypanosoma brucei* and *Trypanosoma cruzi*, respectively.

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and in yeast a leucine. In addition, loop 3 of human TIM is one residue longer than those of TIMs from *T. brucei* (TbTIM), *T. cruzi* (TcTIM), and *L. mexicana*. Thus, this region of the interface appears to be a good target for the design of molecules that affect the association between monomers in TIM from parasites. This is of importance, since millions of people are infected by the aforementioned parasites.

The interfaces of TbTIM, TcTIM, and leishmanial TIM have one cysteine per monomer in the dimer interface, and its derivatization with thiol reagents causes irreversible structural alterations and total inactivation of the three enzymes (*24*). The thiol reagents that inactivate TIMs from trypanosomatids exert a small effect or have no effect at all on TIMs that lack a cysteine in position 14, the latter including human TIM (*5*). Therefore, these observations suggest that it is indeed possible to design molecules that perturb specifically the interface of TIMs from the trypanosomatids.

In studies on the inactivating effect of thiol reagents on TIMs from parasites, it was observed that the interface cysteine of TcTIM is about 2 orders of magnitude more sensitive to thiol reagents than that of TbTIM and *L. mexicana* (*23*, *24*). This is somewhat remarkable, since the amino acid sequences of the three TIMs have an identity of 68% and their X-ray structures are strikingly similar (*9*, *24*). The picture becomes more intriguing if it is considered that the identity of the residues that form the dimer interface is 82% and that the buried surface areas of the cysteines of TbTIM and TcTIM are almost identical (see Table 1 in ref *9*). The packing of the sulfur of Cys14 by the residues of loop 3 in TbTIM and TcTIM is also markedly similar.

Because of the importance of this portion of the interface in the design of molecules that induce destabilization of TIMs from parasites, we carried out a systematic analysis to ascertain why very similar enzymes exhibit such marked differences in regions that appear to be nearly identical. This involved a stepwise approach in which, initially, the amino acid sequence of TbTIM was conferred to TcTIM. This was followed by studies in which the reactivities of the thiol groups of the interface cysteines were compared at the same protonation state. The results illustrate that the local environment and events that are distant to the interface cysteine affect its reactivity with a thiol reagent. The data also show that as each of these factors is equalized, the difference in reactivity of the interface cysteine of TbTIM and TcTIM becomes progressively smaller.

MATERIAL AND METHODS

Enzymes. Recombinant TIM from *T. cruzi*, *L. mexicana*, *T. brucei*, and the Cys14Ser mutant of TbTIM were purified as described elsewhere (*24*, *25*, *29*). The mutant enzymes of TcTIM described below were purified following the methodology described for wild-type TcTIM. All enzymes were stored as suspensions at 4 °C in 100 mM triethanolamine, 10 mM EDTA, 1 mM dithiothreitol, 1 mM sodium azide, pH 8.0, and 70% saturation ammonium sulfate. For the experiments, the enzymes were dialyzed against 100 mM triethanolamine and 10 mM EDTA, pH 7.4.

The Glu18Gln, Gln81Pro, and Thr69Ala/Arg70Lys/Gln81Pro TcTIMs were prepared by polymerase chain reaction (PCR) using the Expand High Fidelity PCR System (Boehringer). The mutagenic oligonucleotides were 5′-CAACGGCTCCCAGAGTTTGCT-3′ (Glu18Gln Fw), 5′-AGCAAACTCTGGGAGCCGTTG-3′ (Glu18Gln Rv), 5′-CTCTCTGCCGATCCTCAAGG-3′ (Gln81Pro Fw), 5′-CCTTGAGGATCGGCAGAGAG-3′ (Gln81Pro Rv), 5′-GAACGCGATCGCSAARTCGGGCGCT-3′ (Thr69Ala/Arg70Lys Fw), and 5′-AGCGCCCGAYTTTSGCGATCGC-GTTC-3′ (Thr69Ala/Arg70Lys Rv) (where R = G/A, S = G/C, Y = C/T). The PCR products were ligated to the pCR 2.1 vector (Invitrogen) and sequenced. Once the genes with the appropriate mutations were identified, they were sequenced completely, subcloned into pET3a, and introduced by transformation into BL21(DE3)pLys cells (Novagen). For expression of the mutant enzymes, cells were grown as described by Borchert et al. and induced with IPTG (*29*).

