Control of the Reactivation Kinetics of Homodimeric Triosephosphate Isomerase from Unfolded Monomers†

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ABSTRACT: Homodimeric triosephosphate isomerases from *Trypanosoma cruzi* (TcTIM) and *Trypanosoma brucei* (TbTIM) have markedly similar catalytic properties and 3-D structures; their overall amino acid sequence identity is 68% and 85% in their interface residues. Nonetheless, active dimer formation from guanidinium chloride unfolded monomers is faster and more efficient in TcTIM than in TbTIM. The enzymes thus provide a unique opportunity for exploring the factors that control the formation of active dimers. The kinetics of reactivation at different protein concentrations showed that the process involved three reactions: monomer folding, association of folded monomers, and a transition from inactive to active dimers. The rate constants of the reactions indicated that, at relatively low protein concentrations, the rate-limiting step of reactivation was the association reaction; at high protein concentrations the transition of inactive to active dimers was rate limiting. The rates of the latter two reactions were higher in TcTIM than in TbTIM. Studies with a mutant of TcTIM that had the interface residues of TbTIM showed that the association rate constant was similar to that of TbTIM. However, the rate of the transition from inactive to active dimers was close to that of TcTIM; thus, this transition depends on the noninterfacial portion of the enzymes. When unfolded monomers of TcTIM and TbTIM were allowed to reactivate together, TcTIM, the hybrid, and TbTIM were formed in a proportion of 1:0.9:0.2. This distribution suggests that, in the hybrid, the characteristics of the TcTIM monomers influence the properties of TbTIM monomers.

Triosephosphate isomerase (TIM) is a ubiquitous enzyme that catalyzes the interconversion of glyceraldehyde 3-phosphate and dihydroacetone phosphate by well-known mechanisms (*1*, *2*). Most TIMs so far described are formed by two identical subunits of about 27 kDa each; in *Pyroccoccus woesi*, TIM is a tetramer (*3*), and in *Thermotoga maritima*, it is part of a fusion product with phosphoglycerate kinase (*4*). All TIMs belong to the family of α/β barrel proteins in which each subunit has eight central β strands surrounded by eight α helices. The strands and helices are joined by loops. A large part of the enzyme is formed by the interface; for example, in the enzymes from *Trypanosoma cruzi* (TcTIM)[1] and *Trypanosoma brucei* (TbTIM) the buried surfaces areas in TcTIM are about 1490 and 1530 Å^2, respectively (*5*, *6*). An important characteristic of TIM is that only the dimers are catalytically active, even though each monomer has the residues that participate in catalysis. Using the numbering of TcTIM, the catalytic residues are Lys 14, His 96, and Glu 168. Lys 14 is next to Cys 15, a residue that is buried within the dimer interface. His 96 is close to Thr 76 of the other subunit, and Glu 168 is part of the mobile loop 6 which closes over the catalytic site.

The denaturation pathway of TIM from several organisms has also been described (*7*−*13*). Some of the works show that denaturation is a three-state process (*7*, *8*, *12*, *13*). First, the dimers dissociate into catalytically inert monomers that have substantial tertiary structure; this state is followed by unfolding of the monomers. The reactivation of TIM from guanidinium chloride (GdnHCl) unfolded monomers has also been the subject of several studies (*14*−*18*). The data show that removal or dilution of the denaturant leads to monomer folding; subsequently, the folded monomers associate to yield the active dimers. As reviewed by Jaenicke and Lilie (*19*), the reactivation of many other oligomeric proteins exhibits a similar reaction sequence. However, in the overall data it is evident that the intimate molecular events that control the rate and extent of formation of oligomers or the pathway into incorrect structures are not entirely known. This is particularly true with respect to the contribution of the

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[1] Abbreviations: EDTA, ethylenediaminetetraacetate; GdnHCl, guanidinium chloride; TbTIM, triosephosphate isomerase from *Trypanosoma brucei*; TcTIM, triosephosphate isomerase from *T. cruzi*.

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noninterfacial portions of the protein to oligomerization or to the side reactions.

Some years ago, we found that the reactivation of TIMs of different species from their unfolded monomers exhibits important differences (*17*). This seemed peculiar because all TIMs have the same basic structure, and the level of amino acid conservation between some TIMs is rather high. One of the most notable differences was between TcTIM and TbTIM. The two enzymes exhibit similar catalytic properties, the Cα traces in their crystal structures superpose with an rms of 0.96 Å (*5*), and their overall amino acid identity is 68% and 85% in their interface residues. Nonetheless, the reactivation of TcTIM was more efficient than in TbTIM. Thus, we reasoned that a study of the kinetics of active dimer formation of these two very similar enzymes could shed light into the mechanisms and structural features that account for their distinct reactivation profiles. Here we describe such studies. We also studied the reactivation of a mutant of TcTIM that had the interface residues of TbTIM to probe the contribution of the interfacial and noninterfacial portions of the enzyme to the kinetics of reactivation. Finally, after ascertaining that heterodimers of TcTIM and TbTIM monomers could be readily formed, we determined the extent to which monomers of one enzyme recruit monomers of the other enzyme. The purpose was to explore if one monomer affects the characteristics of the other.

MATERIALS AND METHODS

Recombinant TIMs from *T. cruzi* and *T. brucei* were purified from *Escherichia coli* in which the enzymes had been expressed. These methodologies have been described (*20*, *21*). The enzymes were maintained at 4 °C as a suspension in 70% saturated ammonium sulfate that also contained 100 mM triethanolamine, 10 mM EDTA, 1 mM dithiothreitol, and 1 mM sodium azide, pH 8.0. For the experiments, the suspension was centrifuged and the pellet dissolved in 100 mM triethanolamine/10 mM EDTA, pH 7.4, and dialyzed against the same buffer.

