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# Effect of a specific inhibitor on the unfolding and refolding kinetics of dimeric triosephosphate isomerase: Establishing the dimeric and similarly structured nature of the main transition states on the forward and backward reactions

Edith González-Mondragón, Rafael A. Zubillaga, Andrés Hernández-Arana *

*Área de Biofisicoquímica, Departamento de Química, Universidad Autónoma Metropolitana-Iztapalapa, Apartado Postal 55-534, Iztapalapa, D.F., 09340, México*

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**Abstract**

2-Phosphoglycolate (PGA), a strong competitive inhibitor of the dimeric enzyme triosephosphate isomerase (TIM), brings about a large decrease in the unfolding rate constant of the protein. The data set of rate constants versus ligand concentration may be equally well explained by regarding either a monomeric or a dimeric transition state (TS). However, if the thermodynamics for binding of PGA to native TIM is taken into account, it becomes clear that a dimeric TS is the right assumption. Furthermore, by studying the effect of the ligand on the second-order refolding reaction, we found results indicating similar PGA-binding affinities to be present in the transition states for the rate-limiting steps of the forward and backward reactions. Most likely, therefore, both TS resemble each other in respect to the active site architecture. It should be mentioned, however, that our data do not rule out the possible occurrence of an unstable, (partially) folded monomeric intermediate, which would rapidly interconvert with the unfolded monomer.


*Keywords:* Unfolding kinetics; Refolding kinetics; Unfolding transition state; Inhibitor-binding; Dimeric transition state

## 1. Introduction

In a previous study [1], it was found that yeast triosephosphate isomerase (TIM), a dimeric enzyme of 53 kDa, unfolds via a first-order reaction at high temperature (i.e., above 52 °C, in solution of pH 7.4). As judged by far-ultraviolet circular dichroism (CD), thermally unfolded TIM retains some residual β-structure; in addition, it is able to refold and recover almost completely its enzymatic activity if quickly cooled down to 49 °C or less. As measured by CD, refolding occurs through a second-order reaction that has a large heat capacity of activation [1]. Therefore, the ensemble of structures representing the main transition state (TS) for refolding must comprise dimeric forms of the molecule. Furthermore, comparisons of activation parameters, derived from kinetic studies, with calorimetrically determined unfolding data seem to indicate, although not conclusively, that unfolding and refolding kinetics share a common TS. Nevertheless, it is conceivable that unfolding and refolding reactions may have different TS ensembles; that is, unfolding may first involve dissociation of the native dimer, followed by the loss of secondary structure of the monomers, which would then be the kinetic step observed by CD. If so, the main TS for unfolding would be a monomeric form of the protein. On the other hand, refolding might actually take place trough rapid formation of a monomeric "folded" intermediate prior to the rate limiting association step. Such a putative intermediate, however, should be unstable to keep in accord with the lack of a noticeable fast phase in kinetic CD curves [1].

Evidence for the presence of a monomeric intermediate has indeed been found in refolding studies in which TIM had been previously unfolded by guanidine hydrochloride [2,3], but the degree of recovery of secondary structure in this intermediate is not known. Folded monomeric intermediates are known to occur in the refolding pathways of other oligomeric proteins. For example, for phosphofructokinase, an intermediate with

* Corresponding author. Tel.: +52 55 5804 4674; fax: +52 55 5804 4666.
*E-mail address:* aha@xanum.uam.mx (A. Hernández-Arana).

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ATP-binding capacity seems to appear before the rate-limiting association step [4]. To address these issues, and also to characterize the degree of structural consolidation in the active site of TS, we carried out a study of the effect of 2-phosphoglycolate (PGA), a potent inhibitor of TIM, on the unfolding and refolding of the enzyme. The effect of specific ligands on the unfolding and refolding kinetics has been reported for a number of small, monomeric proteins. In some cases, such as those of α-lactalbumin [5] and barnase [6], the main transition state has been found to have significant ligand-binding affinity which is about 10- to 70-fold less than the affinity of the native protein. In some other cases, however, the ligand-binding capacity seems to be completely lost upon formation of the transition state [7–9]. Owing to the ubiquitous nature of the so-called "TIM barrel fold", studies such as the one presented here may add further insight into the understanding of the folding mechanism and the stability-activity trade-off in a large number of both monomeric and oligomeric proteins. It should be mentioned that the knowledge of the TIM-PGA-binding thermodynamics, a process that has been studied by a variety of methods [10], was essential to discriminate between alternative unfolding mechanisms postulated in this work.