Assay of Activity. Activity in the direction of glyceraldehyde 3-phosphate to dihydroxyacetone phosphate was measured at 25 °C as described elsewhere (*21*). The 1-mL reaction mixture at pH 7.4 contained 100 mM triethanolamine, 10 mM EDTA, 1 mM substrate (except when the catalytic constants were determined), 0.9 U α-glycerophosphate dehydrogenase, 0.2 mM NADH, and 5 ng of TIM. Activity was calculated from the decrease in absorbance at 340 nm.

Effect of MMTS. The standard conditions for measuring the rate of chemical modification by MMTS were as follows. The enzymes were incubated at 25 °C at a concentration of 5 μg/mL of 100 mM triethanolamine and 10 mM EDTA that had the indicated pH and MMTS concentrations. Aliquots were withdrawn periodically and diluted with the same buffer that had pH 7.4 in order to measure the residual activity. The pseudo-first-order k_1 was determined from plots of percent of activity versus time. The second-order kinetic constant k_2 was then calculated from plots of the first-order rate constant versus MMTS concentration.

Determination of the pK_a of the Interface Cysteines. To determine the pK_a of the cysteine, the enzymes were incubated at a concentration of 5 μg/mL in 100 mM triethanolamine and 10 mM EDTA adjusted to the desired pH; MMTS at the concentrations indicated under Results was also included. The ionic strength of mixtures was maintained constant by inclusion of the appropriate amounts of NaCl. At different times aliquots were withdrawn, diluted with buffer pH 7.4 and activity measured as described above. The apparent pK_a of the interface of the various TIMs was determined from plots of ln of percent remaining activity versus pH. Data were fitted to a model derived from the Henderson−Hasselbalch equation:

$$\ln(\%\ \text{activity}) = \frac{Y_h + Y_l \times 10^{pK_a - pH}}{1 + 10^{pK_a - pH}} \qquad (1)$$

where Y_h and Y_l represent the initial and final activities, respectively.

In the experiments the incubation of MMTS and the indicated TIMs was less than 1 min. This was because at relatively alkaline pH, MMTS undergoes spontaneous decomposition. In fact, at pH 9.7, the highest pH assayed, the decomposition of MMTS is about 60% in 1 min; its rate constant of decomposition is 44 M^{-1} s^{-1}. Thus, at this

Table 1: Kinetic Constants of Wild-Type and Mutant TcTIMs[a]

TIM	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} mM^{-1})
TcTIM	0.48 ± 0.01	2.6×10^5	5.4×10^5
E18Q TcTIM	0.62 ± 0.06	1.8×10^5	2.9×10^5
Q81P TcTIM	0.72 ± 0.05	2.9×10^5	4×10^5
triple mutant of TcTIM	0.56 ± 0.05	2.2×10^5	3.9×10^5

[a] Activity was determined with various concentrations of glyceraldehyde 3-phosphate (0.05−4 mM) as described under Materials and Methods. K_m (±SEM) and k_{cat} were calculated from nonlinear regression plots.

particular pH, the rate constant of inactivation of TbTIM (see data in Figure 2) was calculated taking into account the rate constant of decomposition of MMTS as described by Gomi and Fujioka (*38*). In the rest of the experimental points, the correction was not made, since the MMTS breakdown was less than 6% in 1 min. In addition, it is noted that at pH 10, the enzymes undergo rapid inactivation. This precluded the determination of the effect of MMTS at pH 10 or higher. At pH 9.7 the enzymes were stable for at least 10 min.

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 (*25*), 33 460 M^{-1} cm^{-1} for TcTIM and mutant TcTIM, and 36 440 M^{-1} cm^{-1} for TIM from *L. mexicana* (*23*).

