The Equilibrium Unfolding of Triosephosphate Isomerase from *T. cruzi* in Guanidinium Hydrochloride is a four State Process. Intrinsic Fluorescence Studies

Edgar Vázquez-Contreras,*[1] Brenda Guadalupe Sánchez-Rebollar,[1] and María Elena Chánez-Cárdenas[2]

[1] Instituto de Química. Departamento de Bioquímica. Universidad Nacional Autónoma de México. Circuito Exterior, Ciudad Universitaria México, D.F. 04510, México. Telephone: (5255) 56224565. Fax: (5255) 56162217. E-mail: vazquezc@servidor.unam.mx.

[2] Laboratorio de Patología Vascular Cerebral, Instituto Nacional de Neurología y Neurocirugía "Manuel Velasco Suárez". México, D.F.

Dedicated to the memory of Dr. Raymundo Cruz Almanza

Abstract. Equilibrium and kinetic folding pathways of several homologous proteins have been studied. Early studies concluded that the folding routes of homologous proteins follow fundamentally similar pathways, and that the folding of a certain conformation is conserved throughout evolution. However, there are examples of homologous proteins that unfold by different routes. Regarding triosephosphate isomerase (*TIM*), unfolding studies with enzymes from different sources, have shown: 1) two-state behaviors and 2) more complex processes (including two equilibrium-unfolding intermediates and inespecific aggregation), in the transition from de native homodimer to the denatured monomers. In this work, we studied the changes in intrinsic fluorescence of *TIM* from *Trypanosoma cruzi* after incubation in guanidinium hydrochloride. Our results show that the reaction is described by a four state process. Finally we discuss the results in terms of the heterogeneity observed in TIM denaturation.
Key words: Triosephoshate isomerase, protein folding equilibrium intermediates.

Resumen. Actualmente existen diversos estudios cinéticos y al equilibrio de proteínas homólogas. Los primeros análisis al respecto concluyeron que las rutas de plegamiento siguen caminos similares para estas proteínas y que ciertas conformaciones se conservan durante la evolución. Sin embargo, existen proteínas homólogas que se pliegan por vías diferentes. Con respecto a los estudios realizados con la triosafosfato isomerasa (*TIM*) la desnaturalización ha mostrado: 1) procesos de dos estados y 2) procesos más complejos (incluyendo dos intermediarios y agregación inespecífica), en la transición del homodímero nativo a los monómeros desnaturalizados. En este trabajo estudiamos los cambios de la fluorescencia intrínseca de la TIM de *Trypanosoma cruzi* después de la incubación en hidrocloruro de guanidina. Nuestros resultados muestran que la reacción es descrita por un proceso de cuatro estados. Finalmente discutimos los resultados en términos de la heterogeneidad observada en la desnaturalización de la TIM.
Palabras clave: Triosafosfato isomerasa, plegamientos intermediarios al equilibrio.

Introduction

Equilibrium and kinetic folding pathways of several homologous proteins have been characterized. Early studies concluded that the folding routes of homologous proteins follow fundamentally similar paths and that the folding of a certain conformation is conserved throughout evolution [1-4]. However, there are examples of homologous proteins which unfold with different patterns [5,6]. Regarding triosephosphate isomerase (*TIM*) unfolding and refolding from several species has been studied extensively [7-25]. Thermodynamic data for unfolding have been reported only for human, rabbit and yeast enzymes [8,17-19], where a three-state model ($N \leftrightarrows 2M \leftrightarrows 2U$) has been proposed. In human TIM (*hTIM*), the unfolding of the wild-type enzyme was modeled as a two-state transition, and information on the properties of the monomer was obtained from the two-state unfolding of monomeric mutants [8,18]. The stability of the TIM homodimer was calculated for rabbit TIM from equilibrium and activation energies for the wild-type enzyme [17]. The same unfolding behavior was found with

yeast TIM *(yTIM)* [9,10,19], although in *y*TIM was first evidenced the presence of a stable equilibrium intermediate in the unfolding of the triosephosphate isomerase [9]. For *y*TIM the thermodynamic of the process was obtained for the denaturation and renaturation reactions [19]. In the present work we show that following changes in intrinsic fluorescence parameters of *Trypanosoma cruzi* TIM (*Tc*TIM) induced by guanidinium hydrochoride (Gdn-HCl), the transition between the homodimeric enzyme and the unfolded monomers follows a four-state process.