## 2. Materials and methods

Overexpression and purification of TIM was carried out as described by Vázquez-Contreras et al. [2]. The preparations of TIM were found to be homogeneous as determined by SDS-PAGE and anion-exchange FPLC analysis. TIM concentration was determined from the absorbance at 280 nm, using the absorption coefficient $A^{1\%}_{1\,cm}=10.0$ [11].

### 2.1. Circular dichroism spectroscopy

All experiments were done in 0.010 mg ml$^{-1}$ TIM solutions, buffered with 10 mM Tris, pH 7.4. CD measurements were carried out in a JASCO J-715 spectropolarimeter (Jasco Inc., Easton, MD) equipped with a PTC-348WI Peltier type cell holder for temperature control; 1.00 cm cells were used to achieve appropriate magnetic stirring. To the cell containing TIM solution small aliquots from concentrated 2-phosphoglycolate (PGA) were added to reach final concentrations in the 0.20–1.50 mM range. Transitions of TIM thermal unfolding were registered by continuous monitoring of the ellipticity at 220 nm while the sample temperature was scanned at 2.0 °C min$^{-1}$; refolding profiles were recorded after the denaturation transition had been completed; cooling was also controlled through the Peltier accessory.

### 2.2. Unfolding and refolding kinetics

The kinetics of TIM unfolding was followed by changes in ellipticity at 220 nm [1]. Briefly, a small aliquot of concentrated TIM solution was injected into a 1.00 cm cell containing PGA solution at different concentrations (0.001–1.80 mM), previously equilibrated at 63 °C and submitted to continuous stirring. For data fitting, a single exponential decay equation was used: $\theta_t=\theta_f+(\theta_o-\theta_f)\exp(-k_u t)$, where $\theta_t$ is the ellipticity measured at time $t$, $\theta_f$ is the final ellipticity value, $\theta_o$ represents the corresponding value at zero time and $k_u$ is the unfolding rate constant. The refolding kinetics was monitored by means of CD at 220 nm according to Benítez-Cardoza et al. [1]. TIM was first denatured at 63 °C during 90 s, time sufficient to attain more than 95% of unfolding. Then, the temperature in the Peltier cell holder was set to a value 3.0 °C below the temperature of the refolding experiment (47.0 °C); this allowed fast cooling (15 °C min$^{-1}$ approximately) of the enzyme solution. When the temperature in the cell was 1.0 °C above the desired value, we added an aliquot of concentrated PGA solution to reach final concentration in the 0.01–1.80 mM range. Finally, when the temperature in the cell was 47.5 °C, the setting was raised to 47.0 °C. Kinetic data was fit with a second-order reaction equation, $\theta_t=\theta_f+(\theta_o-\theta_f)/(2C_o\ k_r t+1)$, where $C_o$ is the total molar concentration of TIM monomers, $k_r$ is the folding rate constant and the other parameters were already described above. All experiments were carried out in triplicate.

## 3. Results and discussion

An overview of the effect caused by PGA can be appreciated in Fig. 1, which shows thermal scannings of TIM in the presence of the ligand at different concentrations. The hysteresis

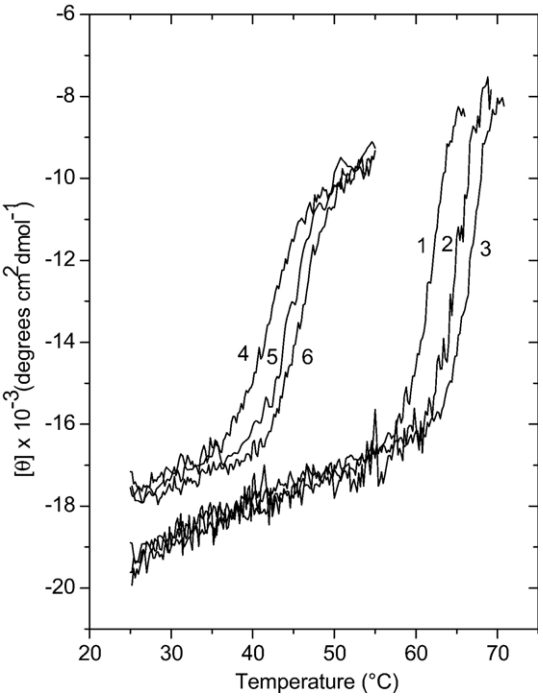



Fig. 1. Thermal unfolding–refolding transitions of *Saccharomyces cerevisiae* triosephosphate isomerase (TIM). Transition curves were obtained at heating or cooling rate of 2.0 °C min$^{-1}$, whereas the CD signal (expressed as mean residue ellipticity) at 220 nm was continuously recorded. Curves 1, 2 and 3 correspond to unfolding transitions at 2-phosphoglicolate (PGA) concentrations of 0.0, 0.2 and 1.5 mM, respectively. After unfolding had been completed, samples were quickly cooled to approximately 55 °C and recording of ellipticity was then started. Refolding curves 4, 5 and 6 correspond to PGA concentrations of 0.0, 0.2 and 1.5 mM, respectively. Solutions of TIM (10 μg ml$^{-1}$) in 0.01 M Tris buffer, pH 7.4, were employed.