Construction of the Interface Mutant. The substitutions of the interface region of TcTIM with the amino acid equivalent to TbTIM were prepared by polymerase chain reaction (PCR) using the Expand High Fidelity PCR System (Boehringer). The mutagenic oligonucleotides were 5′ GGCTCCCAGCAGTTGCTTGTA 3′ [S20Q forward (Fw)] and 5′ TACAAGCAACTGCTGGGAGCC 3′ [S20Q reverse (Rv)], 5′ CCCACCTTTGTGCACATCCCA 3′ (L47V Fw) and 5′ TGGGATGTGCACAAAGGTGGG 3′ (L47V Rv), 5′ ACCTTTGTGCACCTGGCGATGACG 3′ (L47V/I49L and P50A Fw) and 5′ CGTCATCGCCAGGTGCACAAAG-GT 3′ (L47V/I49L and P50A Rv), and 5′ AAGGACTTTG-GAGTGAGCTGG 3′ (Y87F/I89V Fw) and 5′ CCAGCT-CACTCCAAAGTCCTT 3′ (Y87F/I89V Rv). The T7 promoter and T7 terminator external oligonucleotides (Novagen) were also used. The PCR conditions were as follows: 30 cycles for 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C and incubation for 10 min at 72 °C. The changes were introduced in the E19Q mutant TcTIM gene (*22*) by separated reactions using the appropriate mutagenic oligonucleotides. The PCR products were cloned in the pET-3a expression vector as NdeI−BamHI fragments and introduced by transformation into Bl21(DE3)pLysS cells (Novagen). The completed interface mutant gene was sequenced. BL21(DE3)pLysS cells transformed with the interface mutant were grown at 37 °C in Luria−Bertani medium supplemented with 100 μg/mL ampicillin. When the absorbance of the culture was $A_{600} = 0.8-1$, 0.4 mM isopropyl β-D-thiogalactopyranoside was added. Growth was continued overnight at 30 °C. The purification of the interface mutant of TcTIM was carried out following the methodology for the purification of TcTIM (*20*). The yield of the enzyme was 8 mg/L of culture.

Activity. The activity of the various TIMs was measured at pH 7.4 in 1 mL of a mixture that contained 100 mM triethanolamine, 10 mM EDTA, 1 mM glyceraldehyde 3-phosphate, 0.2 mM NADH, and 0.9 unit of α-glycerol phosphate dehydrogenase, pH 7.4; usually 5 ng of protein was used for determination of activity. Activity was determined from the decrease in absorbance at 340 nm as a function of time in an HP8452 spectrophotometer with a multicell attachment, thermostated at 25 °C. The kinetic parameters of the enzymes in the direction of glyceraldehyde 3-phosphate to dihydroxyacetone were determined in the same conditions, except that the concentration of substrate was varied from 0.1 to 5 mM. The reaction was started by the addition of TIM. Protein content of the various TIMs was calculated from their absorbance at 280 nm according to Pace et al. (*23*) using the molecular absorption coefficients ϵ of 34950 M^{-1} cm^{-1} for TbTIM and 36440 M^{-1} cm^{-1} for TcTIM and the interface mutant.

Denaturation and Reactivation of the Enzymes. The enzymes were denatured by incubation of 0.5 or 1 mg of protein/mL at 25 °C for 1 h in 100 mM triethanolamine, 10 mM EDTA, 1 mM dithiothreitol, and 6 M guanidinium chloride, pH 7.4. For reactivation, aliquots of the latter mixture were diluted 100-fold into media that contained 100 mM triethanolamine, 10 mM EDTA, and 1 mM dithiothreitol, at pH 7.4; the concentration of protein in the reactivation mixture ranged between 0.2 and 10 μg/mL (8−400 nM monomer concentration). After dilution, aliquots were withdrawn at different times and immediately added to the mixture for activity measurements. The activity traces were linear with time (until NADH was exhausted), indicating that no reactivation took place during the activity measurements. In all experiments, the concentration of GdnHCl in the media at which the enzymes were allowed to reactivate was 60 mM. The exposure of the control enzymes to this concentration of denaturant for the length of the experiments did not affect their activity.

Determination of the Rate Constants in the Reactivation of TIM. The rate constants of reactivation were calculated from ln plots of activity at different protein concentrations versus time, considering that the reactivation involves the reaction sequence

$$2M_u \underset{k_{-1}}{\overset{1,\,k_1}{\rightleftharpoons}} 2M_f \underset{k_{-2}}{\overset{2,\,k_2}{\rightleftharpoons}} D_{inact} \xrightarrow{3,\,k_3} D_{act}$$

where M_u, M_f, D_{inact}, and D_{act} are unfolded monomers, folded monomers, inactive dimers, and active dimers, respectively. Under all conditions, the rate of the first unimolecular reaction, k_1, was faster than the other two reactions (see Results). The step from M_f to D_{inact} is a bimolecular reaction that is followed by the unimolecular reaction in which D_{inact} is transformed to D_{act}. The last reaction was considered

irreversible. Under steady-state conditions, the Briggs−Haldane equation may be used to determine the rates of steps 2 and 3 (*24*, *25*). Thus, the following equation which corresponds to that of a rectangular hyperbola describes the observed rate constant at any given protein concentration

$$k_{obs} = k_3[\text{TIM}][K/(1 + K[\text{TIM}])] \quad (1)$$

where $K = (k_{-2} + k_3)/k_2$, and thus k_3 is the maximal observed rate constant. Since, in our experimental conditions, k_{-2} is much smaller than k_3, it follows that $K \cong k_3/k_2$, and hence, k_2 is the product of k_3/K.