Results and discussion

Changes in tertiary structure of *Tc*TIM after equilibrium unfolding

To characterize the equilibrium unfolding of the homodimer of Triosephosphate isomerase from *Trypanosoma cruzi* (*Tc*TIM) changes in intrinsic fluorescence spectra was studied
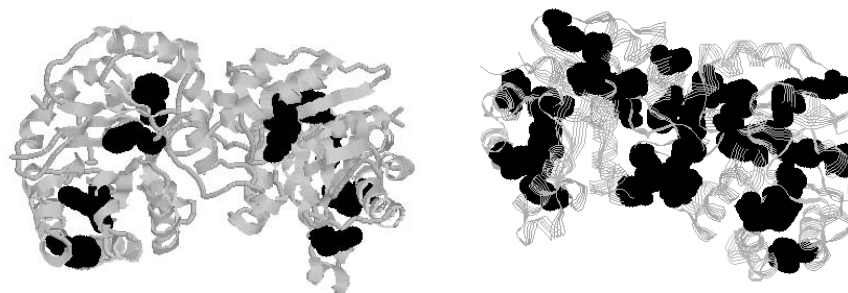
Fig. 1. Diagram of TcTIM homodimer showing the position of the W residues (A) and WYF residues (B). The W13, W91, and W171 are located in loop 1, β strand 4, and the catalytic loop (loop 6), respectively. W194 is in α helix 6 and W160 is located in a short 3_{10} helix before β strand 6. The coordinates for the diagram were taken from the PDB file 1TCD [27].

Table 1. Spectroscopic properties of *Tc*TIM equilibrated in Gdn-HCl. The concentrations shown are representative of the conformers found for the denaturation model of *Tc*TIM (equation 1). A: excitation 280 nm; B: excitation 295 nm. Spectral center of mass (SCM) was fitted to Eq. (2) in experimental section; wavelength of maximal emission ($\lambda_{máx}$); maximum fluorescence intensity (FI); fluorescence intensity at native λmax (FI-$\lambda_{máx}$).

A				
280 nm	FI	$\lambda_{máx}$ (nm)	SMC (nm)	FI-$\lambda_{máx}$
Native	48334 ± 452	317 ± 1	332.0 ± 1	48334 ± 235
0.5 M	50025 ± 426	318 ± 1	331.7 ± 2	47376 ± 248
1.5 M	37909 ± 368	325 ± 2	336.8 ± 5	36173 ± 356
6 M	26724 ± 251	352 ± 2	350.2 ± 3	13374 ± 159
B				
295 nm	FI	$\lambda_{máx}$ (nm)	SMC (nm)	FI-$\lambda_{máx}$
Native	37230 ± 326	323 ± 1	342.2 ± 2	37230 ± 269
0.5 M	39311 ± 289	324 ± 1	339.6 ± 3	39247 ± 312
1.5 M	29395 ± 248	326 ± 2	342.5 ± 5	28711 ± 298
6 M	22340 ± 312	349 ± 2	357.7 ± 6	11551 ± 272

at increasing guanidinium hydrochloride (Gdn-HCl) concentrations. In an average protein[1] 8.5 % of the total amino acids residues are aromatic [26]. In *Tc*TIM every monomer is composed by 251 amino acids residues, 18 of which are aromatics (7.17 %), 5 are tryptophan (1.99 %), 6 tyrosine (2.39 %) and 7 phenylalanine (2.79 %) residues (Figure 1). This number of fluorescent residues is enough to adequately study fluorescence changes in this enzyme as a function of denaturant concentration.

