Refolding of Triosephosphate Isomerase in Low-Water Media Investigated by Fluorescence Resonance Energy Transfer†

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ABSTRACT: The refolding and reassociation of rabbit muscle triosephosphate isomerase (TPI) monomers unfolded with guanidine hydrochloride (GdnHCl) was studied in aqueous media and in reverse micelles (RM) formed with hexadecyltrimethylammonium bromide and *n*-octane/hexanol. Fluorescence resonance energy transfer (FRET) studies were carried out using TPI labeled at Cys-217 with 5-((2-((iodoacetyl)-amino)ethyl)amino)naphthalene-1-sulfonic acid (1,5-IAEDANS) and TPI labeled at one of the free amino groups with fluorescein 5′-isothiocyanate (FITC). Efficient FRET between monomers of AEDANS−TPI and FITC−TPI in aqueous media was detected 2−3 min after denaturant dilution and remained constant for hours. The distance between AEDANS and FITC in a labeled, renatured hetero-TPI dimer calculated from FRET results was 48 Å, in reasonable agreement with estimates based on the crystal structure of TPI. In RM, recovery of enzyme activity during renaturation correlates with the appearance of a high-intrinsic fluorescence intermediate believed to be a partially folded monomer (Fernández-Velasco et al., 1995). Nevertheless, when AEDANS- and FITC-labeled monomers were mixed in RM, FRET occurred as soon as GdnHCl was diluted (FRET efficiency = 0.36), preceding the changes in TPI intrinsic fluorescence and reactivation. Thereafter, the efficiency of FRET increased during the next hour up to ∼0.50, where it remained after 24 h, when 80% of the enzyme activity was recovered. The high initial FRET seen in RM could indicate the formation of an inactive dimer within the first minutes after denaturant dilution. The further increase in FRET observed over the next hour could reflect conformational rearrangements of the protein and transition from the inactive to the active dimer.

Although water is fundamental to the stability and function of proteins (Kauzmann, 1959; Privalov & Grill, 1988; Rupley & Careri, 1991), its influence on protein folding is not well understood. Reverse micelles offer the possibility of controlling the amount of water available to proteins found in their interior (Luisi et al., 1988). Indeed, in reverse micellar systems it has been shown that by varying the water content of the system, it is possible to control the kinetics of reactivation of the homodimeric enzyme triosephosphate isomerase (TPI) (Fernández-Velazco et al., 1995). Thus, studies of protein folding and protein−protein interactions in such systems may yield insight into the steps and mechanisms that operate in the formation of the catalytic active enzymes from unfolded protein structures.

TPI is an enzyme well suited for such studies. It is a homodimer that catalyzes the interconversion of dihydroxyacetone phosphate (DHAP) and D-glyceraldehyde-3-phosphate (GAP) through mechanisms that have been well characterized (Albery et al., 1976, 1977; Knowles, 1991; Wierenga et al., 1991, 1992; Noble et al., 1993; Mande et al., 1994). Of particular relevance is that TPI dissociates and denatures in the presence of GdnHCl and renatures upon denaturant removal in standard aqueous media, recovering its catalytic activity (Sawyer & Gracy, 1975; Zabori et al., 1980). Reactivation of TPI from its unfolded monomers has also been studied in reverse micelles (Garza-Ramos et al., 1992; Fernández-Velasco et al., 1995). These experiments, both in aqueous media (Waley, 1973; Zabori et al., 1980) and in reverse micelles (Garza-Ramos et al., 1992), showed that TPI reactivates through the following sequence of events:

$$M_u \overset{\text{I}}{\leftrightarrow} M_f \overset{\text{II}}{\leftrightarrow} D$$
$$\downarrow$$
$$M_x$$

where M_u is an unfolded monomer that is first transformed into M_f (monomers that are able to dimerize forming the catalytically active dimer D). M_x denotes conformations unable to generate active dimers. In aqueous medium and in reverse micelles it has been shown that, at relatively low protein concentrations, dimerization (step II) is rate limiting.

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Nevertheless, in reverse micelles the rate of reactivation is orders of magnitude slower than in aqueous media. Moreover, the reactivation process can be reversibly arrested by changes in the amount of water in the system (Fernandez-Velasco et al., 1995).

Until now subunit association in reverse micelles has been monitored indirectly through the appearance of enzyme activity. This approach, however, does not allow detection of inactive dimers which can subsequently transform into catalytically active dimers. In this work, we have used fluorescence resonance energy transfer (FRET) to explore the renaturation of TPI from unfolded labeled monomers and the possible existence of inactive and active dimers in reverse micelles. We labeled rabbit muscle TPI with 1,5-((2-((iodoacetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid (1,5-IAEDANS) in the SH group of Cys-217 and with fluorescein 5′-isothiocyanate (FITC) in one of its free amino groups. This strategy provides direct information on distance changes between protein-attached donor−acceptor groups and hence on protein−protein interactions (Cheung, 1991).