of unfolding–refolding cycles has been reported in previous studies of yeast TIM [1,10]; this phenomenon has been attributed to the slowness of the unfolding and refolding kinetics of TIM within a temperature region where the unfolded monomer and the native dimer are both expected to be significantly populated at equilibrium [1]. As can be seen in Fig. 1, increasing concentrations of PGA cause both, unfolding and refolding, transition curves to shift to higher temperatures, indicating that native TIM is stabilized by the ligand. A quantitative study of the effect of PGA on the unfolding kinetics was carried out at 63 °C. At this temperature, the enzyme unfolds rapidly in the absence of PGA but the presence of the ligand markedly slows down the reaction (Fig. 2A); in all cases, first-order kinetics is obeyed. Experimental data were analysed, in first place, by assuming that the time course of the circular dichroism signal monitors the unfolding of TIM monomers with native-like structure. Then, the effect of PGA can be accounted for according to the model (Scheme 1), where M is the (folded) monomer and TS the transition state for unfolding; $K_{a,M}$ and $K_{a,TS}$ are affinity constants for the binding of the ligand, L, to M and TS, respectively. In the transition state theory, reaction rates are proportional to the fraction of reactant molecules in the transition state. Those fractions are represented by $K^{\ddagger}$ and $K^{\ddagger}_{L}$,

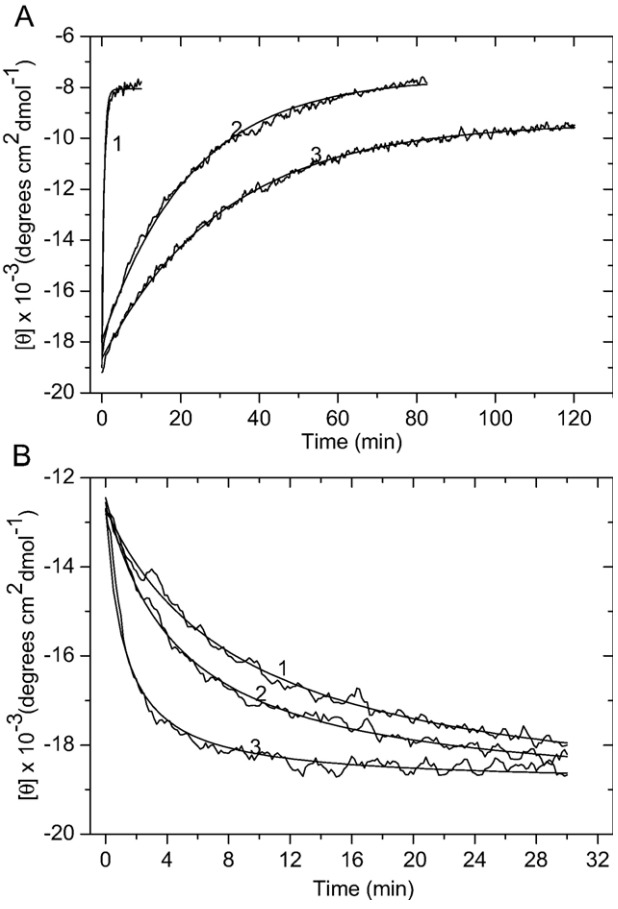
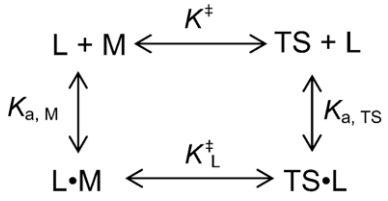



Fig. 2. (A) Kinetics of TIM unfolding (63 °C, pH 7.4) in the absence of PGA (curve 1), and in the presence of the inhibitor at concentrations of 0.8 (curve 2) and 1.8 mM (curve 3). (B) Kinetics of refolding (48 °C) at PGA concentrations of 0.0, 0.1 and 0.8 mM (curves 1, 2 and 3, respectively).