Formation and Purification of the Heterodimer of TcTIM and TbTIM Monomers. We followed the methodology of Sun et al. (*26*) for formation of heterodimers from yeast and rabbit TIM monomers. Native TcTIM and TbTIM at a concentration of 500 μg/mL were incubated in 100 mM triethanolamine, 10 mM EDTA, 1 mM dithiothreitol, and 6 M GdnHCl, pH 7.4, for 1 h at 25 °C. Equal volumes of the denatured enzyme solutions were mixed, usually 0.5 mL of each. This was dialyzed against 250 mL of the same buffer without the denaturant at 25 °C for 4 h and against another 250 mL of the same buffer for about 12 h at 4 °C. The same procedure was followed with TcTIM or TbTIM alone.

The dialyzate was applied to a 14 cm × 1.3 cm column of CM-Sepharose fast flow (Pharmacia Biotech). The column was previously equilibrated with 25 mM triethanolamine, 1 mM EDTA, and 1 mM dithiothreitol, pH 8.0. After loading, 25 mL of the buffer was allowed to pass through the column; no protein was detected in this eluate. Thereafter, the column was eluted successively with 50 mL of buffer that had 30, 50, and 80 mM NaCl. Fractions of 5 mL were collected. As shown in the Results section, each of the latter buffers brought about the respective elution of TcTIM, the hybrid of TcTIM and TbTIM, and TbTIM. The eluates were concentrated in Centricon filters to approximately 1 mg/mL. The activity and the electrophoretic profile in native gels of the hybrid did not change after a month of storage at 4 °C.

Native Gel Electrophoresis. The enzymes were analyzed in nondenaturing electrophoretic acrylamide gels. The concentration of acrylamide was 10%. The gel was prerun for about 1 h at 160 V at 4 °C and run for 2.75 h. The buffer was 153 mM imidazole/400 mM HEPES, pH 7.0. The cathode was at the bottom of the gel. Since TcTIM and TbTIM have a p*I* of 8.2 and 9.8, respectively, the migration of TbTIM was faster than that of TcTIM. The gels were stained with Coomassie blue.

Circular Dichroism. CD spectra were recorded on a Jasco J-715 spectropolarimeter in 0.1 mm quartz cells at 25 °C from 260 to 190 nm. For the experiments, the enzymes were dialyzed against 25 mM phosphate buffer, pH 7.2. Prior to recording the spectra, the enzymes were filtered through a 0.22 μm membrane. Each spectrum shown is the average of two independent preparations. Blanks without protein were also recorded. The latter spectra were subtracted from the experimental.

Intrinsic Fluorescence. The emission fluorescence spectra of the enzymes were recorded in a RF5000U Shimadzu spectrofluorometer in 4 mm quartz cuvettes at room temperature. The excitation wavelength was 280 nm. The concentration of the enzymes was 5 or 20 μg/mL in the buffer used for the reactivation experiments. Before use, the buffer was filtered through 0.22 μm membranes. Blanks without protein were also recorded; these spectra were subtracted from the experimental. The spectral center of mass (SCM) was calculated according to the equation:

$$\text{SCM} = \sum \lambda I(\lambda) / \sum I(\lambda)$$

where $I(\lambda)$ is the fluorescence intensity at wavelength λ.

Table 1: Intrinsic Fluorescence of TcTIM, TbTIM, and the Interface Mutant with and without 6.0 M GdnHCl and after Dilution of the Denaturant[a]

enzyme concn		TcTIM		TbTIM		interface mutant	
		SCM	% FI	SCM	% FI	SCM	% FI
5 μg/mL	native	332	100	339	100	341	100
5 μg/mL	GdnHCl	370	20	369	20	380	10
5 μg/mL	dilution	341	102	343	90	344	103
20 μg/mL	native	337	100	339	100	341	100
20 μg/mL	GdnHCl	363	29	362	20	378	10
20 μg/mL	dilution	343	95	348	74	344	85

[a] The intrinsic fluorescence spectra of the indicated enzymes at the concentrations shown were determined at an excitation wavelength of 280 nm. Where shown the media contained 6.0 M GdnHCl. The mixtures in which the enzymes were denatured were diluted 100-fold, and 1 min later the spectra were recorded. From the spectra of the enzymes in the indicated conditions, the spectral center of mass (SCM) and fluorescence intensity (FI) were calculated. The fluorescence intensity of the native enzymes at the wavelength of maximal emission was considered 100%; the data with the denatured and diluted samples at the latter wavelengths are shown as percent of the fluorescence of the native enzymes.

RESULTS

The kinetics of the reactivation of TIM from unfolded monomers of rabbit and chicken TIMs have been previously studied (*14*−*16*). From their data, the authors concluded that the first step of reactivation is monomer folding; subsequently, the folded monomers associate and form the active dimer. We studied if TcTIM and TbTIM follow the same reaction sequence. The folding events of the two enzymes were followed by changes in intrinsic fluorescence. The two trypanosomal enzymes have five equivalent tryptophans per monomer (13, 91, 160, 171, and 194). Trp 171 is in the mobile loop 6; the rest are buried within the structure (*8*). TcTIM and TbTIM have five equivalent tyrosines. TcTIM has an additional tyrosine (Tyr 87); it is in the dimer interface. The intrinsic fluorescence spectra of TcTIM and TbTIM in their native state and after they were denatured by 6.0 M GdnHCl were recorded. In the two enzymes, the denaturant brought about a red shift of their spectral centers of mass and a decrease of fluorescence intensity (Table 1). This reflected the solvent exposure of the aromatic residues of the enzymes as a consequence of protein unfolding. Table 1 also shows the relative intrinsic fluorescence of the proteins 1 min after denatured TcTIM and TbTIM were diluted 100-fold. Dilution brought about a blue shift and an increase in fluorescence intensity. The same results were observed when the denatured proteins were diluted to a concentration of 5 or 20 μg/mL (Table 1). Note, however, that there were differences between the intrinsic fluorescence spectra of the diluted samples and the native enzymes. This is because the diluted samples have a mixture of folded monomers, dimers, and, in some cases, protein aggregates (see below). Nevertheless, the data indicate that, within 1 min, the aromatic