MATERIALS AND METHODS

Materials. Rabbit muscle triosephosphate isomerase, cetyltrimethylammonium bromide, *n*-octane, hexanol, α-glycerophosphate dehydrogenase, NADH, and glyceraldehyde-3-phosphate diethyl acetal were obtained from Sigma. Glyceraldehyde-3-phosphate was prepared from glyceraldehyde-3-phosphate diethyl acetal as described by the supplier. 1,5-IAEDANS and FITC were obtained from Molecular Probes (Eugene, OR). Other reagents were of the highest analytical grade commercially available.

Determination of Enzyme Activity and Concentration. Enzyme activity in aqueous medium was measured as described previously (Rozacky et al., 1971) and was around 4000 μmol mg^{-1} min^{-1} with glyceraldehyde-3-phosphate as substrate. TPI concentration was determined spectrophotometrically by its absorbance at 280 nm (Lu et al., 1984). The concentrations of IAEDANS or FITC were spectrophotometrically determined by using their molar extinction coefficients (5.7 mM^{-1} at 336 nm or 76 mM^{-1} at 495 nm, respectively). In the case of FITC-labeled TPI, protein concentration was corrected for absorption by FITC. The absorbance of FITC at 280 nm equals 40% of its absorbance at 495 nm. 1,5-IAEDANS absorbs at 280 nm 20% of its absorbance at 336 nm. These values were subtracted accordingly from the measured absorbance at 280 nm of labeled TPI.

Labeling of TPI with 1,5-IAEDANS or FITC. TPI was dissolved in 50 mM Tris-HCl, pH 8.5, at a concentration of 20 mg/mL (0.74 mM monomer). 1,5-IAEDANS (or FITC) was added to this solution at a 5-fold (or 3.5-fold for FITC) molar excess over TPI monomers. The reaction was allowed to proceed at room temperature in the dark for various times (as described under Results); free label was then removed by filtration centrifugation (Penefsky, 1976) in Sephadex G-10 columns (1 cm^3) equilibrated with 40 mM triethanolamine, 10 mM EDTA, and 1 mM dithiothreitol, pH 7.4 (buffer A). When labeling was performed using different ratios of fluorophore:protein (see Results), reaction time was kept constant at 15 h.

Reverse Micelles. The reverse micellar system used was formed with 200 mM cetyltrimethylammonium bromide dissolved in *n*-octane:hexanol (8.7:1, v/v). Reverse micelles were obtained by adding a few microliters of buffer A (see above) to give the desired water concentration (from 2.0% to 7%, v/v). This was followed by vigorous vortexing. Phase diagrams for this system of reverse micelles have been described (Hilhorst et al., 1984). Reverse micelles containing entrapped TPI, AEDANS−TPI, or FITC−TPI were stable for at least 1 week.

Enzyme Activity in Reverse Micelles. Activity was measured in reverse micelles (6% water content) containing buffer A plus 3.3 mM glyceraldehyde-3-phosphate, 0.2 mM NADH (overall concentration), and 5−7 mg/mL α-glycerophosphate dehydrogenase. Ammonium sulfate was removed from the latter enzyme by filtration−centrifugation (Penefsky, 1976) in Sephadex G-50 columns equilibrated with buffer A. To this micellar solution was added an aliquot (10−20 μL) of reverse micelles containing either native or fluorescent-labeled TPI. The decrease in optical density at 340 nm was used to follow activity. The activity of TPI in reverse micelles with 6% water was 400−450 μmol mg^{-1} min^{-1}.

Proteolytic Digestion. Digestion of AEDANS−TPI using V_8 protease was carried out at 37 °C for 30 min in 20 mM Tris-HCl, pH 8.5, at a TPI:protease molar ratio of 100:1. Proteolysis was monitored by changes in electrophoretic mobility in SDS−PAGE according to Laemmli (1970). Gels were stained with Coomassie blue R-250. IAEDANS fluorescence in the peptides separated by electrophoresis was detected by placing the gels on top of a UV transilluminator (λ_{exc} = 310 nm) before staining. Gels were then photographed using a Corning 3-70 cut-off filter placed in front of the camera lens.

