# Persistent Conformational Heterogeneity of Triosephosphate Isomerase: Separation and Characterization of Conformational Isomers in Solution†

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ABSTRACT: Subunit dissociation of dimeric rabbit muscle triosephosphate isomerase (TIM) by hydrostatic pressure has previously been shown not to follow the expected dependence on protein concentration [Rietveld and Ferreira (1996) *Biochemistry 35*, 7743−7751]. This anomalous behavior was attributed to persistent conformational heterogeneity (i.e., the coexistence of long-lived conformational isomers) in the ensemble of TIM dimers. Here, we initially show that subunit dissociation/unfolding of TIM by guanidine hydrochloride (GdnHCl) also exhibits an anomalous dependence on protein concentration. Dissociation/unfolding of TIM by GdnHCl was investigated by intrinsic fluorescence and circular dichroism spectroscopies and was found to be a highly cooperative transition in which the tertiary and secondary structures of the protein were concomitantly lost. A procedure based on size-exclusion chromatography in the presence of intermediate (0.6 M) GdnHCl concentrations was developed to isolate two conformational isomers of TIM that exhibit significantly different stabilities and kinetics of unfolding by GdnHCl. Complete unfolding of the two isolated conformers at a high GdnHCl concentration (1.5 M), followed by refolding by removal of the denaturant, completely abolished the differences in their unfolding kinetics. These results indicate that such differences stem from conformational heterogeneity of TIM and are not related to any chemical modification of the protein. Furthermore, they add support to the notion that long-lived conformational isomers of TIM coexist in solution and provide a basis for the interpretation of the persistent heterogeneity of this protein.

Triosephosphate isomerase (EC 5.3.1.1; TIM)[1] is a glycolytic enzyme that catalyzes the interconversion between D-glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. Rabbit muscle TIM is a homodimer of 26 kDa subunits, and its catalytic mechanism has been extensively studied (*1*−*5*). The primary sequences and crystal structures of TIM are considerably conserved in most of the different source organisms (*6*−*12*). Unfolding of TIM by guanidine hydrochloride (GdnHCl), as well as refolding following dilution of the denaturant, has been investigated in considerable detail (*13*−*21*).

Previous studies have shown that the pressure dissociation of dimeric proteins is generally predictably dependent on protein concentration, as expected from the law of mass action (*22*−*27*). On the other hand, for higher order oligomers such as trimers (*28*) and tetramers (*24*, *29*), a reduced dependence on protein concentration has been observed for subunit dissociation. Dissociation of larger, multisubunit oligomers shows very little or no dependence on protein concentration (*30*, *31*). The association of subunits in such oligomers has been described as a deterministic process, in analogy with the behavior exhibited by macroscopic objects (*32*). The lack of dependence on protein concentration in the dissociation of large oligomers has been attributed to long-lived conformational heterogeneity in the ensemble of molecules (*32*). According to this view, each individual oligomer is believed to have its own characteristic free energy of subunit association and is not in fast exchange with other oligomers in the ensemble. However, the structural basis of this putative conformational/energetic heterogeneity is not clear, and conventional methods used to separate proteins have not allowed the characterization of conformational isomers.

Previous studies of subunit dissociation and unfolding of TIM by hydrostatic pressure have revealed a lack of the expected dependence on protein concentration for subunit association (*33*). This anomalous behavior has been described as a deterministic process and has been explained on the basis of a very slow rate of subunit exchange between different dimers in the ensemble of molecules in solution due to the slow kinetics of TIM dissociation (*21*). The lack of effective subunit exchange between dimers prevents conformational averaging and leads to a persistent conformational heterogeneity (i.e., the coexistence of long-lived conformational isomers). In the present work, we show that subunit dissociation/unfolding of TIM by GdnHCl exhibits an anomalous reduced dependence on protein concentration.

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[1] Abbreviations: TIM, triosephosphate isomerase; GdnHCl, guanidine hydrochloride; DTT, dithiothreitol; SEC, size-exclusion chromatography; MALDI/TOF MS, matrix-assisted laser desorption ionization/time-of-flight mass spectrometry.