We studied the change in intrinsic fluorescence parameters *i.e.* spectral center of mass (SCM), wavelength of maximal emission ($\lambda_{máx}$), maximum fluorescence intensity (FI) and fluorescence intensity at native $\lambda_{máx}$ (FI-$\lambda_{máx}$) (see below), after exposition of W, Y and F residues (exciting the samples at 280 nm) and after the exposition of only W residues (exciting the samples at 295 nm) that occurs during the dissociation and/or unfolding of the homodimer of *Tc*TIM, we use this evidence as a prove of protein packing.

The equilibrium denaturation of *Tc*TIM (50 µg mL^{-1}) was induced by the addition of different concentrations of guani-

dinium hydrochloride (Gdn-HCl) at 25 °C for equilibrium time (48 h) [20]. After incubation of the homodimer Gdn-HCl induces a red shift in SCM and $\lambda_{máx}$ (Table 1). As the Gdn-HCl concentration increases, the unfolding of TcTIM produced a nonmonophasic pattern; this behavior is observed both when following the exposure of WYF residues and when following only the exposure of W residues. This behavior is observed for all the spectroscopic characteristics obtained from fluorescence spectra *i.e.* SCM, $\lambda_{máx}$, FI and FI-$\lambda_{máx}$ (Table 1). This result indicates that the denaturation process of *Tc*TIM is a non two-state process as suggested previously [20].

Four state model of denaturation of *Tc*TIM

The analysis of the results presented here suggest that the model that describes de denaturation pattern of *Tc*TIM is a four state process. This reaction departs with the native enzyme (N_2) which at low Gdn-HCl concentrations (≈ 0.5 M) partially unfold to produce a first intermediate (I_1). After excitation at both 295 or 280 nm, this conformer show an increase in FI relative to native enzyme (Table 1). This increase in FI has not been no reported for any other TIM denaturation, and

it may be explained by either the effect of ionic strength on the fluorescence intensity or by the change in polar surroundings of the aromatic residues of the protein at low Gdn-HCl concentration. At medium denaturant concentrations (1.0 - 2.0 M Gdn-HCl) the first intermediate partially unfolds to produce a second equilibrium conformer (I_2) characterized by a plateau region in the unfolding pattern. At high denaturant concentrations I_2 totally unfolds to produce the unfolded monomers (2U). From the overall observation in changes in intrinsic fluorescence, the suggested pattern for *Tc*TIM equilibrium denaturation in Gdn-HCl is described by:

$$N_2 \leftrightarrows I_1 \leftrightarrows I_2 \leftrightarrows 2U \tag{1}$$

The equilibrium denaturation of homologous TIMs

The amino acid sequences and the three dimensional structures of oligomeric TIM from different species are similar. In spite of these structural similarities, the equilibrium-unfolding studies with enzymes from different species show heterogeneous behaviour. As we report here for the equilibrium unfolding of *Tc*TIM, the four state behavior was earlier described for the denaturation with Gdn-HCl of TIM from *Plasmodium falciparum* [11]. In this process one of the states is described as an aggregate and the process is irreversible. This fact complicates the study of the thermodynamics of the unfolding. The equilibrium unfolding of TIM from *T. brucei* (TbTIM) is even more complex [21], in this transition the irreversible aggregation of both non-native dimer and monomeric expanded intermediates was detected. Three-state process with a monomeric intermediate was reported for the enzyme of yTIM [9,10,19]. Two-state process has been observed for the enzymes of *B. stearothermophilus* (BsTIM) [18], rabbit (rTIM) [17,22,23] and *L. mexicana* (LmTIM) [12].