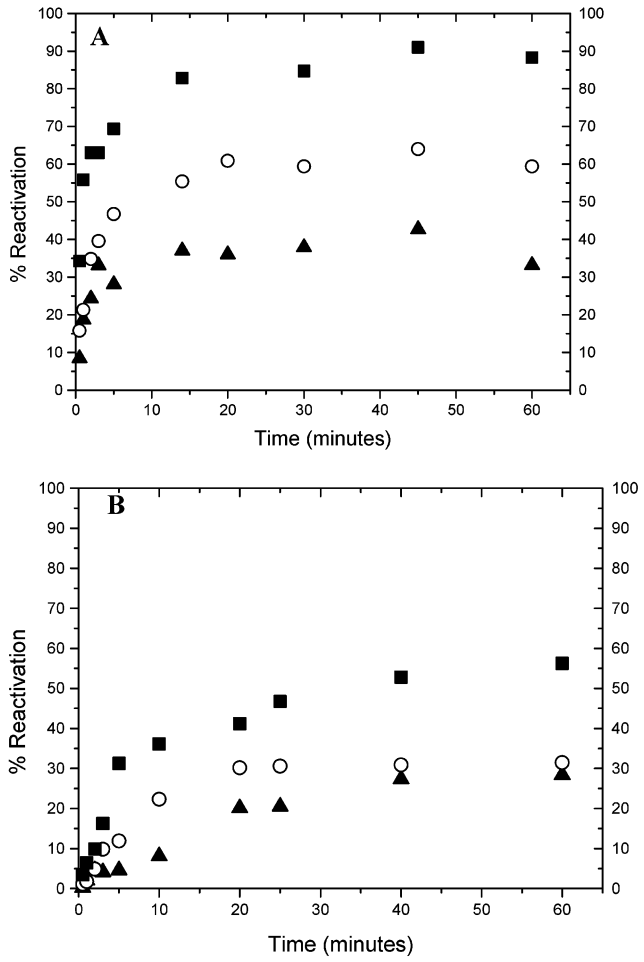

FIGURE 1: Reactivation of TcTIM (A) and TbTIM (B) at different protein concentrations. The enzymes were denatured at a concentration of 500 μg/mL with 6.0 M GdnHCl for 1 h at 25 °C. At time zero, the enzymes were diluted so as to give the indicated concentrations 1 μg/mL (▲), 2.5 μg/mL (○), and 5 μg/mL (■) at pH 7.4 and 25 °C; all of the latter mixtures contained 60 mM GdnHCl. At the times indicated aliquots were withdrawn, and their activity was determined. The results are expressed as percent of the activity recovered in which 100% is the activity of the native enzymes.

residues of the enzymes became buried within the structure of the protein.

Dilution of the mixtures in which the enzymes had been denatured also brought about the progressive appearance of activity; however, the times in which activity progressively appeared were much longer than those in which the aromatic residues became buried (Figure 1). In both TcTIM and TbTIM, the rate and extent of reactivation increased with protein concentration. Thus, in the reactivation reaction, the two proteins exhibited the same pattern. Nonetheless, the time curves showed that, at equal protein concentrations, TcTIM reactivation was higher and faster than in TbTIM (panels A and B of Figure 1, respectively). Recordings of the activities after 24 h showed that, in the span of 1−24 h, the activities increased by about 5% at the most. In all of the experiments described here, the activities observed after 24 h of incubation were considered as the maximal extent to which the enzymes reactivated.

From ln plots of the appearance of activity in the initial times of reactivation, the pseudo-first-order constants of active dimer formation at different concentrations of TbTIM and TcTIM were calculated. The values were plotted against protein concentration in order to obtain the second-order rate constants for active dimer formation (Figure 2). Instead of the ideal linear behavior, the points fell on a curve that had a downward deviation until the process became independent of protein concentrations. The shape of the curve indicates that, at relatively low protein concentrations, the rate is limited by a second-order reaction (monomer association), whereas at high concentrations the rate-limiting step is a unimolecular reaction. This first-order rate constant (Table 2) of the latter uni reaction (k_3) was calculated from the data of Figure 2 as described under Materials and Methods.

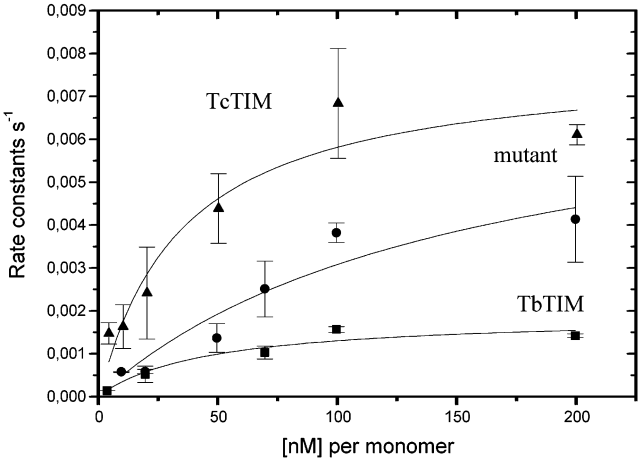

FIGURE 2: Plots of the first-order rate constants versus protein concentration. The experiments were performed as in Figure 1, except that reactivation was carried out at the indicated protein concentration. The ln of the activities in the first minutes of reactivation was plotted against time, and the first-order rate constants were calculated. The latter values were plotted against protein concentration to obtain the second-order rate constant. Each point is the average of at least two determinations; the bars show the extreme values.