Scheme 1.

for the ligand-free and ligand-bound forms of M, respectively. If it is assumed that rapid preequilibrium between M and M • L is achieved, the following equation can be derived [5,12] (see also Appendix A):

$$\frac{k_{u,app}}{k_u^o} = \frac{1 + K_{a,TS}[L]}{1 + K_{a,M}[L]} \quad (1)$$

where $k_u^o$ is the unfolding constant in the absence of ligand and $k_{u,app}$ is the same constant when the concentration of unbound ligand is [L]. A plot of $k_{u,app}/k_u^o$ versus log[L] is shown in Fig. 3; the dashed line is the best fit of Eq. (1) to experimental data, which gave $K_{a,M} = 29 \pm 1.9 \times 10^3$ $M^{-1}$ and $K_{a,TS} = 0$ ($K_{a,TS}$ was constrained to be non-negative in the fitting procedure). Though Eq. (1) allows for a good fit to experimental data, the value of $K_{a,M}$ is certainly too large when compared to the value expected for the binding constant of native TIM: from the calorimetric study of PGA-binding to the native enzyme [5], the value of the

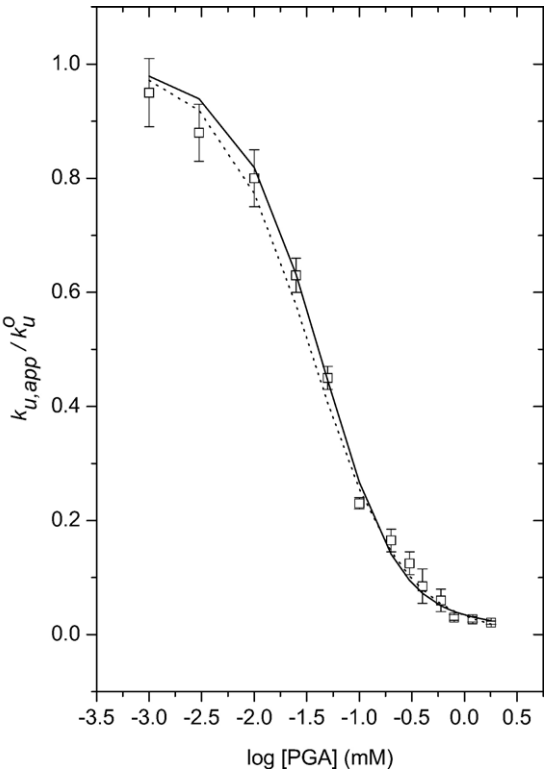



Fig. 3. Effect of PGA concentration on the (first-order) rate constant for TIM unfolding. Values of the rate constant, $k_u$, were obtained from fitting of a single exponential decay equation to kinetic traces such as those in Fig. 2(A); $k_u^o$ refers to the rate constant in absence of the ligand. The dashed line is the best fit of Eq. (1) to $k_{u,app}/k_u^o$ data; the solid line is the best fit according to Eq. (2).

binding constant for each identical site (at 63 °C) is calculated to be only $9\pm2\times10^3$ $M^{-1}$, much less than the $K_{a,M}$ above. Then, we considered a model in which the transition state for unfolding is dimeric (Scheme 2).

In Scheme 2, $N \cdot L_1$ and $N \cdot L_1'$ indicate microstates of native TIM with one molecule of bound PGA, whereas $N \cdot L_2$ stands for the enzyme with both binding sites occupied. All binding events in N are characterized by the same constant, $K_{a,N}$, because reported results [10,13] have shown that regarding both binding sites as identical and independent adequately reproduces binding isotherms of TIM. For simplicity, the same assumption was made for the binding of ligand to TS. As shown in Appendix A, the equation governing the behaviour of $k_{u,app}/k_u^o$ is

$$\frac{k_{u,app}}{k_u^o} = \left\{\frac{1 + K_{a,TS}[L]}{1 + K_{a,N}[L]}\right\}^2 \quad (2)$$

Best fit of Eq. (2) to unfolding data (solid curve in Fig. 3) was obtained with $K_{a,N}=12\pm1.0\times10^3$ $M^{-1}$ and $K_{a,TS}=1.4\pm0.3\times10^3$ $M^{-1}$. Because this value of $K_{a,N}$ closely approaches that which is expected from calorimetric binding data ($9\pm2\times10^3$ $M^{-1}$), it is clear that our results agree better with the transition state for unfolding being a dimer.