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In addition, we have used size-exclusion chromatography in the presence of GdnHCl to achieve physical separation of two energetically distinct, noninterconverting conformers of TIM. These results support the notion that the anomalous dissociation behavior of TIM is caused by persistent conformational heterogeneity of this protein.

## EXPERIMENTAL PROCEDURES

Rabbit muscle TIM (type X) and GdnHCl were from Sigma (St. Louis, MO). The purity and homogeneity of TIM samples were verified by nondenaturing and SDS−PAGE, as previously described (*33*), and by MALDI/TOF masss spectrometry using an Applied Biosystems Voyager spectrometer (see Results). All other reagents used were of the highest analytical grade available.

*Intrinsic Fluorescence Measurements.* Emission spectra were measured on an ISS PC1 spectrofluorometer (ISS Inc., Champaign, IL) at 23 °C. Excitation was at 280 nm, and emission spectra were recorded from 300 to 420 nm. The band-passes for excitation and emission were 8 and 16 nm, respectively.

The spectral center of mass ($\lambda_{av}$, intensity-weighted average emission wavelength) was calculated as

$$\lambda_{av} = \sum \lambda I_\lambda / \sum I_\lambda \quad (1)$$

where $I_\lambda$ is the fluorescence intensity at wavelength $\lambda$. Red shifts in the spectral center of mass were converted into fraction of dissociated/unfolded protein ($\alpha$) according to Weber (*34*):

$$\alpha_G = [1 + Q(\lambda_G - \lambda_U)/(\lambda_N - \lambda_G)]^{-1} \quad (2)$$

where $\lambda_N$ and $\lambda_U$ are the spectral centers of mass of completely native and completely unfolded TIM, respectively, $\lambda_G$ is the spectral center of mass at a given GdnHCl concentration, and $Q$ is the ratio between the fluorescence quantum yields of unfolded and native TIM.

*Circular Dichroism.* Far-UV CD spectra were recorded at 23 °C on a Jasco J-715 spectropolarimeter using a 0.1 cm path cell. The spectral range was from 240 to 200 nm, and the protein concentration used was 2 $\mu$M.

*Size-Exclusion Chromatography.* Size-exclusion chromatography was performed using a Superdex G-200 column (Pharmacia, Uppsala, Sweden) connected to a Shimadzu LC-10AS high-performance liquid chromatography apparatus. The column was previously equilibrated with the appropriate concentration of GdnHCl (see Results) in buffer containing 1 mM DTT and 100 mM Tris-HCl, pH 7.6. The flow rate was 0.7 mL/min, and the runs were monitored by absorption at 220 nm. In the absence of GdnHCl, native TIM eluted from the calibrated column at a retention time corresponding to 52 kDa.

## RESULTS

The dissociation/unfolding of TIM induced by GdnHCl was followed by the red shift in intrinsic fluorescence emission and by the decrease in ellipticity at 222 nm of samples equilibrated in the presence of different concentrations of GdnHCl for 48 h at room temperature (Figure 1A). Control measurements showed that no further changes in fluorescence emission or CD signal took place after 48 h for the entire range of GdnHCl concentrations investigated (see also ref *21*). TIM dissociates/unfolds in a sharp, highly cooperative transition between 0.4 and 0.7 M GdnHCl (Figure 1A). The CD and intrinsic fluorescence data are virtually superimposable, showing the same values for GdnHCl concentration at the transition midpoint ($C_{1/2} = 0.55$ M). This indicates the concomitant loss of the secondary and tertiary structures of TIM with no detectable partially folded intermediate states in the transition, in agreement with our previous report (*21*).