Table 2: Rate Constants of the Reactivation of TcTIM, TbTIM, and the Interface Mutant[a]

	TcTIM	TbTIM	interface mutant
k_1 (s^{-1})	0.08	0.08	0.08
k_2 ($M^{-1}\,s^{-1}$)	2.6×10^5	0.2×10^5	0.2×10^5
k_3 (s^{-1})	8.1×10^{-3}	1.9×10^{-3}	7.8×10^{-3}

[a] The rate constants were calculated from the data in Figure 2 as described under Materials and Methods. For further details see text. It is noted that k_1 was obtained from the changes in intrinsic fluorescence that occurred in 1 min after GdnHCl was diluted 100-fold. Our experimental system did not allow measurements of changes that occurred in less than 1 min. Thus, it is very likely that monomer folding is much faster.

In the reaction sequence of TIM reactivation (see scheme in the Materials and Methods section, above), there are two unimolecular reactions: monomer folding and the transformation of inactive to active dimers. To ascertain which of the two is rate limiting for formation of active dimers, the first-order rate constant for monomer folding (k_1) was calculated from the data in Table 1 that show that, in 1 min, the unfolded monomers were transformed into folded monomers. This gives a first-order rate constant of 8×10^{-2} s^{-1}. The rates are probably faster, and there may be differences between TcTIM and TbTIM; however, our instrumental setup did not allow precise measurements of faster rates. Nonetheless, the rate of monomer folding of the

Table 3: Differences of the Residues That Establish Intersubunit Contacts in TcTIM and TbTIM and Their Position in the Crystal Structures of the Enzymes[a]

TcTIM	TbTIM	location
Glu 19	Gln 18	helix 1
Ser 20	Gln 19	helix 1
Leu 47	Val 46	helix 2
Ile 49	Leu 48	helix 2
Pro 50	Ala 49	helix 2
Tyr 87	Phe 86	helix 3
Ile 89	Val 88	helix 3

[a] The numbering of the amino acid sequences of TcTIM and TbTIM was used. TcTIM has one more residue than TbTIM; it is at position 2.

Table 4: Kinetics of Native TcTIM, TbTIM, the Hybrid of TcTIM and TbTIM Monomers, and the Interface Mutant of TcTIM[a]

TIM	K_m (mM)	$k_{cat} \times 10^5$ (min^{-1})
TcTIM	0.45 ± 0.03	2.7
TbTIM	0.46 ± 0.01	3.1
Tb:TcTIM	0.31 ± 0.02	3.0
interface mutant	0.40 ± 0.01	3.1

[a] The kinetics of the indicated enzymes were assessed in the direction of glyceraldehyde 3-phosphate to dihydroxyacetone phosphate as described in the Materials and Methods section. The concentration of glyceraldehyde 3-phosphate ranged from 0.1 to 5 mM. The data are the average of at least three independent determinations. The standard error of the K_m of the enzymes is shown. The standard errors in k_{cat} for TcTIM, TbTIM, the TcTIM:TbTIM hybrid, and the interface mutant were respectively 1.9%, 4.8%, 2.2%, and 2.6% of the values shown.

two enzymes was faster than k_3, the first-order reaction that limits active dimer formation at high protein concentrations (Table 2). In consequence, at high protein concentrations the rate-limiting step of the overall reactivation process is the unimolecular reaction in which D_{inact} is transformed into D_{act}. In the data of Table 2, it is also evident that the rate constants of the bimolecular and unimolecular D_{inact} to D_{act} reactions are higher in TcTIM than in TbTIM.

Reactivation Kinetics of a Mutant of TcTIM in Which the Interfacial Residues Are Identical to Those of TbTIM. The previous findings raised the question of how the structural differences of the two enzymes account for their differences in the kinetics of reactivation. To address this issue, we constructed a mutant of TcTIM in which the residues that form its interface were made identical to those of TbTIM. The two trypanosomal enzymes differ in 7 of the 32 residues that establish contacts with the other subunit. The position of these residues is also shown (Table 3). This mutant enzyme will be referred to as the *TcTIM interface mutant.* The interface mutant and the wild types exhibited similar catalytic properties (Table 4).

The kinetics of reactivation of the interface mutant were determined following the protocol used with the wild types. Within the limits of our experimental approach, the transition of M_u to M_f of the interface mutant was as fast as in the wild types (Table 1). Similarly to the wild-type TIMs, the rate of reactivation of the interface mutant increased with protein concentration until a plateau was reached (Figure 2). From the data, the second-order rate constant and the rate-limiting first-order constant were calculated and compared to those of the wild types (Table 2). The comparison showed two notable features. One is that the second-order rate constant (monomer association) of the interface mutant is similar to that of TbTIM. Because the mutant had the interface of TbTIM and the noninterfacial portion of TcTIM, the data are strongly suggestive that the formation of D_{inact} from folded monomers is predominantly controlled by the interfacial residues. The other salient point is that the rate of the transition of D_{inact} to D_{act} of the mutant is significantly higher than in TbTIM and only slightly lower than in TcTIM. This suggests that the noninterfacial portion of the enzyme exerts a strong contribution on the rate at which D_{inact} is transformed to D_{act}.