RESULTS

Effect of MMTS on the Glu18Gln Mutant of TcTIM. Although the structures of TbTIM and TcTIM are markedly similar, the enzymes differ in the portion of the interface formed by loop 1 of one monomer and helix 3 of the other subunit (*9*). At the end of loop 1, TbTIM has glutamine in position 18. Its amide group is H-bonded to the peptide oxygen between Asp85 and Phe86 of helix 3 of the other subunit. At variance with TbTIM, TcTIM has glutamic acid in position 18 and its side chain points toward the solvent. Thus, in the respective three-dimensional structures this portion of the interface is more open in TcTIM than in TbTIM. To test if this difference is related to the accessibility of thiol reagents to Cys14, we constructed a mutant of TcTIM in which its glutamic acid was exchanged for a glutamine. The enzyme was purified and characterized. The K_m of the mutant enzyme for glyceraldehyde 3-phosphate was similar to that of wild-type TcTIM, its k_{cat} was about 30% lower (Table 1). The second-order rate constant of the inhibition (k_2) induced by MMTS was determined from plots of the pseudo-first-order rate constants (k_1) at various concentrations of MMTS and compared to those in TcTIM and TbTIM (Table 2). The mutant enzyme exhibited a slightly higher sensitivity to MMTS. Hence, it would appear that the existence of a glutamic acid or glutamine in position 18 does not account for the marked differences in sensitivity to thiol reagents between TbTIM and TcTIM.

Properties of the Gln81Pro Mutant of TcTIM and a Mutant of TcTIM with Its Loop 3 Identical to That of TbTIM. TIM from *L. mexicana* also has a cysteine in position 14 and its side chain is surrounded by the residues of loop 3 of the other subunit (*30*). The derivatization of this cysteine by MMTS or other thiol reagents causes its inactivation (*24*).

Table 2: Second-Order Rate Constants for the Reaction of MMTS with the Interface Cysteine of Various TIMs at pH 7.4[a]

TIM	k_2 (M^{-1} s^{-1})
TbTIM	0.6 ± 0.05[b]
TcTIM	40 ± 2[b]
E18Q TcTIM	90 ± 3.6
Q81P	32 ± 2
TcTIM triple mutant	18 ± 3
L. mexicana	0.16 ± 0.005

[a] The enzymes were incubated in the standard conditions at pH 7.4 with MMTS, and at various times aliquots were withdrawn, diluted and their activity measured. From the data, pseudo-first-order rates constants were determined from plots of percent activity versus time fitted by nonlinear regression analysis. The second-order rate constants k_2 (±SEM) for the reaction were calculated as described under Materials and Methods. The concentrations of MMTS used with the various TIMs were: for E18Q TcTIM, 20, 30, 40, and 50 μM MMTS; for Q81P TcTIM, 30, 40, and 50 μM MMTS; for the triple mutant of TcTIM, 5, 10, 15, and 20 μM MMTS; for TIM from *L. mexicana*, 0.4, 0.8, 1.2, and 1.6 mM MMTS. [b] Data from Pérez-Montfort et al. (*25*).

At pH 7.4, the k_2 of inactivation by MMTS of leishmanial TIM was about 3 times lower than in TbTIM and about 250 times lower than in TcTIM (Table 2). To understand the causes of the significantly higher sensitivity of TcTIM when compared to TbTIM and leishmanial TIM, their amino acid sequences were examined, particularly in loop 3 and in the neighboring residues (Figure 1). At the end of loop 3, TbTIM and leishmanial TIM have proline in position 81, the corresponding amino acid in TcTIM is glutamine. Another relevant feature of the sequences is that the amino acid compositions of loop 3 of TbTIM and leishmanial TIM are identical and differ from TcTIM in residues 69 and 70. The former two TIMs have alanine and lysine in these positions, whereas in TcTIM the residues are threonine and arginine, respectively.

To determine if these differences account for the high susceptibility of TcTIM to thiol reagents, we prepared two mutants of TcTIM. In one of them, Gln81 was exchanged for proline. This Gln81Pro TcTIM was used to construct another mutant in which its Thr69 and Arg70 were replaced by the residues that exist in TbTIM and leishmanial TIM. This Thr69Ala/Arg70Lys/Gln81Pro TcTIM will be referred throughout as the triple mutant. The enzymes were purified to homogeneity and characterized. The kinetics of these two mutants did not differ significantly from those of wild-type TcTIM (Table 1). However, in Gln81Pro TcTIM, the second-order rate constant of inhibition by MMTS was about 30% lower than in the wild-type TcTIM (Table 2). In the triple mutant, the second-order rate constant of inactivation by MMTS was even lower (Table 2). These findings indicate that the amino acid sequences of loop 3 and adjacent residues contribute partially to the susceptibility of the interface cysteine to thiol reagents. Nonetheless, it is somewhat remarkable that the triple mutant, which has loop 3 identical to that of TbTIM and leishmanial TIM, has a sensitivity to MMTS that is 31 and 114 times higher than that of TbTIM and leishmanial TIM, respectively.

