Reactivation of triosephosphate isomerase from three trypanosomatids and human: effect of Suramin

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The reactivation of the homodimeric triosephosphate isomerases (TIMs) from *Trypanosoma brucei*, *T. cruzi*, *Leishmania mexicana* and humans was determined after their denaturation with guanidine hydrochloride. In the range of 2–32 µg of *T. brucei* TIM per ml and 0.2–5 µg of the other enzymes per ml, the rate and extent of TIM reactivation depended on protein concentration, indicating that at these protein concentrations, the rate-limiting step of reactivation is monomer association and not monomer folding. The rate of monomer association was more than one order of magnitude lower in the *T. brucei* enzyme than in the other three enzymes. Suramin is a drug of choice in the treatment of sleeping sickness, but its mechanism of action is not

known. At micromolar concentrations, Suramin inhibited the reactivation of the four enzymes, but the extent of inhibition by Suramin decreased with increasing protein concentration as consequence of a diminution of the life time of the folded monomer. Since the life time of the monomer of *T. brucei* TIM is longer than that of the other enzymes, Suramin is a more effective inhibitor of the reactivation of TIM from *T. brucei*, particularly at monomer concentrations above 1 µg of protein per ml (monomer concentration approx. 37 nM). Compounds that are structurally related to Suramin also inhibit TIM reactivation; their effect was about five times more pronounced in the enzyme from *T. brucei* than in human TIM.

INTRODUCTION

Triosephosphate isomerase (TIM) is a homodimeric housekeeping enzyme that catalyses the interconversion of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. The kinetics, energetics [1] and reactivation [2–4] of TIM have been thoroughly studied. The reactivation data show that unfolded monomers fold to catalytically inactive monomers, which subsequently associate to form the active dimer.

Suramin is a negatively charged molecule that has been extensively used in the treatment of sleeping sickness, an African parasitosis caused by *Trypanosoma brucei* [5]. It is ineffective in the treatment of Chagas disease [6], which is caused by the closely related *T. cruzi*. The mechanism of action of Suramin is unknown, but it affects the function of many proteins [7–11], including that of TIM and other enzymes of the glycolytic pathway of *T. brucei* [12,13]. It was suggested that the latter effects could be related to its trypanocidal action [14]. In *T. brucei*, TIM is synthesized in cytoplasmic polysomes, but it is entrapped in membrane-bound microbodies called glycosomes [15]. In a recent review, Wang [16] suggested that Suramin could affect the import of TIM into the glycosomes. Since the isoelectric point of TIM from *T. brucei* is 9.85, Wang's suggestion seems worthy of attention. However, we thought that Suramin could interfere with TIM formation as well, by preventing either monomer folding or monomer association. Therefore, we determined the effect of Suramin on the reactivation of TIM from *T. brucei* that had been denatured with guanidine hydrochloride. Its effect on the reactivation of TIMs from *T. cruzi*, *Leishmania mexicana* and *Homo sapiens* was also examined.

Suramin inhibited the reactivation of the four TIMs. However, the inhibiting effect of Suramin depended on the rate of monomer association. Since the rate of association of TIM from *T. brucei* is lower than in the other TIMs, Suramin is a more potent inhibitor of the reactivation of TIM from *T. brucei* than of the

other TIMs. The effect of Suramin-like molecules on the reactivation of TIMs from *T. brucei* and human was also determined.

MATERIALS AND METHODS

Suramin was provided by Bayer de México, Mexico City, Mexico. The rest of the reagents were from Sigma or Aldrich. Recombinant TIM from *T. brucei*, *T. cruzi*, *L. mexicana* and human were purified from *Escherichia coli*, in which the enzymes had been expressed. The details for the cloning, expression and purification of *T. brucei*, *T. cruzi*, *L. mexicana* and human TIM were as described elsewhere ([17–20] respectively). The enzymes were maintained at 4 °C as a suspension in 70 % saturation ammonium sulphate. For the experiments, the suspension was centrifuged and the pellet dissolved in 100 mM triethanolamine/10 mM EDTA, pH 7.4, and dialysed against the same buffer.

Activity

TIM activity was measured [21] 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, 0.9 units of α -glycerol phosphate dehydrogenase, and 5 ng of protein for TIM from *T. brucei* and *T. cruzi* and 2.5 ng for leishmanial and human TIM. Activity was determined from the diminution of absorbance at 340 nm as function of time in an HP 8452 spectrophotometer with a multicell attachment, thermostatted at 25 °C. The reaction was started by the addition of TIM. The average activities of *T. brucei*, *T. cruzi*, *L. mexicana* and human TIM were 3151, 3569, 5498 and 5948 µmol/min per mg, respectively. In different experiments the activities did not vary by more than 10 %. Protein content of the various TIMs was calculated from their absorbance at 280 nm using the molecular absorption coefficients ($\epsilon^{M^{-1}\cdot cm^{-1}}$)

Abbreviation used: TIM, triosephosphate isomerase.