Amino Acid Sequencing. Peptides generated by V_8 protease digestion were resolved by SDS−PAGE and electroblotted onto immobilon-P membranes (Millipore) at 0.5 A for 15 min and sequenced by automated Edman degradation using an ABI 475A gas phase instrument.

Absorption and Fluorescence Measurements. Absorption spectra were measured on a Hewlett Packard 8548 spectrophotometer. Fluorescence spectra (excitation band pass 2 nm; emission band pass 8 nm) were measured on an Aminco SLM 8000 spectrofluorometer. For intrinsic fluorescence measurements excitation was at 286 nm. For AEDANS−TPI or in energy transfer experiments (see below), excitation was at 360 nm and emission spectra were acquired from 370 to 600 nm. Temperature was maintained at 25 °C with a thermostated cell holder.

Fluorescence spectral center of mass (SCM; average emission wavelength) was calculated as:

$$\mathrm{SCM} = \Sigma\lambda I(\lambda)/\Sigma I(\lambda) \qquad (1)$$

where $I(\lambda)$ is the fluorescence intensity at wavelength λ. Fluorescence spectra of identical control samples not containing protein were recorded and subtracted from the experimental samples to correct for background interference, which was significant (up to 20%) in the reverse micelles system.

Fluorescence anisotropy measurements were made on the same fluorometer equipped with polarizers and quartz Polacoat filters. Fluorescence was excited at 360 nm for AEDANS−TPI (or 494 nm for FITC−TPI) and observed at 470 nm (or 530 nm for FITC−TPI). Fluorescence polariza-

tion was calculated from the equation:

$$P = [I_{VV} - I_{VH}(I_{HV}/I_{HH})]/[I_{VV} + I_{VH}(I_{HV}/I_{HH})] \quad (2)$$

where I represents fluorescence intensity and the subscripts V and H represent vertically or horizontally aligned polarizers for excitation or emission, respectively. Polarization was then converted to anisotropy (r) according to $r = 2P/3 - P$.

Fluorescence Resonance Energy Transfer. The efficiency E of FRET between probes was determined by measuring the fluorescence intensity of the donor (AEDANS−TPI) in both the absence (F_D) and presence (F_{DA}) of acceptor (FITC−TPI), as given by:

$$E = 1 - F_{DA}/F_D \quad (3)$$

The efficiency of FRET depends on the inverse sixth power of the distance between donor and acceptor (Lakowicz, 1983). This allows FRET measurements to be used with high sensitivity to follow reassociation of TPI monomers during refolding of the enzyme in aqueous medium and reverse micelles. From the value of E, the average distance, R, between donor and acceptor was calculated as:

$$R = R_0[(1 - E)/E]^{1/6} \quad (4)$$

The Förster critical distance (R_0) is an intrinsic constant for each energy transfer pair, corresponding to the distance at which the efficiency of energy transfer is 50%, and was determined (Cheung, 1991) as:

$$R_0 = 9786(Jn^{-4}k^2Q_D)^{1/6}\ \text{Å} \quad (5)$$

where n is the refractive index of the medium (1.4 for aqueous medium), k^2 is a factor describing the relative orientation of the transition dipoles of the donor and acceptor chromophores (2/3 for probes exhibiting isotropic, dynamic orientational averaging during the excited state lifetimes; Cheung, 1991), and Q_D is the fluorescence quantum yield of the donor in the absence of acceptor. For AEDANS−TPI, a Q_D value of 0.68 was used, based on comparison of emission intensities of samples containing identical concentrations of AEDANS−TPI or IAEDANS alone in water or ethanol (Q_D values for IAEDANS in water or ethanol are 0.27 and 0.69, respectively; Molecular Probes). J is the spectral overlap integral between donor emission [F(λ)] and acceptor absorption [$\epsilon(\lambda)$] spectra and can be calculated by:

$$J = \int F(\lambda)\epsilon(\lambda)\lambda^4\, d\lambda / \int F(\lambda)\, d\lambda \quad (6)$$

J was calculated by overlapping the donor (AEDANS−TPI) emission and acceptor (FITC−TPI) absorption spectra. A summation was performed at 2 nm steps for the overlapping area (Mallender et al., 1994):

$$J = \Sigma I_{FD}\epsilon_A\lambda^4/\text{area}_{FD} \quad (7)$$

where I_{FD} is the donor fluorescence intensity (in arbitrary units) at wavelength λ and ϵ_A is the acceptor absorption (in absorbance units) at wavelength λ of the overlapping area. Area_{FD} is the area (in fluorescence arbitrary units) of the donor emission spectrum. The overlap integral J was calculated to be 8.48×10^{-14} cm^3 M^{-1}. Using the above indicated values, R_0 for the AEDANS−TPI/fluorescein−TPI pair was calculated to be 8.48×10^{-14} cm^3 M^{-1}.