   In the extensively studied unfolding of TIM, irreversibility has been often observed [11,21,24,25]; on the other hand, for *Tc*TIM the renaturation reaction is fully reversible [28]. This observation suggests that in *Tc*TIM unfolding induced by Gdn-HCl, the observed intermediates are not aggregates. This conclusion is important because the denaturation pattern of the triosephosphate isomerase from *T. brucei* [21] (*Tb*TIM) a notably similar protein to *Tc*TIM [20] is irreversible. In *Tb*TIM denaturation induced by Gdn-HCl the intermediates where characterized as a non native dimer and as expanded monomer, it was also demonstrated that both intermediates are prone to aggregate. We are performing experiments in order to characterize the nature of I_1 and I_2. This information allows us to construct a model to address the thermodynamic characterization of the process.

Final remarks

Even the final folding and activity are conserved in TIM enzyme, the changes in the amino acids sequence causes very heterogeneous unfolding patterns in enzymes from different species. Even so, and despite the irreversibility and complexi-

ty often found in the unfolding of oligomeric proteins, the study of the energetic properties of these biomolecules are necessary because of the constant participation of oligomers in metabolism.

Experimental

Overexpression and purification of recombinant protein

E.coli BL21DE3 cells with recombinant TcTIM gene were a generous gift of Dr. Ruy Pérez-Montfort (Instituto de Fisiología Celular, UNAM, México). Overexpression and purification of TcTIM was performed as described by Ostoa-Saloma [29]. Purified fractions showed a single band by SDS-PAGE.

Gdn-HCl unfolding experiments monitored by fluorescence

Denaturation experiments were performed incubating 50 µg mL^{-1} of *TcTIM* at 25 °C in 100 mM TEA /10 mM EDTA /1.0 mM DTT, pH 7.4 for at least 48 h. Gdn-HCl concentration was increased from 0 to 6.0 M. The fluorescence changes were performed using an ISS PCI Photon Counting Spectrofluorometer (ISS, Urbana, IL) at 25 °C using both excitation wavelengths 280 and 295 nm (slit width = 1 mm). The spectral center of mass (SCM) of each spectrum defined as

$$SCM = \Sigma \lambda I(\lambda)/\Sigma I(\lambda) \tag{2}$$

was calculated with the ISS software. In equation (2) I(λ) is the fluorescence intensity at wavelength λ. Reference samples were subtracted from all the experimental measurements.

Acknowledgments

References

1. Hollecker. M.; Creighton, T. E. *J. Mol. Biol.* **1983**, *168*, 409-437.
2. Krebs H, Schmid FX, Jaenicke R. *J. Mol. Biol.* **1983**, *169*, 619-635.
3. Stackhouse TM, Onuffer JJ, Matthews R, Ahmed SA, Miles EW. *Biochemistry* **1988**, *27*, 824-832.
4. Kragelund, BB; Hojrup, P; Jensen, M. S.; Schjerling, C. K.; Juul, E.; Knudsen. J.; Poulsen, F.M. *J. Mol. Biol.* **1996**, *256*, 187-200.

5. Ferguson, N.; Capaldi, A.P.; James, R.; Kleanthous, C.; Radford, S.E.; *J. Mol. Biol.* **1999**, *286*,1597-1608.

6. Zerovnik, E.; Virden, R.; Jerala, R.; Turk, V.; Waltho, J.P. *Proteins* **1998**, *32*, 296-303.

7. Zabori, S.; Rudolph, R.; Jaenicke, R. *Z. Naturforsch.* **1980**, *35c,* 999-1004.

8. Mainfroid, V.; Terpstra, P.; Beauregard, M.; Frère, J.M.; Mande, S.C.; Hol, W.G.J.; Martial, J.A.; Goraj, K. *J. Mol. Biol.* **1996**, *257,* 441-456.