It remained to be shown whether the transition states for unfolding and refolding might possess similar structure or not. Thus, the effect of PGA on the refolding reaction of TIM was studied at 48.0 °C. At this temperature, refolding goes essentially to completion, without complications that arise from aggregation of the unfolded monomers [1]. Fig. 2B shows the accelerating action of PGA on the refolding reaction; separate studies performed at various protein concentrations (results not shown) indicated that the presence of ligand does not alter the (second) order of the reaction. The effect of PGA concentration on the refolding rate constant, $k_{r,app}$ is shown (squares) in Fig. 4; the dashed line is the best fit obtained with the equation:

$$\frac{k_{r,app}}{k_r^o} = \left\{\frac{1 + K_{a,TS}[L]}{1 + K_{a,U}[L]}\right\}^2 \quad (3)$$

which was derived by envisioning that PGA binds to the unfolded-TIM monomer, U, with an affinity constant $K_{a,U}$: $U + L \leftrightarrow U \cdot L$. Afterwards, dimeric transition-state species are formed as in Scheme 3.

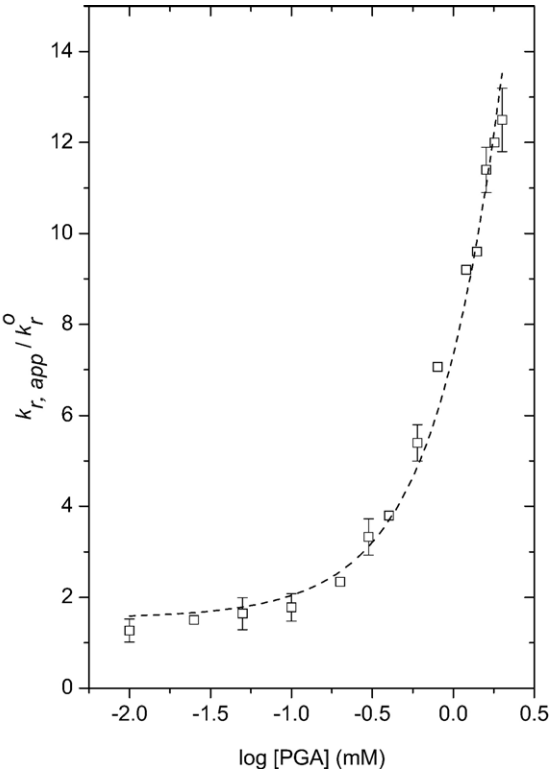
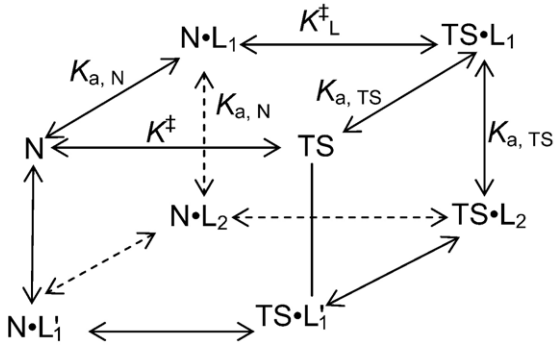
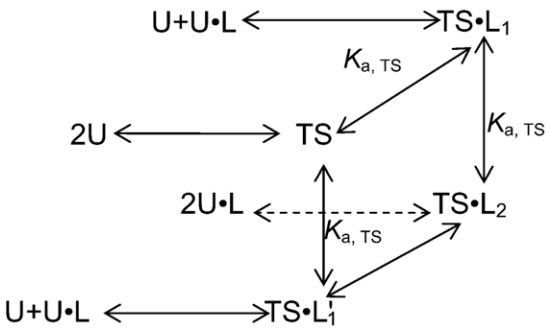



Fig. 4. Dependence of the rate constant for TIM refolding on the concentration of PGA. Values of the rate constant, $k_r$, were obtained from fitting of refolding traces (Fig. 2(B)) to a kinetic equation for a reaction that is of second order with respect to the concentration of unfolded monomers (see Ref. [1]); $k_r^o$ is the value of $k_r$ in the absence of ligand. The dashed line is the best fit of Eq. (3) to experimental data.

Best fit of Eq. (3) resulted in $K_{a,TS}=4.1\pm0.3\times10^3$ $M^{-1}$ and $K_{a,U}=0.8\pm0.1\times10^3$ $M^{-1}$. Thermodynamic parameters for TIM-PGA-binding may be used to compute that $K_{a,N}$ would reach a value of $1.5\times10^4$ at 48.0 °C; the ratio of this value to that for $K_{a,TS}$, obtained from refolding studies, indicates that the affinity for PGA of TS would be four-fold reduced with respect to that of the native enzyme; in comparison, unfolding studies (see above) indicate an affinity reduction of eight- to nine-fold; despite uncertainties introduced by extrapolation of thermodynamic binding data and experimental errors, such a




Scheme 2.