Ionization of the Interface Cysteine of TbTIM and TcTIM. The X-ray structures of TcTIM and TbTIM show that their interface cysteines are not exposed to the solvent (*9*). This is in accord with data that indicate that the derivatization of the cysteine is relatively slow (*20*, *25*). Since the transfer of ionized groups from the solvent to the interior of proteins is

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                                    *                                      60
T. brucei    -MSKPQPIAA ANWKCNGSQQ SLSELIDLFN STSINHDVQC VVASTFVHLA MTKERLSHPK
T. cruzi     MASKPQPIAA ANWKCNGSES LLVPLIETLN AATFDHDVQC VVAPTFLHIP MTKARLTNPK
L. mexicana MSAKPQPIAA ANWKCNGTTA SIEKLVQVFN EHTISHDVQC VVAPTFVHIP LVQAKLRNPK

                      * *          *                                      120
T. brucei    FVIAAQNAIA KSGAFTGEVS LPILKDFGVN WIVLGHSERR AYYGETNEIV ADKVAAAVAS
T. cruzi     FQIAAQNAIT RSGAFTGEVS LQILKDYGIS WVVLGHSERR LYYGETNEIV AEKVAQACAA
L. mexicana YVISAENAIA KSGAFTGEVS MPILKDIGVH WVILGHSERR TYYGETDEIV AQKVSEACKQ

                                                                            180
T. brucei    GFMVIACIGE TLQERESGRT AVVVLTQIAA IAKKLKKADW AKVVIAYEPV WAIGTGKVAT
T. cruzi     GFHVIVCVGE TNEEREAGRT AAVVLTQLAA VAQKLSKEAW SRVVIAYEPV WAIGTGKVAT
L. mexicana GFMVIACIGE TLQQREANQT AKVVLSQTSA IAAKLTKDAW NQVVLAYEPV WAIGTGKVAT

                                                                            240
T. cruzi     PQQAQEVHEL LRRWVRSKLG TDIAAQLRIL YGGSVTAKNA RTLYQMRDIN GFLVGGASLK
T. brucei    PQQAQEAHAL IRSWVSSKIG ADVRGELRIL YGGSVNGKNA RTLYQQRDVN GFLVGGASLK
L. mexicana PEQAQEVHLL LRKWVSENIG TDVAAKLRIL YGGSVNAANA ATLYAKPDIN GFLVGGASLK