9. Vázquez-Contreras, E.; Zubillaga, R.; Mendoza-Hernández, G.; Costas, M.; Fernández-Velasco, D. A. *Protein Peptide Lett.* **2000**, *7*, 57-64

10. Morgan, C. J.; Wilkins, D. K.; Smith, L. J.; Kawata, Y.; Dobson, C. M. *J. Mol. Biol.* **2000** 300, 11-16

11. Gokhale, R. S.; Ray, S. S.; Balaram, H.; and Balaram, P. *Biochemistry* **1999** 38, 423–431.

12. Lambeir, A. M.; Backmann, J.; Ruiz-Sanz, J.; Filimonov, V.; Nielsen, J. E.; Kursula, I.; Norledge, B. V.; Wierenga, R. K. *Eur. J. Biochem.* **2000** 267, 2516-2524

13. Borchert, T.V.; Abagyan, R.; Jaenicke, R.; Wierenga, R.K. *Proc. Natl. Acad. Sci. USA,* **1994**, *91*, 1515-1518.

14. Schliebs, W.; Thanki, N.; Jaenicke, R.; Wierenga, R.K. *Biochemistry* **1997**, *36*, 9655-9662.

15. Fernández-Velasco, D.A.; Sepúlveda-Becerra, M.; Galina, A.; Darszon, A.; Tuena de Gómez-Puyou, M.; Gómez-Puyou, A. *Biochemistry* **1995**, *34*, 361-369.

16. Waley, S. *Biochem. J.* **1973**, *135*, 165-172.

17. Rietveld, A. W.; Ferreira, S. T. *Biochemistry* **1998**, *35*, 7743-7751

18. Mainfroid, V.; Mande, S.C.; Hol, W.G.J.; Martial, J.A.; Goraj, K. *Biochemistry* **1996**, *35*, 4110-4117.

19. Najera, M.; Costas, M.; Fernández-Velasco, D. A. *Biochem. J.* **2003**, *370*, 785-792

20. Chánez-Cárdenas, M. E.; Vázquez-Contreras, E. *Rev. Soc. Quím. Méx.* **2002**, *46, 3, 219-222*

21. Chánez-Cárdenas, M. E.; Fernández-Velasco, D. A.; Vázquez-Contreras, E.; Coria, R.; Saab-Rincón, G.; Pérez-Montfort, R. *Arch. Biochem. Biophys.* **2002**, *399*, 117-129

22. Moreau, V. H.; Rietveld, A.W. M.; Ferreira S. T. *Biochemistry* **2003**, *42*, 14831-14837

23. Pan, H.; Raza, A. S.; Smith, D. L. *J. Mol. Biol.* **2004**, *336*, 1251–1263

24. Alvarez, M.; Zeelen, J. P.; Mainfroid, V.; Rentier-Delrue, F.; Martial, J. A.; Wyns, L.; Wierenga, R. K.; Maes, D. *J. Biol. Chem.* **1998**, *273*, 2199-2206

25. Beaucamp, N.; Hofmann, A.; Kellerer, B.; Jaenicke, R. Protein Sci. **1997**, *6*, 2159-2165

26. Doolittle, R.F. Redundante in protein sequences. In *prediction of protein structure and the principles of protein conformation* (Fastman, G.D eds.) Plenum Press NY, **1989**, pp 599-623.

27. Maldonado, E.; Soriano-García, M.; Moreno, A.; Cabrera, N.; Garza-Ramos, G.; Tuena de Gómez-Puyou, M.; Gómez-Puyou, A.; Pérez-Montfort, R. *J. Mol. Biol.* **1998**, *283*, 193–203.

28. Zomosa-Signoret, V.; Hernández-Alcántara, G.; Reyes-Vivas, H.; Martínez-Martínez, E.; Garza-Ramos, G.; Pérez-Montfort, R.; Tuena de Gómez-Puyou, M.; Gómez-Puyou, A. *Biochemistry* **2003**, *42*, 331

29. Ostoa-Saloma, P.; Garza-Ramos, G.; Ramírez, J.; Becker, I.; Berzunza, I.; Landa, A.; Gómez-Puyou, A.; Tuena de Gómez-Puyou, M.; Pérez-Montfort, R. *Eur. J. Biochem.* **1997**, *244*, 700-705.