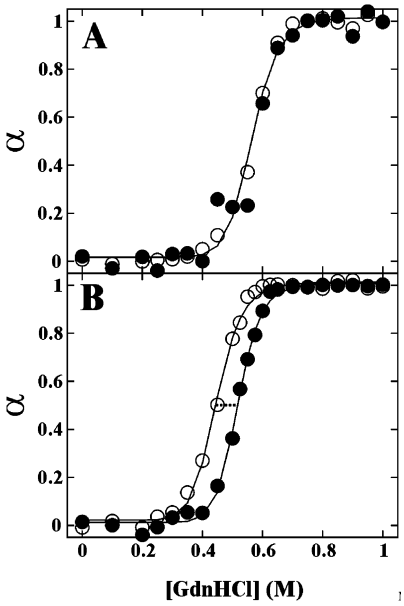


FIGURE 1: Dissociation/unfolding of TIM by GdnHCl. Panel A: Extent of reaction ($\alpha$) followed by the red shift in the fluorescence spectral center of mass (open circles) and by the decrease in ellipticity at 222 nm (closed circles). Panel B: Extent of reaction followed by the red shift of fluorescence emission using 1.2 $\mu$M (closed circles) or 0.12 $\mu$M (open circles) TIM. The dotted line indicates $\Delta C_{1/2}$ (see text). In both panels, samples were incubated for 48 h at room temperature in the presence of the indicated concentrations of GdnHCl before measurements.

Figure 1B shows the dependence of dissociation/unfolding by GdnHCl on the concentration of TIM. Subunit dissociation/unfolding was analyzed according to the linear extrapolation method (*35*) as

$$\Delta G = \Delta G_{H_2O} - m[\text{GdnHCl}] \quad (3)$$

where $\Delta G$ refers to the Gibbs free energy of subunit dissociation/unfolding in the presence of a given GdnHCl concentration, $\Delta G_{H_2O}$ is the free energy of subunit dissociation/unfolding in the absence of denaturant, and $m$ is a parameter that measures the steepness of the dependence of dissociation/unfolding on GdnHCl concentration. Following this approach, the expected dependence on protein concentration for subunit dissociation/unfolding induced by GdnHCl (21) is given by

$$\Delta C_{1/2} = RT/m \ln C_2/C_1 \quad (4)$$

where $C_1$ and $C_2$ are the two protein concentrations used in the experiments, $\Delta C_{1/2}$ is the difference in GdnHCl concentrations at the midpoint of the transitions, and $R$ and $T$ have their usual meanings. For a 10-fold change in protein concentration (such as shown in the experiment of Figure

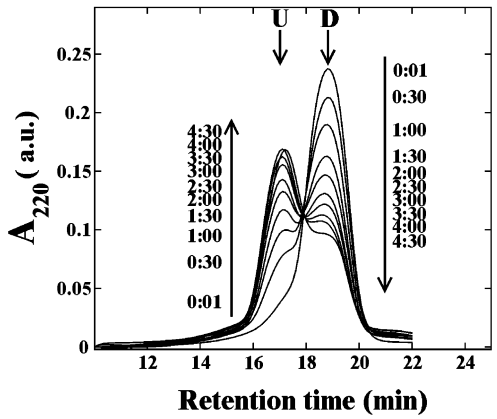


FIGURE 2: Size-exclusion chromatography analysis of the dissociation/unfolding of TIM. The peak labeled D, at a longer retention time (~19 min), corresponds to native dimeric TIM, and the peak at ~17 min, labeled U, corresponds to unfolded monomers. Note the existence of an invariant point at ~18 min of elution time, which indicates that the unfolding of TIM by GdnHCl is a two-state transition between native dimers and unfolded monomers. Sample injections (100 $\mu$L of a 2 $\mu$M TIM solution) were carried out at the indicated times of incubation in the presence of 0.7 M GdnHCl. The native dimer peak decreases with time as the unfolded monomer peak increases. Time is expressed in hours:minutes. The *y*-axis represents absorbance at 220 nm in arbitrary units.

1B), the predicted $\Delta C_{1/2}$ value thus calculated is 0.19 M. However, the experimentally obtained $\Delta C_{1/2}$ value under such conditions was ~0.08 M (Figure 1B). It is important to note that eqs 3 and 4 are based on the assumption of a true equilibrium between the species in solution. Therefore, the discrepancy between the experimental value of $\Delta C_{1/2}$ and the value calculated according to eqs 3 and 4 is a clear indication of the inadequacy of this analysis to properly describe the dissociation/unfolding behavior of TIM and suggests that a true equilibrium between native dimers and unfolded monomers is not observed. As a consequence, our criterion for defining the completion of dissociation/unfolding transitions of TIM is based on the fact that no additional spectrocopic changes can be detected after a prolonged period of incubation in the presence of denaturant (as noted above). This result is also in line with the reduced dependence on protein concentration previously described for subunit dissociation/unfolding of TIM by hydrostatic pressure (*33*).