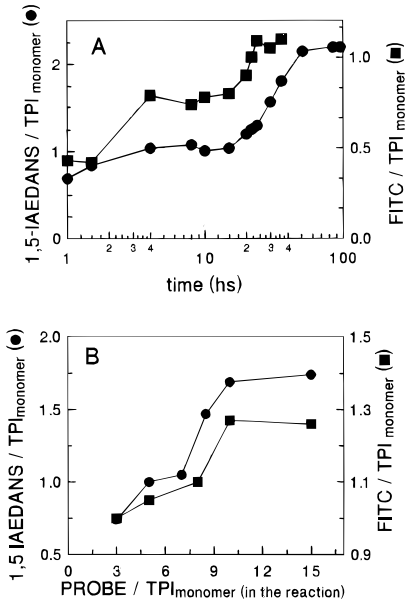

FIGURE 1: Labeling of TPI with 1,5-IAEDANS and FITC as a function of incubation time and probe:protein ratio. See Materials and Methods for the fluorophore/TPI monomer stoichiometry determination: (A) labeling ratio (1,5-AEDANS and FITC/monomer of TPI) versus time of incubation of TPI with 1,5-IAEDANS (5-fold molar excess/monomer) and FITC (3.5-fold molar excess/monomer) and (B) labeling ratio (1,5-AEDANS and FITC/monomer of TPI) after incubation of TPI with various molar ratios of 1,5-IAEDANS and FITC for 15 h.

RESULTS

TPI can be reversibly denatured into unfolded monomers by incubation with guanidine hydrochloride (GdnHCl) (Waley, 1973; Zabori et al., 1980). In previous studies the kinetics of reactivation of TPI following dilution of denaturant were investigated in the reverse micelles system used here (Garza-Ramos et al., 1992; Fernández-Velasco et al., 1995). Measurements of the intrinsic fluorescence of TPI during reactivation showed a parallelism between structural changes and appearance of catalytic activity (Fernández-Velasco et al., 1995). In this work TPI was covalently labeled with extrinsic fluorescent probes in order to further characterize the steps (i.e., monomer folding, subunit association, structural rearrangements of the dimer) involved in reactivation of the enzyme after unfolding by GdnHCl.

Fluorescent Labeling of TPI. Figure 1A shows the time course of reaction of TPI with a 5-fold molar excess of 1,5-IAEDANS (IAEDANS). The labeling stoichiometry (AEDANS−TPI monomer) increased with time and reached a level of about 1 mol of AEDANS/mol of TPI monomers between 4 and 15 h. At longer reaction times the stoichiometry of labeling increased to a total of about 2 mol of IAEDANS/mol of monomer (Figure 1A). The dependence of the labeling stoichiometry on the molar ratio AEDANS:TPI is shown in Figure 1B. With a constant reaction time of 15 h, it was possible to selectively label TPI with 1 mol of IAEDANS/monomer using a ratio of 5:7 probes:monomer in the reaction (Figure 1B).

Labeling of TPI with FITC was carried out under similar conditions as those used for IAEDANS. Figure 1A also shows the time course of reaction of TPI with FITC using a

3.5-fold molar excess of probe relative to monomer. Reaction of TPI with FITC was slower than with 1,5-IAEDANS, and the stoichiometry of labeling reached about 1−1.2 mol of FITC/mol of TPI monomer after 20 h of incubation. The dependence of the labeling reaction on FITC concentration at a constant reaction time of 15 h is shown in Figure 1B. In a range of FITC:monomer ratios between 3:1 and 15:1, the labeling stoichiometry remained around 1−1.2 mol of FITC/mol of monomer.

TPI labeled at ~1:1 mol of 1,5-IAEDANS or FITC/mol of monomer usually retained ~80% of its activity (see Materials and Methods).

Determination of the IAEDANS Binding Site. TPI was labeled at a 1:1 molar ratio of 1,5-IAEDANS:monomer. The fluorescent protein sample was then digested with V_8 protease (see Materials and Methods), which gave two proteolytic fragments resolved by SDS−PAGE (data not shown). The smallest fluorescent fragment of 8 kDa was electroblotted onto immobilon-P (Millipore) membranes and used for automated Edman degradation amino acid sequencing. This analysis revealed a sequence which exactly matched the sequence of Glu-165 to Val-184 of rabbit TPI (Corran & Waley, 1973). In the region from amino acid 194 to amino acid 248 (carboxy terminal), there is only one cysteine at position 217. Therefore, cysteine 217 was the only candidate to be covalently modified by IAEDANS. Interestingly, trypanosomal TPI has three cysteines but does not contain a cysteine at position 217 (Swinkels et al., 1986). We tried to label trypanosomal TPI with 1,5-IAEDANS, but it did not react with the fluorophore. Taken together these observations indicated that Cys-217 was the most reactive cysteine with 1,5-IAEDANS in rabbit TPI.