TIM Reactivation and Aggregation. In the experiments on the reactivation of the TIMs studied, it is clear that the extent of active dimer formation from unfolded monomers was not 100% (Figure 1). Except with TcTIM at concentrations of 5 μg/L (200 nM monomer concentrations) in which reactivation was about 90%, the extent of reactivation was around 50%, or less at lower protein concentrations. This is a common observation in studies on the reactivation of enzymes. In fact, it is well documented that the fraction of the protein that fails to form catalytically competent enzymes undergoes aggregation through a second-order reaction that competes with the reactions of the reactivation pathway (*27*). Thus, as discussed by Jaenicke and Lillie (*19*), there is a kinetic partition between the reactions that lead to formation of active enzymes and those that end in aggregation. In this context, it is illustrative that TcTIM reactivated around 90% at 200 nM monomer concentrations, whereas with 400 nM the extent of active dimer formation was about 50% (not shown). Thus, it would appear that monomer concentration is central in the pathway that the intermediates of the reaction follow.

Formation of Hybrids of TbTIM and TcTIM Monomers. The reactivation experiments with TcTIM and TbTIM indicate that the former reactivates faster than TbTIM and that this is due to the higher rate of the reactions that involve monomer association and the transition of the inactive to the active dimer. In light of these data, it was considered of interest to study the characteristics of the formation of hybrids from monomers that have distinct reactivation kinetics. Accordingly, we first studied if TcTIM and TbTIM monomers hybridize. To this end, a mixture of equal amounts of TcTIM and TbTIM was denatured in 6.0 M GdnHCl. Thereafter, the mixture was extensively dialyzed, and the products were analyzed by native gel electrophoresis. We used this system because the isoelectric points of TcTIM and TbTIM are 8.2 and 9.8, respectively, and hence, the mobility of TbTIM toward the cathode was faster than that of TcTIM (Figure 3, inset). Under the conditions used, there was clear separation between TbTIM and TcTIM, and thus, the hybrid could be easily identified as a protein band that migrates between TcTIM and TbTIM.

The analysis of the proteins that were formed when unfolded monomers of TcTIM and TbTIM were allowed to reactivate together showed two proteins (Figure 3, inset). One had the mobility of TcTIM. The other protein migrated between TcTIM and TbTIM; therefore, it corresponded to the hybrid. An important point of the experiment is that the gel did not show the protein band that corresponds to TbTIM (in some preparations, we have observed a very faint band). In this respect, it is important to note that, during dialysis of denatured TbTIM alone, the protein underwent extensive aggregation. However, when denatured TbTIM and TcTIM

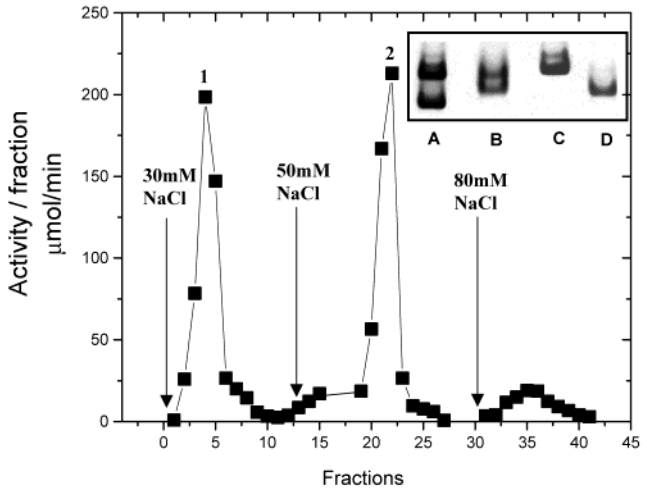

FIGURE 3: Formation and isolation of hybrids of TcTIM and TbTIM. A mixture of equal amounts of unfolded monomers of TcTIM and TbTIM was dialyzed against 25 mM triethanolamine/10 mM EDTA, pH 7.4, and thereafter applied to a column of carboxymethyl-Sepharose and eluted with buffer that contained the indicated salt concentrations. Activity in the fractions was determined. The fractions that showed activity were pooled and concentrated. They were subsequently analyzed by native gel electrophoresis. The inset shows the gel stained with Coomassie blue. Please note that the calculated isoelectric points of TbTIM, TcTIM, and the hybrid are 9.8, 8.2, and 9.0, respectively. Thus, they showed distinct migration in native gel electrophoresis. Lane A had a mixture of native TcTIM and TbTIM, lane B the products of dialysis of the mixture that contained unfolded TcTIM and TbTIM monomers, lane C the protein that eluted with 30 mM NaCl (peak 1), and lane D, the protein that eluted with 50 mM NaCl (peak 2); note that the migration of this protein is intermediate between TcTIM and TbTIM.

were dialyzed together, the extent of protein aggregation was much lower. These observations together with the electrophoretic data indicated that TcTIM monomers recruited a significant amount of TbTIM monomers and, thereby, diminished their aggregation.

The distinct migration of the hybrid in native gels suggested that it could be isolated from the original enzymes by ion-exchange chromatography. Accordingly, the mixture in which TcTIM and TbTIM had been denatured was dialyzed and applied to a column of carboxymethyl-Sepharose. After adsorption of the proteins, three clearly distinguishable activity peaks were eluted with buffer that contained 30, 50, and 80 mM NaCl (Figure 3). In native gels, the protein that eluted with 30 mM salt exhibited the mobility of TcTIM. The protein that eluted with 50 mM salt migrated between TcTIM and TbTIM; in this lane, no other protein bands were apparent. Finally, with 80 mM NaCl, a small activity peak was eluted. This small peak corresponded to TbTIM, since control experiments showed that the elution of TbTIM required NaCl concentrations of 80 mM.