Subunit dissociation/unfolding of TIM were also directly analyzed by size-exclusion chromatography (SEC) in the presence of GdnHCl (Figure 2). In this experiment, samples containing 2 $\mu$M TIM were incubated in the presence of 0.7 M GdnHCl, a concentration of denaturant that led to complete unfolding of the protein (see Figure 1). As shown in Figure 2, incubation for increasing periods of time at room temperature in the presence of 0.7 M GdnHCl led to a progressive decrease in the peak that eluted at ~19 min, corresponding to native dimeric TIM (52 kDa). Conversely, the peak that eluted at ~17 min, corresponding to unfolded monomers exhibiting a larger hydrodynamic radius, increased in parallel. It is interesting to note the presence, in the various chromatograms shown in Figure 2, of an invariant point at ~18 min of elution time. The existence of this invariant point supports the notion that only two species are present along the entire unfolding transition, indicating that unfolding of TIM proceeds directly from native dimers to unfolded

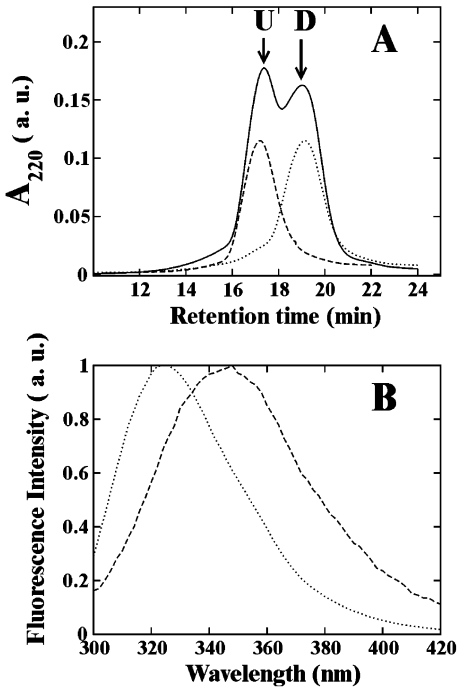


FIGURE 3: Lack of measurable exchange between isolated TIM fractions. Panel A: Size-exclusion chromatography of TIM (200 $\mu$M) incubated in the presence of 0.6 M GdnHCl for 48 h at room temperature (solid line). The two peaks (native dimers and unfolded monomers, labeled D and U, respectively) were separately collected, kept at room temperature for 4 h still in the presence of 0.6 M GdnHCl, and reinjected into the column. In this second chromatographic run, the sample that had originally been isolated as native dimers remained as a single peak with a retention time characteristic of native TIM (dotted line), while the sample that had originally been isolated as unfolded monomers eluted as a single peak with a retention time characteristic of unfolded monomers (dashed line). Panel B: Normalized fluorescence emission spectra of the isolated samples after 4 h of incubation in the presence of 0.6 M GdnHCl at room temperature. In both panels, the *y*-axis represents arbitrary units.

monomers, without detectable intermediates, consistent with the spectroscopic data presented above (Figure 1).

The results shown in Figure 2 indicated that SEC could be a useful tool to isolate folded and unfolded TIM fractions obtained by incubation in the presence of GdnHCl. To obtain sufficient quantities of protein in both folded and unfolded states for subsequent biophysical characterization (see below), we have carried out preparative SEC separations using samples of high protein concentration. Figure 3A shows the chromatographic separation of a 200 $\mu$M TIM sample that had been previously incubated in the presence of 0.6 M GdnHCl for 48 h at room temperature. Control experiments showed that no further spectroscopic (fluorescence and CD) changes took place in the sample after 48 h of incubation at room temperature. Furthermore, at 200 $\mu$M TIM the value of $C_{1/2}$ was shifted to 0.6 M GdnHCl. Thus, under these conditions approximately 50% of unfolded monomers and native dimers were found in solution (Figure 3A). To investigate the rate of exchange between native dimeric and unfolded monomeric species, the two peaks separated by SEC were collected, kept at room temperature for an additional 4 h, still in the presence of 0.6 M GdnHCl, and reanalyzed by SEC. As shown in Figure 3A, the fraction that had originally been isolated as native dimers (labeled "D") remained native, and the fraction corresponding to