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of 34950 for *T. brucei*, 36440 for *T. cruzi* and *L. mexicana*, and 33460 for human TIM.

Denaturation and reactivation of TIMs

In many of the experiments, the various TIMs were denatured [2–4] by incubating the enzymes (500 μ g of protein per ml) for 1 h in a mixture at pH 7.4 which contained 100 mM triethanolamine, 10 mM EDTA, 1 mM dithiothreitol and 6 M guanidine hydrochloride. For reactivation, an aliquot was diluted 100-fold in a mixture (pH 7.4) of 100 mM triethanolamine, 10 mM EDTA and the concentrations of Suramin or other compounds that are indicated in the Results section. Thus in these experiments, reactivation took place at a protein concentration of 5 μ g per ml of reactivation buffer which contained 60 mM guanidine hydrochloride. When the concentration of protein in the reactivation reaction was varied, the concentration of TIM in the denaturation buffer was adjusted so that the dilution step yielded the protein concentration desired. We took care that reactivation was carried out at a concentration of 60 mM guanidine hydrochloride. The exposure of control enzymes to this concentration of denaturant did not affect their activity.

For measuring reactivation, aliquots were withdrawn from the aforementioned mixture (at the times indicated), diluted in the same buffer, and an aliquot that contained 5 or 2.5 ng (see above) of protein was added to the reaction mixture for measuring activity. This procedure took about 20 s. The activity traces were linear with time (until NADH was exhausted), indicating that no reactivation took place during the activity measurements.

It has been reported that Suramin inhibits the activity of TIM [12]. These observations were confirmed (results not shown). It is pointed out, however, that after the above-mentioned dilutions of the reactivation mixture, the concentration of Suramin in the assay mixture was always less than 1 μ M, a concentration that did not affect TIM activity. Furthermore, the experiments in which the native enzymes were treated with Suramin at the concentrations used in the reactivation step, and subsequently diluted for an assay of activity, showed that activity was not modified by preincubation with Suramin.

RESULTS

The effect of Suramin on the reactivation of recombinant TIMs from *T. brucei*, *T. cruzi*, *L. mexicana* and human that had been denatured with guanidine hydrochloride was determined. At a concentration of 5 μ g of protein per ml in the reactivation mixture, Suramin effectively prevented the reactivation of TIM from *T. brucei* (Figure 1); half-maximal inhibition was attained with about 60 μ M. Suramin also diminished reactivation of the other TIMs, but to a significantly lesser extent, e.g. 200 μ M Suramin inhibited their reactivation by about 25% or less.

Effect of Suramin on TIM reactivation at various protein concentrations

Reactivation of denatured TIM involves an initial step in which the unfolded monomers (M_u) undergo intramolecular arrangements yielding catalytically inactive, partially folded monomers (M_{pf}). In a second step, the partially folded monomers associate and form catalytically active dimers [2–4]:

Step I is an intramolecular reaction, whereas step II is a bimolecular reaction; thus, its rate increases with protein con-

Figure 1 Effect of Suramin on TIM reactivation

TIMs from *T. brucei*, *T. cruzi*, *L. mexicana* and human were denatured at pH 7.4 in 100 mM triethanolamine, 10 mM EDTA, 1 mM dithiothreitol and 6 M guanidine hydrochloride for 1 h. At that time, the enzymes were diluted 100-fold in the same buffer (without guanidine hydrochloride) that contained the indicated concentrations of Suramin. The concentration of the enzymes was 5 μ g/ml. After 1 h, the mixtures were diluted and activity was measured with 5 or 2.5 ng of protein per ml of reaction mixture. The extent of reactivation was calculated from the activity of controls that had not been exposed to Suramin. The activities of the *T. brucei*, *T. cruzi*, *L. mexicana* and human TIMs after reactivation that had not been exposed to guanidine hydrochloride were 46, 85, 68 and 78%, respectively; these latter values were standardized to 100% in the Figure.