Scheme 3.

discrepancy between both results might be indicative of different structure, around the active site, of TS ensembles for the forward and backward reactions. On the other hand, although some residual secondary structure is present in the unfolded monomer, the value of $0.8\times10^3$ $M^{-1}$ for $K_{a,U}$ seems to be too large. For instance, competitive inhibitors, such as phosphoenolpyruvate and DL-glycerol 1-phosphate, bind to native TIM enzymes with apparent constants of about $1.0\times10^3$ $M^{-1}$, and less specific inhibitors (arsenate, sulphate and phosphate) display even smaller affinities (80 to 200 $M^{-1}$) [14]. With this in mind, we tried a more complex mechanism for the analysis of refolding data. A "folded" but unstable, monomeric intermediate, I, in rapid preequilibrium with U, was postulated to be present on pathway: $U\leftrightarrow I\rightarrow TS$. Fast equilibration between U and I (governed by a constant $K_e$), and a poorly populated I state ($K_e \ll 1$) were necessary assumptions to keep in accord with the observed overall second-order kinetics and the absence of any significant fast phase on CD-measured refolding curves. Of course, an additional binding constant, $K_{a,I}$, has to be introduced. It can be shown (see Appendix A) that the expression for the dependence of $k^o_{r,app}/k^o_r$ on [L] is

$$\frac{k_{r,app}}{k_r^o} = (1+K_e)^2\,\frac{1+K_{a,TS}[L]\left\{1+\frac{K_{a,U}}{K_{a,I}}\left\{1+K_{a,TS}[L]\right\}\right\}}{\left\{1+K_{a,U}[L]+K_e\left\{1+K_{a,I}[L]\right\}\right\}^2} \quad (4)$$

When we used Eq. (4), which has four adjustable parameters, results from data fitting showed overparameterization; that is, there was a strong dependency of two parameter values on the values of the other parameters, as well as very large errors associated to the parameters. Because this situation means that there are more parameters in Eq. (4) than are needed to express the data, the values of two parameters had to be fixed on the fitting procedure. As we were more interested in observing what effect the more complex model would have on $K_{a,U}$ and $K_{a,TS}$, we fixed the values of $K_e$ and $K_{a,I}$; besides, both of the varying parameters were constrained to ensure that, when used in combination with the values of the two fixed parameters, the amount of "folded" intermediate (I) formed in the preequilibrium phase did not exceed a small (5%) fraction of the total monomer concentration, even at the highest concentration of PGA employed in the experiments (i.e., the values of the equilibrium constants involved in Eq. (4) had to be consistent with the absence of a significant fast refolding phase). With these restrictions, the following parameters gave the best fit: $K_e=0.01$, $K_{a,I}=0.35\times10^3$ $M^{-1}$, $K_{a,TS}=2.5\pm0.1\times10^3$ $M^{-1}$ and $K_{a,U}=0.20\pm0.05\times10^3$ $M^{-1}$. As can be seen, use of Eq. (4) instead of Eq. (3) led to a decrease in both $K_{a,TS}$ and $K_{a,U}$. These latter results now indicate a transition state with six times lees affinity towards PGA than the native enzyme, in better agreement with the affinity reduction computed from unfolding studies. That is, both sets of results point out the similar structure of TSs for the rate-limiting steps of the forward and backward reactions. Likewise, the low value of $K_{a,U}$ seems to be in accord with the notion that a "thermally" unfolded TIM monomer should have very low affinity towards the ligand. However, because our experimental data can be equally well explained by either Eq. (3) or Eq. (4), the occurrence on the TIM folding pathway of unstable but "folded" monomer(s) should be regarded only as a reasonable possibility.

In summary, this work intended to show that studies on the effect of specific ligands in the unfolding–refolding kinetics of proteins, when used together with complete data sets of the corresponding binding thermodynamics, can be of help to discriminate between alternative folding mechanisms. Particularly, for the dimeric enzyme triosephosphate isomerase, kinetic data alone could be equally well explained by assuming either a monomeric or dimeric TS for the unfolding reaction. Nevertheless, the latter possibility agrees better with the already known thermodynamics of protein–ligand-binding.