Similar experiments were performed with FITC−TPI labeled at a 1:1 molar ratio. A 7 kDa fragment obtained after V_8 digestion matched the rabbit muscle TPI sequence from Glu-165 to Ala-176. Since there are five lysines in the carboxy terminal region from Glu-176 to Gln-248, one of them must be labeled by FITC.

Unfolding of TPI by GdnHCl. The main purpose of the present study was to investigate the kinetics and mechanism of refolding of TPI both in aqueous medium and in reverse micelles. To gain insight into the mechanism of unfolding induced by GdnHCl, we initially carried out equilibrium unfolding studies by monitoring the intrinsic fluorescence of TPI and the environment-sensitive fluorescence of AEDANS−TPI derivative in aqueous medium. Incubation of TPI with increasing concentrations of GdnHCl promoted a marked (23 nm) red shift of the intrinsic fluorescence emission (Figure 2A), revealing a major increase in exposure of tryptophan residues to water in the unfolded state. The midpoint of intrinsic fluorescence changes occurred at 0.7 M GdnHCl. For AEDANS−TPI, a similar red shift (21 nm) of the emission was observed upon unfolding by GdnHCl (Figure 2A). The red shift in AEDANS−TPI emission and significant (ca. 55%) fluorescence quenching (Figure 3A) indicate that Cys-217 became increasingly exposed to water at higher GdnHCl concentrations. It was observed that the fluorescence of AEDANS−TPI, but not of native TPI, was affected by 0.5 M GdnHCl (Figure 2A). This suggested local unfolding of the structure of TPI in the vicinity of Cys-217 prior to overall structural changes produced by total unfolding of the enzyme.

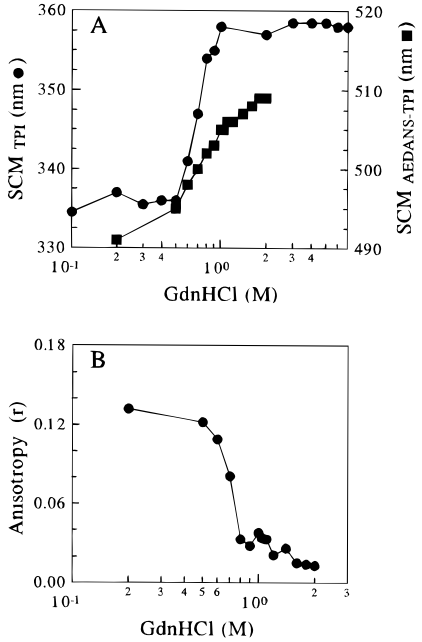

FIGURE 2: Effect of GdnHCl concentration on (A) the spectral center of mass (SCM) of TPI intrinsic fluorescence (●) and AEDANS−TPI fluorescence (■) and (B) AEDANS−TPI anisotropy. TPI (or AEDANS−TPI) at a concentration of 22 mg/mL was incubated in 40 mM triethanolamine/10 mM EDTA/1 mM DTT, pH 7.4, buffer containing the indicated GdnHCl concentrations. Emission spectra of TPI or AEDANS−TPI samples were measured with excitation at 286 or 360 nm, respectively. For fluorescence anisotropy measurements of AEDANS−TPI, samples were excited at 336 nm, and the emission was measured at 470 nm. In the absence of GdnHCl TPI had a SCM = 335 nm, and AEDANS−TPI had a SCM = 488 and an anisotropy of 0.165.

Figure 2B shows changes in fluorescence anisotropy of AEDANS−TPI that occur upon unfolding of the enzyme by GdnHCl. The fluorescence anisotropy decreased from 0.165 for native AEDANS−TPI to 0.015 in the presence of high concentrations of GdnHCl. The decrease in anisotropy reflects a marked increase in mobility of the fluorescent probe, which is compatible with the existence of unfolded monomers in guanidine solution (Sawyer & Gracy, 1975). For TPI−AEDANS, the three fluorescent parameters monitored (fluorescence intensity, spectral center of mass and anisotropy; Figure 2) indicated a similar midpoint value of about 0.65 M GdnHCl for the unfolding transition.