Figure 2 Effect of different concentrations of Suramin on the reactivation of TIM from *T. brucei*

The experiment was as in Figure 1, except that the concentrations of protein during reactivation were 2 and 32 μ g/ml.

centration. Suramin could inhibit reactivation by preventing monomer folding or the association of the folded monomers. To examine these possibilities, the effect of Suramin was determined on the reactivation of TIM from *T. brucei* at two protein concentrations (Figure 2). Suramin inhibited more effectively when reactivation was carried out with 2 than with 32 μ g per ml.

Figure 3 Effect of Suramin on TIM reactivation at various protein concentrations

In (A) the denaturation and reactivation conditions were as in Figure 1, except that denaturation was carried out with 4.0 M guanidine hydrochloride, and that the reactivation mixture contained 150 μM Suramin and the indicated protein concentrations. In (B) the extent of reactivation is plotted against the $t_{1/2}$ values of the monomers at the protein concentrations of (A). See also data in Table 1.

Thus, the effect of Suramin on the reactivation of TIM from *T. brucei* is protein-concentration dependent.

These observations raised the question of whether there is a range of protein concentration for TIMs from *T. cruzi*, *L. mexicana* and human in which Suramin inhibits their reactivation. Hence, the effect of 150 μM Suramin at several protein concentrations was studied (Figure 3A). At protein concentrations below 1 μg per ml, Suramin effectively inhibited reactivation; however, as protein concentration increased, the inhibiting action of Suramin diminished. It is noteworthy that at protein concentrations at which Suramin produced a small inhibition of reactivation of TIMs from *T. cruzi*, *L. mexicana* and human, Suramin still strongly inhibited the reactivation of *T. brucei* TIM (Figure 3A).

Half-life of the monomers prior to dimerization and the effect of Suramin on TIM reactivation

Since the rate of the association reaction (or its equivalent, the life time of the monomer) depends on protein concentration, the latter findings suggested that the inhibiting effect of Suramin on the reactivation of the four TIMs is related to the life time of the monomers before they associate to form the active dimer. Therefore, the life time of the folded monomer at several protein concentrations was determined. To this end, the four enzymes were denatured in guanidine hydrochloride and transferred to the reactivation buffer; the final reactivation mixture contained different concentrations of protein and 60 mM guanidine hydrochloride. Hence, the only variable in the reactivation reaction was protein concentration. After dilution, aliquots were withdrawn at various times for assay of activity (Figure 4). Activity was also measured after 24 h of incubation. The activities observed at these times were considered to be the maximal reactivation that the enzymes had undergone. Except for TIM from *T. cruzi*, in which an approx. 15% decrease in activity was observed at a concentration of 0.2 μg per ml, the activity of the controls was not affected in a 24 h incubation time.

The values obtained from the equation:

$$\log \left[\frac{(\text{maximal reactivation}) - (\text{reactivation at time } x)}{(\text{maximal reactivation})} \right] \times 100$$

were plotted against time. At initial times, the plots were linear, but at later times the points tended to curve upwards, particularly at relatively low protein concentrations. We have no explanation for this behaviour, but from the plots the point at which 50% maximal reactivation took place was extrapolated to obtain the $t_{1/2}$ of the monomer.

The $t_{1/2}$ values of the monomers at various protein concentrations are shown in Table 1. Except for the human enzyme with 5 and 1 μg of protein per ml, the $t_{1/2}$ decreased as protein concentration was increased. Apparently, with human TIM at protein concentrations of 5 and 1 μg per ml, the rate of the association reaction is close to, or faster than, that of monomer folding [2]. On the other hand, in the other enzymes, the life times of the monomer decreased with protein concentration (Table 1), indicating that the rate-limiting step of reactivation was the dimerization step. The data also showed that the four enzymes exhibited different reactivation rates, but it is noteworthy that the life time of the *T. brucei* monomers was significantly longer than those of the other TIMs.

The amino acids that form the interface between the two monomers of *T. brucei*, *T. cruzi* and *L. mexicana* are identical at 28 of the 32 residues [18,22]. Furthermore, the overall identity of the monomers is close to 70% [18]. Thus, differences of more than one order of magnitude in the rate of reactivation between TIM from *T. brucei* and the other two trypanosomatid enzymes were rather unexpected. This suggests that apparently small variations in the amino acid composition of TIM have significant impact on monomer association.

It has been documented that in the formation of multimeric enzymes, the monomers can follow two kinetically competing pathways, i.e. they can form the active dimeric structure, or undergo incorrect association yielding inactive aggregates

Figure 4 Reactivation of TIMs from *T. brucei*, *T. cruzi*, *L. mexicana* and human as a function of time at various protein concentrations

The experiment was carried out under the standard conditions of denaturation and reactivation, except that reactivation was carried out at the concentrations of protein indicated on each graph. At the times shown, aliquots were withdrawn for assay of activity. Note that for *T. brucei* TIM, higher protein concentrations were used.