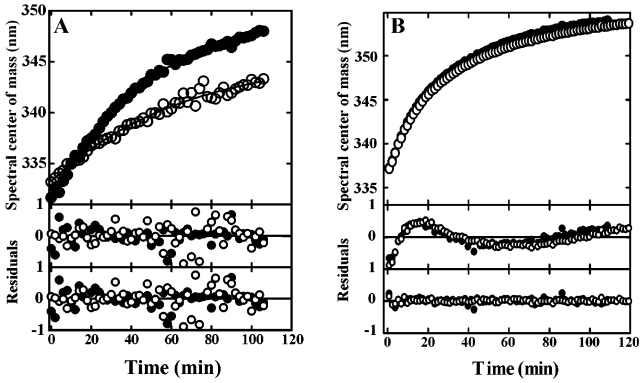


FIGURE 4: Kinetics of dissociation/unfolding of TIM in the presence of 0.7 M GdnHCl (followed by the red shift in the fluorescence spectral center of mass). Panel A: Native dimers (strong conformers) and unfolded monomers (weak conformers) were isolated by SEC as shown in Figure 3. Both samples were then run through a small desalting column for removal of GdnHCl. The unfolding kinetics of the strong (open circles) and weak (closed circles) samples were followed after addition of 0.7 M GdnHCl at time 0. Panel B shows the unfolding kinetics of a native TIM sample (closed circles) or a TIM sample that was previously submitted to a complete unfolding/refolding cycle as described in Results (open circles). The middle and lower panels show residuals plots of the fits of the kinetic data to single- or double-exponential models, respectively.

unfolded monomers (labeled "U") remained unfolded in the second chromatographic run. In addition, intrinsic fluorescence emission spectra of both samples were recorded. Figure 3B shows that the spectrum of the sample originally isolated as native dimers retained a blue-shifted emission characteristic of native TIM dimers, whereas the sample isolated as unfolded monomers exhibited a red-shifted fluorescence emission characteristic of unfolded TIM. These results indicate the lack of measurable exchange between native TIM dimers and unfolded monomers, even after 4 h of incubation at room temperature in the presence of 0.6 M GdnHCl. Similar results were observed when the isolated D and U fractions were kept overnight at 4 °C in the presence of 0.6 M GdnHCl (data not shown).

On the basis of their different stabilities to unfolding by GdnHCl, we classified the two TIM fractions isolated by SEC as "strong" (i.e., the fraction that remained native after incubation for 48 h in the presence of 0.6 M GdnHCl) and "weak" (i.e., the fraction that was completely unfolded). Upon removal of GdnHCl from the samples (using a desalting column), both strong and weak fractions exhibited fluorescence spectra characteristic of native TIM dimers (data not shown), indicating complete refolding of the weak TIM fraction. The kinetics of unfolding of such refolded strong and weak fractions were then investigated (Figure 4A). For this experiment, the protein concentrations of both fractions were adjusted to 2 $\mu$M, and 0.7 M GdnHCl was used to guarantee complete unfolding of both fractions (according to Figure 1). Although the two samples exhibited very similar initial fluorescence spectra (indicating that indeed both consisted of native TIM dimers), they showed significantly different kinetics of dissociation/unfolding by GdnHCl (Figure 4A). The refolded weak TIM sample (closed symbols) exhibited significantly faster unfolding kinetics than the strong sample (open symbols), which had remained native and dimeric throughout the chromatographic isolation step.