                   250
T. cruzi     PEFVEIIEAT K
T. brucei    PEFVDIIKAT Q
L. mexicana PEFRDIIDAT R
```

FIGURE 1: Sequence alignment of TbTIM, TcTIM, and *L. mexicana* TIM. The residues that form loop 3 are in bold. The residues of TcTIM that were substituted are marked with an asterisk (*).

energetically unfavorable, buried acidic groups exhibit a relatively high pK_a (*31*, *32*). It is also well-documented that sulfhydryl agents react almost exclusively with the thiolate form of cysteine (*33*−*37*). To explore if the different sensitivity of TbTIM and TcTIM to sulfhydryl reagents is related to differences in the protonation level of the interface cysteines, the apparent pK_a of their thiol groups was determined. TbTIM and TcTIM were incubated with MMTS at various pH, and aliquots were withdrawn to measure the remaining activity under standard conditions at pH 7.4. The results showed that as the pH of the media was increased, the inactivating effect of MMTS increased (Figure 2). From plots of the remaining activity at various pH, the pK_a of the interface cysteines was calculated (Table 3). Regarding the result at pH 9.7, it is noted that at this pH, there is an important spontaneous decay of MMTS with a rate constant of 44 M^{-1} s^{-1}. Therefore, the rate of inactivation of TbTIM was calculated taking into account the rate of decomposition of MMTS (see ref *38* and Materials and Methods). The correction was not made for the rest of the points in Figure 2, because the loss of MMTS was less than 6%, in the course of the experiments.

The experiments on the effect of MMTS at various pH included another control. In addition to the interface cysteine, TbTIM has cysteines at positions 40 and 126, and TcTIM has three at positions 40, 117, and 126. Although at pH 7.4, inactivation of the two enzymes by MMTS results from the initial derivatization of their interface cysteine (*25*), there was the possibility that at relatively high pH, inactivation by MMTS was due to derivatization of cysteines, other than Cys14. Therefore, we determined the effect of MMTS on

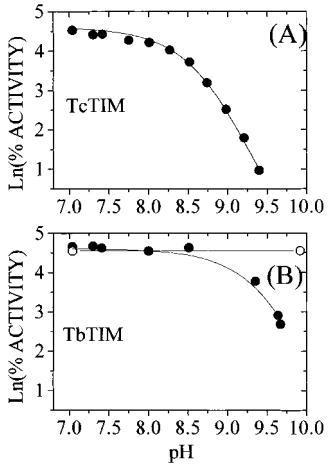

FIGURE 2: pH dependence of the effect of MMTS on the activity of (A) TcTIM and (B) wild-type TbTIM (●) and Cys14Ser TbTIM (○). The enzymes were incubated at a concentration of 5 μg/mL in a mixture of 100 mM triethanolamine and 10 mM EDTA at the indicated pH. The concentration of MMTS used was 10 μM (A) and 80 μM (B). After 1 min of incubation, an aliquot was withdrawn and diluted in order to measure activity; the latter was measured at a concentration of 5 ng/mL (see Materials and Methods). The results are expressed as ln(% activity) in which 100% was 3000, 2900, and 3000 μmol min^{-1} mg^{-1} for TcTIM, TbTIM, and Cys14Ser mutant TbTIM, respectively. The lines are fitted according to eq 1.

the activity of a mutant of TbTIM in which its interface cysteine was replaced by serine. At all pH, the latter mutant was insensitive to MMTS (Figure 2B). Thus, inactivation by MMTS of trypanosomal TIMs is due to alkylation of their interface cysteine.

Table 3: Apparent pK_a of the Interface Cysteine of Wild-Type TcTIM and Its Triple Mutant and TbTIM in the Absence (−) or Presence (+) of Phosphoglycolate[a]

TIM	− phosphoglycolate	+ phosphoglycolate
TcTIM	9.28 ± 0.07	9.33 ± 0.02
TcTIM triple mutant	9.33 ± 0.04	9.44 ± 0.03
TbTIM	10.08 ± 0.03	10.1 ± 0.04

[a] The pK_a were calculated as described under Materials and Methods. For calculation of the pK_a of the interface cysteine of TcTIM and TbTIM in the absence of phosphoglycolate the data of Figure 2 were used. The same protocol with 10 μM MMTS was used for determination of the pK_a of the interface cysteine in the triple mutant of TcTIM. Where indicated, the pK_a was calculated with 5 mM phosphoglycolate and MMTS at a concentration of 5, 10, and 30 μM for TcTIM, the triple mutant, and TbTIM, respectively.

Table 4: Second-Order Rate Constants for the Reaction of the Interface Cysteine with MMTS When 33% of the Interface Cysteines Were in the Thiolate Form: Effect of Phosphoglycolate[a]

	k_2 (M^{-1} s^{-1})	
TIM	− phosphoglycolate	+ phosphoglycolate
TcTIM	3380 ± 212	1695 ± 18
TcTIM triple mutant	654 ± 30	218 ± 23
TbTIM	420 ± 16	266 ± 2