Refolding of AEDANS−TPI. The fluorescence of AEDANS−TPI and regain of enzymatic activity were used to follow reactivation of TPI from unfolded monomers both in aqueous medium and in reverse micelles. In experiments in aqueous solution, native or denatured TPI was diluted (90-fold) in buffer A (in order to dilute GdnHCl to subdenaturing concentrations). Comparison of intrinsic fluorescence spectra obtained in the presence of 4 M GdnHCl and immediately following dilution in buffer A showed that refolding of the enzyme was immediate (i.e., within the dead time of a few minutes required for spectra acquisition), as revealed by the return of the spectral center of mass to the value corresponding to native TPI (Figure 3A, Table 1). The intrinsic fluorescence intensity of renatured TPI also showed a fast increase toward the value observed for native TPI, although in this case recovery was not complete.

Fluorescence anisotropy measurements during refolding of AEDANS−TPI in aqueous medium are shown in Figure 4A. Immediately after dilution of the denaturant the ani-

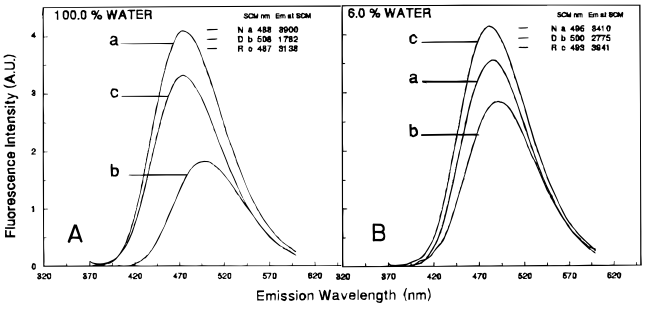

FIGURE 3: Emission spectra of native (a), denatured (b), and renatured (c) AEDANS−TPI in all-aqueous medium (A) and reverse micelles (B). AEDANS−TPI at a concentration of 11.25 mg/mL was incubated in buffer containing 40 mM triethanolamine/10 mM EDTA/1 mM DTT, pH 7.4, and 4 M GdnHCl. After 1 h an aliquot of 2 μL was transferred to all-aqueous medium (A) or reverse micelles formed with 6.0% water (B) (residual guanidine concentration was 44.4 mM).

Table 1: Fluorescent Characteristics of TPI−AEDANS[a]

TPI−AEDANS	SCM, nm	relative fluorescence at the SCM	fluorescence anisotropy
in buffer A	488	100	0.148 ± 0.003
in RM with 6.0% water	495	87	0.120 ± 0.003
in 4.0 M GdnHCl	508	46	0.021 ± 0.003
in RM (6.0%) with 4.0 M GdnHCl	500	71	0.076 ± 0.003
renatured in buffer A	488	90	0.151 ± 0.003
renatured in RM with 6.0% water	495	100	0.115 ± 0.002

[a] SCM is the spectral center of mass.

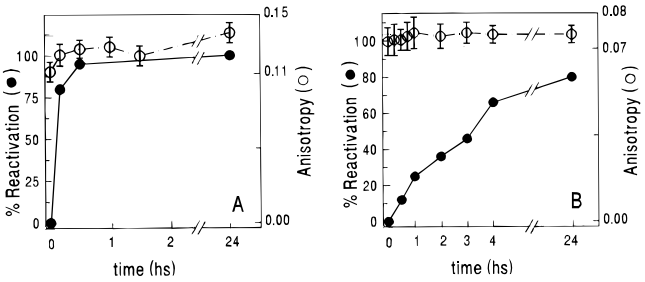

FIGURE 4: Renaturation of AEDANS−TPI in all-aqueous medium (A) and reverse micelles containing 6.0% water (B) measured by fluorescence anisotropy (○) and of enzymatic activity recovery (●). AEDANS−TPI (12 mg/mL) was incubated in buffer containing 40 mM triethanolamine/10 mM EDTA/1 mM DTT, pH 7.4, and 4 M GdnHCl. After 1 h an aliquot of 2 μL was transferred to all-aqueous medium (A) or reverse micelles formed with 6.0% water (B) (residual guanidine concentration was 44.4 mM). At the indicated times activity and fluorescence anisotropy of the samples were measured.

sotropy was about 0.111 (up from a value of 0.021 in the presence of GdnHCl), and this was accompanied by approximately 80% recovery of enzyme activity. At longer times, recovery of activity approached 100%, and the anisotropy further increased to about 0.151 (a value very similar to the anisotropy of 0.148 exhibited by native TPI, Table 1).