Table 1: Analysis of Unfolding Kinetics of TIM[a]

| species | $k_1$ (min$^{-1}$) | $k_2$ (min$^{-1}$) |
|---|---|---|
| native | 0.021 ± 0.001 | 0.097 ± 0.007 |
| strong[b] | 0.015 ± 0.002 | |
| weak[b] | | 0.042 ± 0.004 |
| refolded | 0.018 ± 0.001 | 0.085 ± 0.003 |
| refolded strong | 0.015 ± 0.001 | 0.059 ± 0.004 |
| refolded weak | 0.013 ± 0.001 | 0.071 ± 0.006 |

[a] Data for native and refolded TIM are from Figure 4B; data for isolated strong and weak fractions are from Figure 4A; data from refolded strong and refolded weak samples are from Figure 5. Data fits employed single- or double-exponential models and were performed using Jandel Scientific Sigma Plot 8. [b] For isolated strong and weak TIM fractions, analysis of the kinetics with a single-exponential model yielded good fits to the data (see Figure 4A), and therefore, a single kinetic constant is reported in these cases.

For both fractions, the kinetic data could be well described by single-exponential fits, as indicated by the residual plots shown in the middle panel of Figure 4A. Rate constants obtained from the fits to the kinetic data are shown in Table 1. Attempts to fit the data for each isolated fraction using a double-exponential model did not result in any improvement relative to the single-exponential fits (Figure 4A, lower panel).

Our previous studies indicated that the conformational heterogeneity of TIM is not related to chemical modification or any other form of covalent heterogeneity of the protein (*33*) but rather to the existence of long-lived conformational isomers (*21*). We have now extended the characterization of the chemical homogeneity of TIM employing MALDI/TOF mass spectrometry analysis. Analysis of a native TIM sample yielded a molecular mass of 26641 Da for the monomer, while analysis of a sample that was first incubated under fully denaturing conditions (1.5 M GdnHCl for 1 h at room temperature) and subsequently refolded by removal of the denaturant in a desalting column yielded a molecular mass of 26638 Da. Both values are also in excellent agreement with the expected molecular mass of TIM monomers. The almost exact coincidence of the molecular mass values found for native and refolded TIM not only argues against the existence of any chemical modification of the protein upon unfolding/refolding,but also excludes the possibility that the heterogeneity in stability of TIM fractions in the presence of GdnHCl could be due to the release of a tightly bound ligand from some or all TIM molecules upon unfolding. Furthermore, the kinetics of unfolding of a sample that was previously fully unfolded and refolded by passage through a desalting column was identical to that of a native sample (Figure 4B), which also argues against covalent modification and/or release of any ligand from TIM upon unfolding. Interestingly, attempts to fit the kinetic data for native and unfolded/refolded samples with a single-exponential model yielded poor results, as indicated by the significant systematic deviations shown in the residual plots (Figure 4B, middle panel). Double-exponential fits resulted in marked improvements in the fits, with randomly distributed residuals (Figure 4B, lower panel). Rate constants obtained from the fits to the kinetic data are shown in Table 1.

Finally, to confirm that the different sensitivities of the weak and strong conformers to GdnHCl were indeed related to conformational differences, both conformers in isolation

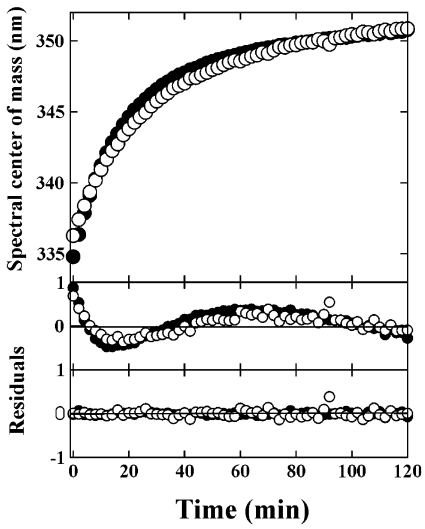


FIGURE 5: Kinetics of dissociation/unfolding of refolded TIM fractions in the presence of 0.7 M GdnHCl (followed by the red shift in the fluorescence spectral center of mass). Strong (open circles) and weak (closed circles) TIM fractions isolated by SEC were submitted to a full unfolding/refolding cycle as described in Results. The unfolding kinetics of the refolded samples were then followed after addition of 0.7 M GdnHCl at time 0. The middle and lower panels show residual plots of the fits of the kinetic data to single- or double-exponential models, respectively.