[a] The experimental conditions and calculation of the rate constant for inactivation (±SEM) were as in Table 2, except that the pH of the incubation mixtures was 9.0 for TcTIM and its triple mutant and 9.7 for TbTIM. For the experiments the following concentrations of MMTS were used: TcTIM without phosphoglycolate, 4, 6, 8, and 10 μM; TcTIM with phosphoglycolate, 8, 12, 16, and 20 μM; triple mutant without phosphoglycolate, 6, 8, 10, and 12 μM; triple mutant with phosphoglycolate, 8, 12, 16, and 20 μM; TbTIM without phosphoglycolate, 25, 50, 75, and 100 μM; TbTIM with phosphoglycolate, 37, 75, 112, and 150 μM.

The pK_a of the thiol group of Cys14 of TbTIM and TcTIM was calculated (Table 3) and was higher in both cases than the reported value for cysteine in aqueous solutions, which is 8.5−8.8. The pK_a of the interface cysteine of TcTIM was about 0.7 unit lower than that of TbTIM.

The pK_a of cysteines may be affected by the properties of neighboring residues, in particular lysines and arginines (*39*). As there are differences in the amino acid sequence of loop 3 of TcTIM and TbTIM, we also determined the pK_a of the interface cysteine of the triple mutant. Its pK_a did not differ significantly from that of the wild-type TcTIM (Table 3).

Inactivation Rate Constant by MMTS of TbTIM, TcTIM, and the Triple Mutant at the Same Ionization Level of the Thiol Groups of Their Interface Cysteine. The lower pK_a of the interface cysteine of TcTIM in comparison to that of TbTIM could account for its higher sensitivity to thiol reagents in media with a pH lower than the pK_a of the cysteine. For example, at pH 7.4, k_2 is about 70 times larger in TcTIM than in TbTIM (Table 2). If the latter difference were solely due to the differences in the pK_a of their cysteines, it would be expected that the three enzymes would exhibit the same inactivation rate constant at equal ratios of thiol to thiolate groups. Accordingly, the rate constant of inactivation by MMTS in the three enzymes was determined in media that had a pH in which 33% of the interface cysteines were in the thiolate form. Under these conditions, the k_2 for MMTS in TcTIM was still significantly higher than in TbTIM (Table 4). In the triple mutant, however, the k_2 of the reaction was only slightly higher than in TbTIM.

Effect of Phosphoglycolate on the Action of MMTS on TcTIM and TbTIM. The latter results indicate that differences in the ionization level of the interface cysteine *and* the amino acid sequence of loop 3 are central in the sensitivity of trypanosomal TIMs to thiol reagents. However, it has been shown (*25*) that the occupancy of the catalytic site by the substrate analogue phosphoglycolate affects the reactivity of the interface cysteine with thiol reagents. Therefore, we examined if this effect of phosphoglycolate is accompanied by changes in the pK_a of the interface cysteine. The results showed that in the three enzymes, phosphoglycolate did not modify the pK_a of interface cysteines (Table 3). We also determined k_2 in the presence of phosphoglycolate under conditions in which the interface cysteines of the three enzymes had the same ionization level. Phosphoglycolate diminished the k_2 of the inactivation reaction by MMTS in the three enzymes (Table 4). However, in wild-type TcTIM and its triple mutant, the decrease of k_2 induced by phosphoglycolate was about 2 times, whereas in TbTIM, the decrease was rather modest. In consequence, the difference in rate constants of inactivation between TcTIM and its triple mutant with TbTIM became smaller. In fact, k_2 in the triple mutant of TcTIM was slightly lower than in TbTIM.

DISCUSSION

TIMs from *T. cruzi* and *T. brucei* are markedly similar in amino acid sequence and three-dimensional structure. Nonetheless, TcTIM is many times more sensitive to agents that derivatize the interface cysteine (*9*, *22*−*25*). The purpose of this work was to determine the cause(s) of these differences. Because in the trypanosomal TIM the side chain of the interface cysteine is closely packed by the amino acids of loop 3 of the other monomer, the studies could also provide information on the factors that control the interactions between subunits in that region of the dimer interface. In this respect it is noted that the K_{diss} of TIM monomers has been reported to range between 10^{-13} and 10^{-14} M (*2*, *16*). In our experiments on the effect of MMTS on TIM, the concentration of the enzyme was 0.1 μM; thus, the effect of the thiol reagent was determined under conditions in which TIM was in its dimeric form.