## Acknowledgements

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## Appendix A

An equation expressing the effect of a ligand, L, on the unfolding rate of a protein with a single binding site can be derived by considering Scheme 1 in text. By assuming rapid equilibrium between ligand-free (M) and ligand-bound (M·L) forms of the protein, one can regard this scheme as a thermodynamic cycle from which the following relationships are obtained:

$$K^{\ddagger}K_{a,TS} = K_{a,M}K_L^{\ddagger} \quad (A1)$$

$$k_uK_{a,TS} = K_{a,M}k_{u,L} \quad (A2)$$

As mentioned in text, $k_u$ and $k_{u,L}$, which represent unfolding rate constants for M and M·L, respectively, are taken as proportional to $K^{\ddagger}$ and $K_L^{\ddagger}$. The observed unfolding rate is thus written as

$$\text{unfolding rate} = k_u[M] + k_{u,L}[M\cdot L]$$

By substituting $k_{u,L}$ by its value from Eq. (A2), and using the equilibrium relationship $[M\cdot L]=K_{a,M}[M][L]$, we can change the above rate expression to

$$\text{unfolding rate} = k_u[M]\{1+k_{a,TS}[L]\} \quad (A3)$$

Due to their high energy, transition states are barely populated; therefore, the partition of protein molecules can be expressed as

$$[M]_{tot} = [M] + [M\cdot L] = [M]\{1+K_{a,M}[L]\} \quad (A4)$$

and Eq. (A3) is then rewritten as follows

$$\text{unfolding rate} = k_u\frac{1+K_{a,TS}[L]}{1+K_{a,M}[L]}[M]_{tot} = k_{u,app}[M]_{tot} \quad (A5)$$

where $k_{u,app}$ is the experimental rate constant when the concentration of unbound ligand is [L]. Finally, the ratio of $k_{u,app}$ to the value of the rate constant in the absence of ligand, $k_u^o$, is given by:

$$\frac{k_{u,app}}{k_u^o} = \frac{1 + K_{a,TS}[L]}{1 + K_{a,M}[L]} \tag{A6}$$

which is Eq. (1) in text.

If unfolding proceeds directly from a native dimer (without prior dissociation into monomers), as in Scheme 2, there would be four unfolding species (i.e., N, N • $L_1$, N • $L_1'$ and N • $L_2$; see text). In this case, the unfolding rate expression becomes

$$\text{unfolding rate} = k_u[N] + k_{u,L}[N \cdot L_1] + k'_{u,L}[N \cdot L'_1] + k'_{u,L_2}[N \cdot L_2]$$

By assuming identical and independent binding sites, the above expression reduces to

$$\text{unfolding rate} = k_u[N] + 2k_{u,L}[N \cdot L_1] + k_{u,L_2}[N \cdot L_2]$$

Relationships between rate constants are obtained from the thermodynamic cycles in Scheme 2 as

$$k_u K_{a,TS} = k_{u,L} K_{a,N} \tag{A7}$$

$$k_{u,L} K_{a,TS} = k_{u,L_2} K_{a,N} \tag{A8}$$

Introducing (A7) and (A8), together with the binding relationships $[N \cdot L_1]=[N \cdot L_1']=K_a[N][L]$, one obtains

$$\text{unfolding rate} = k_u[N]\{1 + 2K_{a,TS}[L] + K_{a,TS}^2[L]^2\} \tag{A9}$$

On the other hand, the equation for the partition of protein species takes the form

$$[N]_{tot} = [N]\{1 + 2K_{a,N}[L] + K_{a,N}^2[L]^2\} \tag{A10}$$

Solving for [N] in the last equation and substituting into Eq. (A9), we have Eq. (A11),

$$\text{unfolding rate} = k_u\left\{\frac{1 + K_{a,TS}[L]}{1 + K_{a,N}[L]}\right\}^2 [N]_{tot} = k_{u,app}[N]_{tot} \tag{A11}$$

from which Eq. (2) in text immediately follows.

Regarding the effect of ligand on the refolding reaction, we note that according to Scheme 3 the second-order rate equation may be written as

$$\text{refolding rate} = -\left\{\frac{d[U]}{dT} + \frac{d[U \cdot L]}{dT}\right\}$$

$$\text{refolding rate} = 2k_u[U]^2 + 4k_{r,L}[U \cdot L][U] + 2k_{r,2L}[U \cdot L]^2 \tag{A12}$$

where $k_r$ is the refolding rate constant for the reaction involving association of two ligand-free unfolded monomers, U. The other rate constants pertain to the association of one ligand-bound with one ligand-free monomer ($k_{r,L}$), and of two ligand-bound monomers ($k_{r,2L}$). As before, the "equilibrium" relationships

$$k_r K_{a,TS} = k_{r,L} K_{a,U} \tag{A13}$$

$$k_{r,L} = K_{a,TS} = k_{r,2L} K_{a,U} \tag{A14}$$

can be introduced into Eq. (A12) to change rate constants for ligand-binding constants in transition states. After rearranging and also using: $[U \cdot L]=K_{a,U}[U][L]$, we obtain:

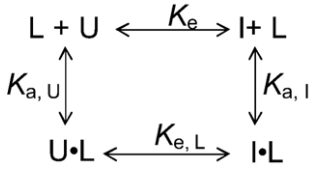
$$\text{refolding rate} = 2k_r[U]^2\{1 + 2K_{a,TS}[L] + K_{a,TS}^2[L]^2\} \tag{A15}$$

Because the total concentration of monomers, $C_o$, is equal to $[U]\{1+K_{a,U}[L]\}$, we can rewrite Eq. (A15) in the form of a second-order rate expression in terms of $C_o$:

$$\text{refolding rate} = 2k_r\left\{\frac{1 + K_{a,TS}[L]}{1 + K_{a,US}[L]}\right\}^2 C_o^2 = 2k_{r,app}C_o^2 \tag{A16}$$

from which Eq. (3) in text is easily derived.

If refolding involves on-pathway formation of a "folded", monomeric intermediate, I, we can derive an expression for the kinetic constant by assuming fast U↔I preequilibrium before the rate-determining association step. The equilibrium constant, $K_e=[I]/[U]$, has to be small enough for the population of I molecules formed in the preequilibrium fast phase to remain at low level. Indeed, four species are present on preequilibrium, as the following cycle shows (Scheme 4).




Scheme 4.

Once U and I are equilibrated with each other, their rates of concentration change should be related by

$$\frac{d[U]}{dt} = \frac{1}{K_e}\frac{d[I]}{dt} \tag{A17}$$

Ligand-bound species, U • L and I • L, are similarly related through $K_{e,L}$. However, regarding the cycle above, we see that $K_{e,L}=(K_eK_{a,I})/K_{a,U}$; thus, the expression equivalent to (A17) becomes

$$\frac{d[U \cdot L]}{dt} = \frac{K_{a,U}}{K_eK_{a,I}}\frac{d[I \cdot L]}{dt} \tag{A18}$$

Because I is assumed to be folded, the actual refolding rate, as reflected in the time course of CD, equals $-(d[U]/dt+d[U \cdot L]/dt)$; that is, according to (A17) and (A18), the refolding rate is thus given by

$$\text{refolding rate} = -\frac{1}{K_e}\frac{d[I]}{dt} - \left(\frac{K_{a,U}}{K_eK_{a,I}}\right)\frac{d[I \cdot L]}{dt} \tag{A19}$$

At this point, derivation of the refolding rate may follow along the same line as in the previous model (i.e., Eqs. (A12)–(A15)),

excepting that the time derivatives (d[I]/d$t$) and (d[I • L]/d$t$) should be weighted as indicated in (A19), and rate constants ($k_r$, $k_{r,L}$ and $k_{r,2L}$) refer in this case to association reactions proceeding from ligand-free and ligand-bound intermediate species. The "equilibrium" relationships expressed in (A13) and (A14) are also applicable here, but $K_{a,U}$ has to be replaced for $K_{a,I}$. Thus, by following the procedure used previously to derive Eq. (A15), we arrive in this case to the following expression for the refolding rate:

$$\text{refolding rate} = \frac{2k_r}{K_e}[I]^2\left\{1 + K_{a,TS}[L] + \frac{K_{a,U}}{K_{a,I}}K_{a,TS}^2[L]^2 + \frac{K_{a,U}}{K_{a,I}}K_{a,TS}[L]\right\} \tag{A20}$$

Finally, we must recall that the population of monomers is partitioned into four different states; therefore, the total concentration of monomers ($C_o$) is given by

$$C_o = [U] + [U \cdot L] + [I] + [I \cdot L]$$

$$C_o = [I]\left\{1 + \frac{1}{K_e} + \frac{K_{a,U}}{K_e}[L] + K_{a,I}[L]\right\} \tag{A21}$$

wherefrom [I] may be put as a function of $C_o$ and ligand concentration; after substituting in (A20) and rearranging, we find the desired form of expressing the reaction rate and defining $k_{r,app}$:

$$\text{refolding rate} = 2k_rK_e\frac{1 + K_{a,TS}[L]\left\{1 + \frac{K_{a,U}}{K_{a,I}}\{1 + K_{a,TS}[L]\}\right\}}{\{1 + K_{a,U}[L] + K_e\{1 + K_{a,I}[L]\}\}^2}C_o^2$$

$$\text{refolding rate} = 2k_{r,app}C_o^2 \tag{A22}$$

By setting [L]=0 in Eq. (A22), we also note that the rate constant in the absence of ligand is

$$k_r^o = \frac{2k_rK_e}{(1 + K_e)^2} \tag{A23}$$

The quotient of Eqs. (A22) and (A23) then gives Eq. (4) in text.

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