were submitted to a complete unfolding−refolding cycle by incubation in the presence of 1.5 M GdnHCl, followed by removal of GdnHCl as described above. After this cycle, the kinetics of unfolding of the two samples were again measured in the presence of 0.7 M GdnHCl (Figure 5, upper panel). Remarkably, the difference in unfolding kinetics of the strong and weak conformers disappeared after this unfolding/refolding cycle. Also of interest, very good fits to the kinetic data were obtained using a double-exponential model, whereas a single-exponential fit yielded poor results (Figure 5, middle and lower panels). This indicates that the differences in stabilities and unfolding kinetics between strong and weak samples are indeed of conformational origin and cannot be explained by covalent modification or chemical heterogeneity of TIM.

## DISCUSSION

The anomalous behavior of subunit association of TIM has been previously demonstrated (*21*, *33*) and was experimentally characterized by a complete lack of dependence of subunit dissociation on protein concentration. This was described in terms of a deterministic behavior of subunit association and was explained on the basis of the persistent conformational heterogeneity of TIM molecules in solution (*21*, *32*, *33*). Those previous studies also indicated that the heterogeneity of TIM was not related to the presence of isoforms, mutations, or other detectable forms of chemical modification of TIM.

In the present work, we have used SEC in the presence of intermediate GdnHCl concentrations to develop an experimental approach for the characterization of the persistent conformational/energetic heterogeneity of TIM dimers. The dissociation/unfolding of TIM induced by GdnHCl was initially followed by the red shift in intrinsic fluorescence emission and by the loss of CD ellipticity at 222 nm. The spectral changes indicated that the tertiary and secondary structures of TIM were lost in parallel in the equilibrium unfolding by GdnHCl. Indeed, a two-state transition between native dimers and unfolded monomers was supported not only by the highly cooperative spectroscopic changes (Figure 1A) but also by SEC analysis of the kinetics of unfolding (Figure 2).

Using 200 $\mu$M TIM, it was possible to obtain a mixture containing approximately 50% native dimers and 50% unfolded monomers after the unfolding reaction reached completion in the presence of 0.6 M GdnHCl (Figure 3). The condition involved incubation of TIM in the presence of 0.6 M GdnHCl for 48 h at room temperature, and control experiments showed that no further spectroscopic changes took place after 48 h of incubation (not shown). These two distinct TIM fractions (native or strong dimers and unfolded or weak monomers) could be separated by SEC. Interestingly, no measurable exchange between native and unfolded species took place in the isolated fractions even after several hours of incubation at room temperature in the presence of 0.6 M GdnHCl (Figure 3). This indicates that the rate of subunit exchange between native dimers and unfolded monomers is very slow, consistent with our previous results on the kinetics of unfolding by GdnHCl (*21*).

After refolding by removal of the denaturant, the TIM samples that had originally been isolated by SEC as strong and weak fractions exhibited significantly different kinetics of dissociation/unfolding induced by GdnHCl (Figure 4A). That such difference in unfolding kinetics was indeed due to conformational differences between the two fractions (and not to any kind of covalent heterogeneity) was confirmed by subjecting both isolated strong and weak TIM samples to a cycle of complete unfolding (in the presence of 1.5 M GdnHCl) followed by refolding. After this complete unfolding/refolding cycle, the fractions that originally behaved as strong and weak no longer exhibited any difference in the kinetics of dissociation/unfolding by GdnHCl (Figure 5). This means that full unfolding led to an effective conformational averaging of TIM dimers and abolished any preexistent conformational heterogeneity in the samples. Mass spectroscopy (MS) analysis provided additional evidence supporting the notion that the heterogeneity in stability of distinct TIM fractions in the presence of GdnHCl was not caused by chemical or covalent heterogeneity. The molecular mass values obtained by MS of native and fully unfolded/refolded TIM samples were almost identical, ruling out the existence of chemical modification of TIM upon unfolding/refolding. This analysis also excluded the possibility that the difference in stabilities of distinct TIM fractions could be due to the release of any tightly bound ligand from some or all TIM molecules upon unfolding.