Table 1 Half-lives of the folded monomers and extent of reactivation of TIMs from *T. brucei*, *T. cruzi*, *L. mexicana* and human at various protein concentrations

The experiment was carried as in Figure 1, except that the protein concentration in the reactivation step was as indicated. The $t_{1/2}$ values for reactivation in minutes were calculated from plots of the log [(maximal reactivation) – (reactivation at time x)/Maximal reactivation] $\times$ 100 against time. Maximal reactivation was the activity observed after 24 h of reactivation; these values are shown in the columns marked %, where 100% is the activity of enzymes that had not been exposed to guanidine hydrochloride.

Species	Protein concentration ($\mu\text{g/ml}$)											
	32		8		5		2		1		0.2	
	$t_{1/2}$	%	$t_{1/2}$	%	$t_{1/2}$	%	$t_{1/2}$	%	$t_{1/2}$	%	$t_{1/2}$	%
<i>T. brucei</i>	6	54	11	63	—	—	40	26	—	—	—	—
<i>T. cruzi</i>	—	—	—	—	0.4	84	—	—	6	72	12	40
<i>L. mexicana</i>	—	—	—	—	4	68	—	—	9	64	19	26
Human	—	—	—	—	1	81	—	—	1	80	4	84

[3,4,23]. In this context, it may be observed (Table 1) that in the lower range of reactivation rates, the extent of reactivation with all enzymes was also relatively low. This would be in consonance with a reaction sequence in which the partially folded monomer

(M_{pt}) can follow two competing pathways; it can either yield active dimers (step II in the reaction sequence above) or fold into non-specific species that subsequently aggregate. When the rate of formation of active dimers becomes relatively slow (at low

	Inhibition %	
	10 μ M	100 μ M
Suramin Sodium	12	74
Direct Yellow 50	11	99
Direct Red 75	11	98
Direct Red 81	28	94
Trypan Blue	50	99
Direct Violet 51	76	97
Direct Red 23	71	99
Direct Orange 31	95	99
Direct Yellow 8	98	98
Congo Red	99	99

Figure 5. Effect of various dyes and Suramin on the reactivation of TIM from *T. brucei*

The experiment was as in Figure 1 with *T. brucei* TIM, except that the indicated compounds were included during the reactivation reaction at concentrations of 10 and 100 μ M.

protein concentrations), the rate of formation of incorrect structures becomes more significant.

Figure 3(B) is a plot of the $t_{1/2}$ values of the monomers of

Table 2 Concentrations of Suramin and other compounds that produce half-maximal inhibition of reactivation of *T. brucei* and human TIMs

The experiment was as in Figure 1. Reactivation was carried out with 5 μ g of protein per ml and various concentrations of the indicated compounds. From the plots, the concentration that produced half-maximal inhibition of maximal reactivation was calculated.

Compound	Concentration (μ M) producing 50% inhibition		Concentration ratio (human: <i>T. brucei</i>)
	<i>T. brucei</i>	Human	
Congo Red	0.5	1.3	2.6
Direct Red 23	6	27	4.5
Direct Red 75	11	72	6.5
Direct Yellow 50	18	150	8.3
Suramin	60	400	6.7

the various TIMs against the inhibition produced by 150 μ M Suramin. It is apparent that the extent of inhibition induced by Suramin diminished as the life time of the monomer became shorter. This suggests that Suramin acts through a reaction that leads to formation of Suramin- M_{pf} complexes that fail to form active dimers. This reaction would be controlled by the concentration of Suramin and by the rate of step II. However, there are some additional points in the data of Figure 3(B) that deserve attention. The human enzyme exhibited similar lifetimes with 1 and 5 μ g of protein per ml, yet the inhibition induced by Suramin was higher at the lower protein concentration. It is possible that the method for determining the life time of the monomer does not detect rather small differences in reactivation rates. Also, it may be noted that at roughly equal life times, Suramin inhibited dimer formation of the four TIMs to different extents, which could indicate that the monomers from different sources have different affinities for Suramin. Nevertheless, the results indicate that Suramin is a rather unusual inhibitor, i.e. it aborts dimer formation only when the monomers exist for a substantial time. In this context, it is important that among the enzymes studied, the enzyme from *T. brucei* exhibited the lowest rate of dimerization (Table 1). For example, the $t_{1/2}$ for maximal reactivation with 2 μ g per ml was around 40 min for *T. brucei* TIM, whereas with 1 μ g of human TIM per ml, the $t_{1/2}$ was in the range of 1 min. Thus, the kinetics of dimer formation confer to *T. brucei* TIM a higher sensitivity to Suramin.