Figure 3 summarizes part of the results of this work. It shows the ratios of the second-order rate constant of inactivation by MMTS (k_2) of TcTIM and that of the triple mutant to the k_2 in TbTIM. The difference in the ratios in the various experimental conditions can be used to estimate the extent to which each of the explored factors contributes to the reactivity of the cysteine. At pH 7.4, the ratio in the sensitivity of TcTIM to TbTIM to MMTS is around 70. At this pH, the ratio decreases to about 30 when the residues of loop 3 of TcTIM are made identical to those of TbTIM. Because the pK_a of the cysteines of the triple mutant and the wild-type TcTIM are almost the same (Table 3), the decrease from 70 to 30 reflects mainly the contribution of the residues in the vicinity of the sulfur of the interface cysteine.

When the sensitivities of the enzymes to MMTS are compared at equal levels of protonation of their interface cysteine, the ratios of k_2 of wild-type TcTIM to TbTIM drops from 70 to 8 and that of the triple mutant to TbTIM from 30 to 1.5. The fact that at equal levels of ionization of the

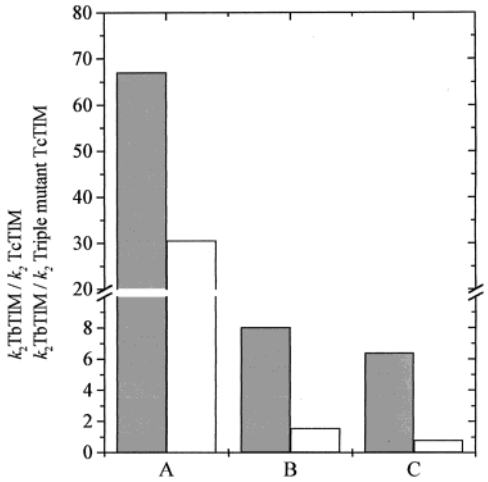

FIGURE 3: Ratio of the second-order rate constant (k_2) of inactivation by MMTS between TcTIM:TbTIM (closed bars) and the triple mutant of TcTIM:TbTIM (open bars). The ratios were calculated from the data in Tables 2 and 4: (A) ratios at pH 7.4; (B) ratios in conditions in which 33% of the interface cysteine is in thiolate form; (C) the latter conditions in the presence of 5 mM phosphoglycolate.

thiol groups the ratios are lower than at pH 7.4 indicates that at the latter pH, the hindrances imposed by the ionization level of the cysteine are higher in TbTIM than in TcTIM and its triple mutant.

It has been shown that the occupancy of the catalytic site by phosphoglycolate affects the reactivity of the interface cysteine to MMTS (*25*). With respect to this action of phosphoglycolate, it is mechanistically important that although phosphoglycolate does not modify the pK_a of the interface cysteine (Table 3), it nonetheless diminishes the k_2 of inactivation in the three enzymes. It is also relevant that at equal levels of ionization of the interface cysteine, phosphoglycolate diminished the difference of k_2 between TcTIM and TbTIM and that in these conditions, k_2 in the triple mutant became lower than that of TbTIM, albeit slightly. The changes in the k_2 ratios induced by phosphoglycolate also illustrate that in very similar enzymes, the occupancy of the catalytic site does not affect to the same extent the reactivity of the cysteine. In summary, the overall data show that the reactivity of the interface cysteine is controlled by several factors: i.e., the structure of loop 3, the pK_a of the cysteine, and the occupancy of the catalytic site. In addition, the data show that the difference between the two trypanosomal TIMs is a consequence of differences in local factors and events or interactions that are far from the interface residue.

From a broader point of view, there are two additional comments. This work illustrates that by probing a property of a particular amino acid residue under different conditions, it is possible to ascertain the factors that govern the behavior of the residue. The other point concerns the properties of regions of enzymes with identical amino acid sequences. In his classical work, Anfinsen (*40*) showed that the structure of proteins is determined by their amino acid sequence. Nonetheless, it has been observed that a protein may acquire a drastically different structure (*41*) depending on the nature of the solvent. Likewise, in an analysis of 5420 proteins, Sudarsanan (*42*) found that the same amino acid sequence could exist in several different structural conformations. In this context, the characteristics of the triple mutant of TcTIM and TbTIM illustrate an aspect of the relationship between structure and function that, as far as we know, has not been described before. We found that two enzymes with identical amino acid compositions in the region that surrounds the interface cysteine exhibit significant differences in the pK_a of the cysteine. This suggests that in proteins, the same amino acid sequence in homologous enzymes may exhibit distinct characteristics that, apparently, are the consequence of distant events or interactions.

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