The observation that the kinetics of unfolding of a TIM sample that had been fully unfolded/refolded was identical to that of a native TIM sample (Figure 4B) indicates that the original distribution of conformers present in native TIM samples is reestablished following a complete unfolding/refolding cycle. Taken together, these observations indicate that the existence of conformational isomers of TIM in the ensemble of dimers is at the origin of the heterogeneity of stabilities against GdnHCl-induced or pressure-induced (*33*) unfolding.

The existence of at least two distinct populations of conformers in native TIM samples is also supported by the analysis of the unfolding kinetic data (Figure 4B). This analysis showed that, for both native and fully refolded TIM, two distinct rate constants were necessary to adequately describe the data. By contrast, the data for each isolated conformer (strong and weak) could be well described using a single-exponential fit (Figure 4A). Interestingly, when both isolated fractions were fully unfolded/refolded, the double-exponential nature of the unfolding kinetis was reestablished (Figure 5), indicating the conformational averaging of the samples upon a full unfolding/refolding cycle.

In addition to our observations with TIM (*21*, *33*), the existence of conformational isomers has recently been reported for several other proteins, including the chaperonin GroEl (*31*), IgG antibodies (*36*), class II MHC (*37*, *38*), myoglobin (*39*), the prion protein (PrP) (*40*), and insect odorand-binding protein (*41*). In most of those cases, conformational isomerism was proposed to be associated with the capacity of the proteins to bind multiple ligands (*31*, *36*−*39*, *41*). Remarkably, in the case of prions, it has been reported that clinically distinct forms of the disease in a single animal species may not be associated with mutations in the PrP gene but rather to long-lived conformational differences between PrP molecules (*40*). Furthermore, such different "strains" of hamster PrP have been shown to exhibit distinct sensitivities to unfolding by GdnHCl (*40*). This behavior is likely due to the existence of multiple conformational isomers of PrP separated by sufficiently high activation free energy barriers, in analogy with our results on TIM.

The conformational heterogeneity and deterministic behavior of proteins have also been implicated in processes in which protein concentration is so low that protein molecules can be considered as isolated particles in solution. This is, for example, the case of the release of virus particles after cell lysis (*42*, *43*). In this case, if the virus were to dissociate readily by dilution into medium, the stochastic chance of subunit (re)association would be extremely small. Therefore, survival of the viral particles is made possible because the rate of dissociation of its subunits is negligibly slow (*43*), which, in turn, leads to the existence of multiple, persistent conformational isomers in the ensemble.

It is interesting to note that a significant parallel exists between the type of slow conformational dynamics and isomerism that we have described and previous results on the existence of nonequilibrium protein conformational substates obtained in low-temperature experiments. As previously pointed out (*43*), studies carried out by Frauenfelder and co-workers have been particularly revealing in characterizing the existence of such conformational states. They showed that the kinetics of rebinding of CO to myoglobin following flash photolysis was markedly nonexponential at subzero temperatures (*44*−*47*). Nonexponential kinetics arises from the existence of several conformational substates of myoglobin with distinct CO binding rates. At room temperature, the rate of interconversion between such multiple substates is usually fast (due to low activation barriers), which explains why the detection of conformational substates is favored at cryogenic temperatures. Furthermore, using time-resolved fluorescence as a probe of protein conformational dynamics, we and others have shown that proteins exhibit significant conformational heterogeneity even at ambient temperature (for early examples, see refs *48*−*51*). These results indicate that the activation free energy barriers for interconversion between substates may be sufficiently high to lead to the observation of conformational heterogeneity at room temperatur, and add further support to the notion of conformational substates advanced by Frauenfelder and co-workers. Thus, the existence of long-lived conformational isomerism of TIM supported by our present results may be seen as an extension of the conformational substates model.

Finally, some of the proteins reported to present conformational isomerism carry out their biological functions at very low concentrations, when subunit association is thermodynamically unfavorable (*36*−*38*). Thus, a complete description of the functional processes carried out by those proteins should take into account the existence of a set of persistent conformational isomers in the ensemble of protein molecules. The persistent conformational isomerism and deterministic behavior of proteins may play a fundamental role in the stability and function of those proteins, since many biological process occur in time scales that are comparable to, or shorter than, the slow dynamics of conformational interconversion between isomers.

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