Effect of various dyes on the reactivation of TIM from *T. brucei* and human

It was also investigated whether molecules with chemical groups similar to those that form the Suramin molecule affect the reactivation of *T. brucei* TIM. Suramin is a symmetric molecule with a central urea moiety; it has naphthalene groups and six negatively charged sulphonates (Figure 5). There are numerous molecules that have these chemical groups, many of which are dyes. In fact, Suramin was synthesized after it was found that some dyes, such as Trypan Red, exhibited trypanocidal action [24,25]. Figure 5 shows the molecules that were tested and their effect at concentrations of 10 and 100 μ M on the reactivation of TIM from *T. brucei*. The two salient features of these results are that all dyes inhibited reactivation and that Suramin was one of the inhibitors with lowest potency.

There are significant structural differences between all the compounds tested, which suggests that there are no precise structural characteristics involved in inhibition. Nonetheless, the data illustrate that the less potent inhibitors are symmetrical

compounds with a central urea moiety. Direct Red 23, a more effective inhibitor of reactivation than Suramin, also has a urea group, but it is not in a symmetrical molecule. Another relevant point is that there is no correlation between the number of negative charges in the molecule and the extent of inhibition: in fact, Direct Yellow 8, which has one net negative charge, was a potent inhibitor of reactivation. Finally, since Congo Red intercalates between β -sheets of amyloid proteins [26], it is interesting that this dye proved to be a powerful inhibitor of reactivation, i.e. the half-maximal inhibition was obtained with 0.5 μ M (see Table 2).

The effectiveness of some of the dyes was tested comparatively on the reactivation of TIMs from *T. brucei* and human (Table 2). All the compounds were more effective on the trypanosomal than the human enzyme, but it is relevant that the concentration required to produce 50% inhibition of reactivation of human and *T. brucei* TIMs varied, but within a limited range. This suggests that similar mechanisms operate in the inhibition of reactivation of *T. brucei* and human TIMs.

DISCUSSION

We have found that Suramin inhibits the reactivation of TIMs from four different sources through a process that increases with the life time of the monomer. The dependency of the inhibiting action of Suramin on the rate of dimerization suggests strongly that it complexes with the monomer prior to the dimerization step. In this context it is noted that extensive efforts are being made to ascertain the structural arrangements that TIM monomers undergo in the transition from inactive to catalytically active structures [27]. Since Suramin interacts with partially folded monomers, it is possible that studies of the Suramin-monomer complex could yield information on the structure of the monomer prior to dimerization. We would like to point out that, to our knowledge, there is only one report of a molecule that inhibits protein oligomerization [28].

Another relevant feature of the results is that, of the enzymes studied, TIM from *T. brucei* exhibits the lowest rate of dimerization. This makes Suramin a more effective inhibitor of *T. brucei* TIM than of the other TIMs. If formation of TIM in *T. cruzi*, *L. mexicana*, human and *T. brucei* takes place in the presence of more than 1 μ g of Suramin per ml, Suramin at micromolar concentrations would be a highly specific inhibitor of *T. brucei* TIM. As this information is not available, the pharmacological action of Suramin may or may not be related to the formation of TIM in *T. brucei*. Along this avenue, it may be relevant that the concentration of TIM within *T. brucei* glycosomes is in the order of 55 μ M [11].

Regarding the pharmacology of Suramin, it is noteworthy that all the dyes studied inhibited TIM reactivation. As noted, Suramin was synthesized after the studies of Paul Ehrlich on the staining of trypanosomes by dyes such as Trypan Red (cited in [24,25]). Moreover, at the beginning of this century, the latter was used to treat Nagana in cattle, which is caused by *T. brucei* [24]. Thus, it is possible that the described higher sensitivity of the reactivation reaction of *T. brucei* TIM to Suramin-like molecules is related to their trypanocidal action.

Most of the current approaches in drug design for treatment of parasitosis are based on the three-dimensional structure of the target protein [29–31]. This is the logical approach to obtain a drug with reduced toxicity and facile adjustments of effective

drug levels. However, the data in this work suggest that protein oligomerization could also be targeted for drug design. Moreover, the present findings indicate that the kinetics of oligomerization reactions may be used advantageously to obtain inhibitors of the formation of multimeric enzymes.

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