On the basis of total activity recovered from the column, the percent of activity that corresponded to TcTIM, the hybrid, and TbTIM was calculated (Table 5). The data showed that the activity recovered in the fractions that corresponded to the hybrid was almost equal to that of TcTIM and substantially higher than that of TbTIM. In fact, the ratio of activities of TcTIM:hybrid:TbTIM was 1:0.9:0.2. This is markedly different from the 1:2:1 ratio expected from a random probabilistic distribution of monomers. These findings indicate that TbTIM monomers prefer to form active

Table 5: Distribution of the Products of the Simultaneous Reactivation of TcTIM and TbTIM Monomers[a]

enzyme	% recovery
TcTIM	48
TbTIM	9
TbTIM + TcTIM	43

[a] TcTIM and TbTIM, 500 mg each, were denatured in 0.5 mL of 6.0 M GdnHCl, and the mixtures were combined and thereafter dialyzed against 25 mM triethanolamine/10 mM EDTA, pH 7.4.The dialyzate was applied to a column of carboxymethyl-Sepharose and eluted as described in Figure 2. The enzymes in the eluates were identified as described in Figure 2 and the text (see Materials and Methods section for details). The activity in each of the three sets of fractions is expressed as percent of the total activity recovered.

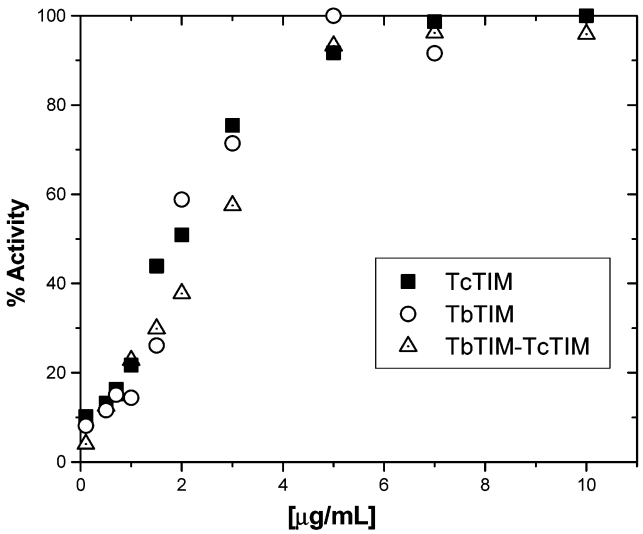

FIGURE 4: Stability of TcTIM, TbTIM, and the hybrid of TcTIM and TbTIM monomers at different protein concentrations. The enzymes were incubated at the indicated concentrations for 2 h at 36 °C. At that time their activity was determined. The results are expressed as percent of the specific activity of the enzymes before incubation: (■) TcTIM, (○) TbTIM, and the hybrid (△).

dimers with TcTIM monomers than with their own counterparts. Likewise, the data show that, through the formation of hybrids, the partition of TbTIM monomers into aggregates is largely overcome.

Characteristics of TcTIM:TbTIM Hybrids. It was necessary to ascertain the kinetic and structural characteristics of the hybrid. The kinetics of the isolated hybrid were assessed in the direction of glyceraldehyde 3-phosphate to dihydroxyacetone phosphate. Like the native enzymes, Lineweaver−Burk plots of the activity data were linear, yielding K_m and k_{cat} values similar to those of the homodimers (Table 4). The stability of the hybrid was also explored. Dimeric proteins dissociate according to the association constant between monomers. Since TIM monomers are inactive, information on the stability of the dimer may be inferred from the specific activity of the enzyme after it is preincubated at different concentrations. Thus, the hybrid was incubated at different concentrations for 2 h. At that time, the specific activity of the various samples was determined. Experiments with TcTIM and TbTIM were carried out in parallel. The results (Figure 4) showed that the stability of the hybrid was similar to that of native TcTIM and TbTIM.

The intrinsic fluorescence of TcTIM and TbTIM at an excitation wavelength of 280 nm differs by 3 nm in their