Native or denatured TPI was also transferred to micelles containing 6% water. As shown in Figure 3B, the spectral center of mass of TPI renatured in reverse micelles (previously denatured in 4.0 M GdnHCl) returned to the value found for native enzyme, while its fluorescence intensity was about 15% higher than for native TPI (Table 1). Fluorescence anisotropy measurements of AEDANS−TPI during refolding in reverse micelles revealed that the anisotropy

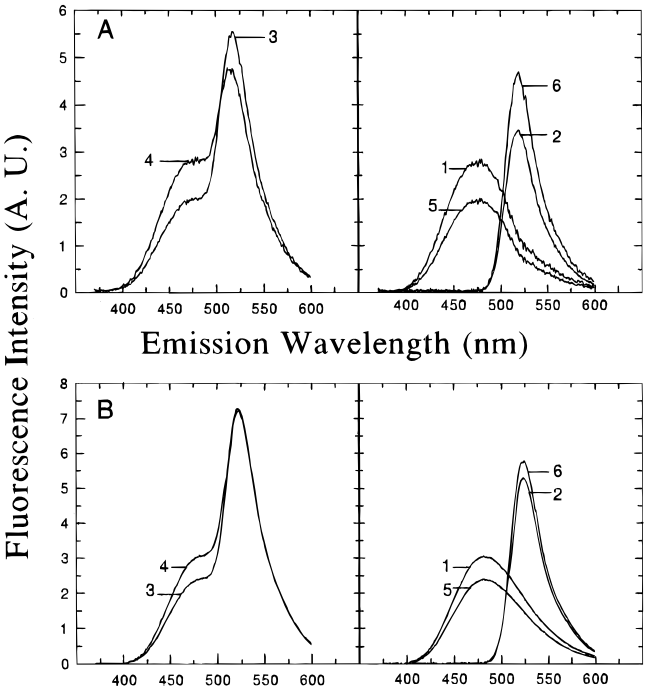

FIGURE 5: Reassociation of AEDANS−TPI and FITC−TPI in all-aqueous medium (A) and reverse micelles formed with 6.0% water (B). Traces correspond to the emission spectrum of the renaturation mixture of AEDANS−TPI and FITC−TPI (3) and the following controls: renaturation mixture of AEDANS−TPI and TPI (1) and renaturation mixture of FITC−TPI and TPI (2). Spectrum 4 corresponds to the mathematical sum of spectra 1 and 2. Spectrum 5 represents the emission of the donor in the mixture of interest and is obtained by multiplying spectrum 1 by the factor F_{DA}/F_A, where F_D is the fluorescence intensity of the donor (AEDANS−TPI) in the absence and F_{DA} in the presence of the acceptor (FITC−TPI). Spectrum 6 corresponds to the emission of the acceptor in the mixture of interest and is obtained by subtraction of spectrum 3 from 5.

(∼0.08) did not change over time for up to 24 h following dilution of denaturant. It is noted that no enzyme activity was detected immediately after transfer of TPI from 4 M GdnHCl to reverse micelles (Figure 4B). Recovery of activity in micelles occurred with a much slower kinetics than in water, over a period of several hours following dilution of denaturant.

Reassociation of TPI Subunits Monitored by FRET. Fluorescence energy transfer between AEDANS and fluorescein was used to monitor reassociation of TPI subunits following dilution of GdnHCl in both aqueous medium and reverse micelles. For these experiments, AEDANS−TPI and FITC−TPI (1:1 fluorophore:monomer) were separately denatured by incubation in the presence of 4.0 M GdnHCl for 1 h at room temperature. Aliquots of the denatured proteins were then mixed 1:1, and subsequently diluted in either buffer A (Figure 5A) or reverse micelles containing 6% water (Figure 5B). In either case, the final concentration of GdnHCl was 66 mM. Fluorescence emission was measured from 370 to 600 nm upon excitation at 360 nm. The resulting fluorescence spectra are shown in Figure 5. In order to account for possible effects of unfolding/refolding on the fluorescence intensity of labeled TPI (which might interfere with energy transfer measurements), we carried out control measurements in which either AEDANS−TPI or FITC−TPI was denatured in 4.0 M GdnHCl, mixed with an equal amount of nonlabeled denatured TPI, and then allowed to refold by dilution of GdnHCl (in aqueous solution or

Table 2: Efficiency of Energy Transfer and Average Distance between Donor (D) AEDANS and Acceptor (A) FITC[a]

molar ratio of D:A	probability to form DA, %	efficiency of energy transfer (*E*)	*r* between AEDANS−FITC, Å
1:1	50	0.54 ± 0	44 ± 0
1:2	66	0.40 ± 0.09	48.7 ± 3.3
1:4	80	0.42 ± 0.16	48.3 ± 3.7
2:1	33	0.28 ± 0.017	53.3 ± 0.9
4:1	20	0.47 ± 0.10	46 ± 3.2