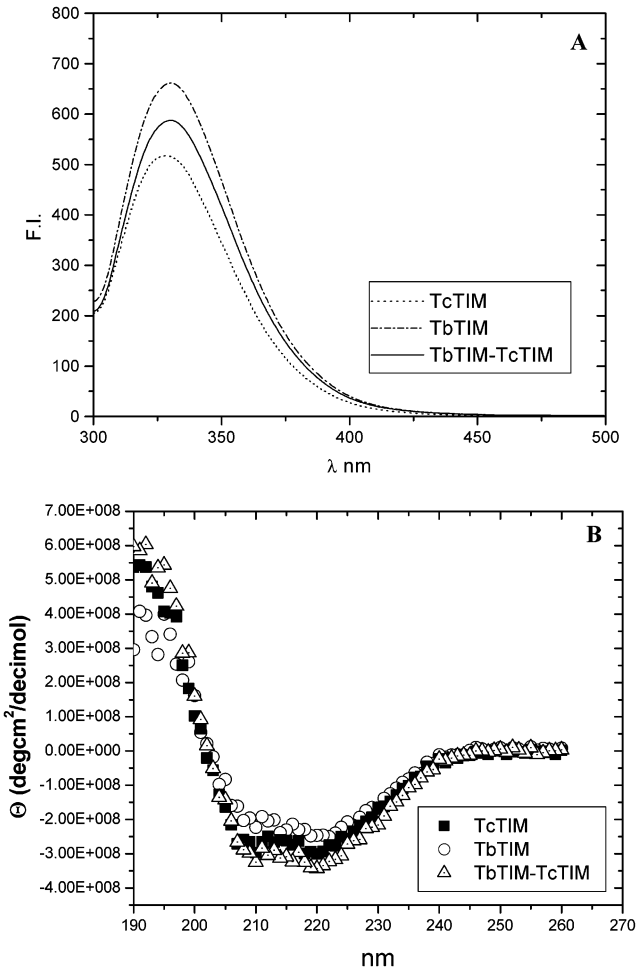

FIGURE 5: Intrinsic fluorescence (A) and circular dichroism (B) spectra of TcTIM, TbTIM, and the hybrid of TcTIM and TbTIM monomers. The spectra of the indicated enzymes were recorded as described in the Materials and Methods section.

spectral center of mass. There are also differences in their fluorescence intensity; it is higher in TbTIM than in TcTIM (Figure 5A). The spectral center of mass of the hybrid was similar to that of TbTIM. However, its intensity at the wavelength of maximal emission was intermediate between that of TcTIM and TbTIM. Apparently, in the hybrid, each of the two monomers conserved the environment of their aromatic residues. The circular dichroism spectra of TcTIM, TbTIM, and the hybrid were markedly similar (Figure 5B). Therefore, the overall data indicate that the hybrid is a catalytically competent enzyme with the structural features of native TIMs.

DISCUSSION

The studies with TcTIM and TbTIM showed that their reactivation from unfolded monomers is a sequence of uni-bi-uni molecular reactions. The first unimolecular reaction involves the folding of the monomers. Subsequently, in the bimolecular reaction, two folded monomers join and form an inactive dimer. This is followed by internal arrangements that end in the catalytically competent dimer. This sequence of reactions seems to be a general pathway for formation of oligomeric proteins. Indeed, in enzymes other than TIM, the existence of the three reactions and the corresponding intermediates of the reactivation pathway has been well documented (*28*−*33*). However, a salient finding of this work is that TcTIM and TbTIM exhibit marked differences in reactivation; albeit they have markedly similar amino acid sequences and three-dimensional structures. Our experiments on reactivation showed that the rate constants of k_2 and k_3 are higher in TcTIM than in TbTIM. Thus, it would appear that the structural differences between the enzymes affect strongly the kinetics of reactivation.

In an attempt to define the structural features that account for the distinct reactivation kinetics of the two trypanosomal enzymes, the reactivation of a mutant of TcTIM that had the interface residues of TbTIM was studied. The results illustrated that the rate of the bimolecular reaction was similar to that of TbTIM, whereas the rate of the D_{inact} to D_{act} reaction was close to that of TcTIM. Therefore, it would appear that the joining of the two monomers depends on the interfacial portion of the enzymes. On the other hand, the higher k_3 of the interface mutant, in comparison with TbTIM, indicates that the internal structural arrangements that occur in the D_{inact} to D_{act} transition are kinetically controlled by the noninterfacial portion of the protein. In this connection, it is also relevant that the susceptibility to proteolytic digestion by subtilisin of TcTIM is much higher than that of TbTIM (*34*). This suggests that TcTIM has a higher intrinsic flexibility. Thus, it seems worthwhile to explore if differences in flexibility are related to reactivation kinetics.

Hybrids. We also studied the formation of active hybrids from unfolded monomers of TbTIM and TcTIM. The studies were an attempt to explore how the interaction of monomers with different characteristics and k_2 and k_3 of reactivation affect the overall formation of catalytically active hybrids. The formation of hybrids is well documented by in vivo and in vitro experiments (*35*−*39*). Several of the studies show that monomers of homologous enzymes can cross-react, yielding catalytic active hybrids; however, this is not always the case. For example, TIM monomers from yeast TIM hybridize with monomers of rabbit and chicken, but rabbit and chicken monomers do not (*26*). Monomers of ornithine decarboxylase from *T. brucei* and mouse also hybridize, but they do fail to do so with the monomers of the enzyme from *Leishmania donovani* (*35*). Likewise, hybrids of thymidylate synthase can be formed from the monomers of the enzymes from *E. coli* and *Lactobacillus casei* but not with monomers of the enzyme from phage T4 (*36*). As discussed by the authors, the level of amino acid conservation in the interface appears to be central for formation of the heterodimer. Thus, in the light of these data, and since the interfaces of TcTIM and TbTIM have 25 identical amino acids out of a total of 32 interfacial residues, it was not surprising to find that hybrids of these two enzymes can be readily formed.

What is unusual in the formation of the trypanosomal hybrids is that when unfolded monomers of TbTIM and TcTIM were allowed to reactivate together, the yield of active hybrids was high and almost equal to that of TcTIM. The yield of TbTIM dimers, on the other hand, was rather modest. Because of the differences in the interfacial residues of TcTIM and TbTIM, it could be expected that folded monomers of each enzyme prefer to dock into their own counterparts or randomly, if the differences are tolerated or irrelevant for dimer formation. However, in our experimental conditions, the ratio of TcTIM:hybrid:TbTIM was 1:0.9:0.2. This indicates that there is a preferential partition of TbTIM

monomers into TcTIM monomers. In this regard, it is relevant that the kinetic data indicate that the noninterfacial portion of the enzyme has a strong influence on k_3, the step that involves the final arrangements in the formation of active dimers. Therefore, it is probable that, in the transition of D_{inact} to D_{act}, the characteristics of one monomer affect the characteristics of the other monomer.

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