[a] These were calculated as described in Materials and Methods.

reverse micelles). Fluorescence emission spectra obtained for AEDANS−TPI or FITC−TPI thus refolded are shown as traces 1 and 2, respectively. Trace 4 shows the arithmetic sum of traces 1 and 2 (i.e., the expected emission in the absence of energy transfer). Trace 3 shows the measured emission spectrum obtained when denatured AEDANS−TPI and FITC−TPI were first mixed and then refolded. Both in aqueous medium and in reverse micelles, the decrease in donor fluorescence indicated the existence of energy transfer (compare spectra 3 and 4). In aqueous medium (Figure 5A), this was accompanied by an increase in acceptor fluorescence, indicating that hybrid dimers had formed containing both donor and acceptor and giving rise to energy transfer (Figure 5A). However in reverse micelles, the increase in acceptor fluorescence is seen only after correcting for the contribution of the donor (compare spectra 6 and 2 in Figure 5B). Thus, the decrease in donor fluorescence and increase in acceptor emission indicated that FRET also occurred in the hybrid dimers refolded in reverse micelles.

FRET was also used to estimate the distance between the IAEDANS and FITC binding sites in the TPI molecule. For this purpose, FRET measurements were carried out using different molar ratios of AEDANS−TPI:FITC−TPI, and the results obtained are summarized in Table 2. With a 1:1 molar ratio of donor:acceptor, the statistical probability of D−A pair formation following refolding of a mixture of denatured monomers is 0.50. The probability of FRET (measured by a decrease in donor emission) increases as the molar ratio of donor:acceptor decreases (e.g., the probabilities of forming a D−A pair are 0.66 or 0.80 for mixtures containing 1:2 or 1:4 donor:acceptor, respectively; Table 2). An increase in donor:acceptor ratio leads to a corresponding decrease in probability of FRET (for D:A ratios of 2:1 or 4:1, the probabilities are 0.33 or 0.20, respectively; Table 2). The efficiencies of FRET (and the calculated D−A distances) measured at these different D:A ratios are shown in Table 2. Based on these results, an average distance of 48 ± 3 Å was calculated between the IAEDANS (Cys-217) and FITC binding sites in TPI.

The kinetics of reassociation of TPI subunits were examined with FRET. In aqueous buffer, dilution of GdnHCl was immediately (i.e., within 2−3 min) followed by appearance of FRET, and its efficiency remained constant, at approximately 0.54, for at least 24 h (data not shown). This rapid appearance of energy transfer is compatible with fast refolding of TPI following dilution of denaturant revealed by recovery of intrinsic fluorescence (Figure 3A), fluorescence anisotropy of AEDANS−TPI, and enzymatic activity measurements (Figure 4A).

In reverse micelles, the kinetics of FRET increase following dilution of GdnHCl were slower than in aqueous buffer and clearly resolvable in the first 30 min. A FRET

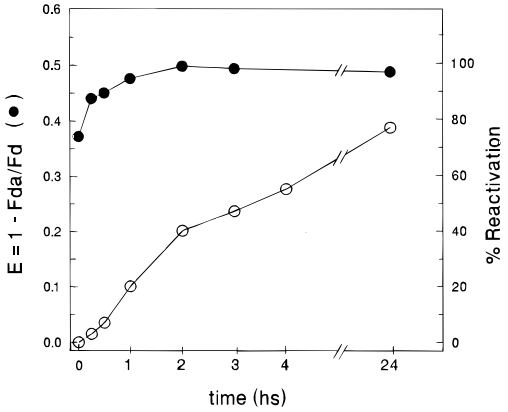

FIGURE 6: Kinetics of activity recovery and efficiency of energy transfer from AEDANS−TPI to FITC−TPI renatured in reverse micelles formed with 6.0% water. AEDANS−TPI and FITC−TPI were separately denatured in 4.0 M GdnHCl. After 1 h of incubation, equal aliquots of both samples were combined and transferred to reverse micelles formed with 6.0% water. Residual guanidine concentration was 66.0 mM (considering that GdnHCl is only distributed in the aqueous phase of the reverse micelles). Protein concentration was 14.5 mg/mL. At the indicated times activity and emission spectra (λ_{exc} = 360 nm) were simultaneously measured. Open circles represent the enzymatic activity recovery of the AEDANS−TPI and FITC−TPI mixture. Closed circles show the value of the energy transfer efficiency calculated at the indicated times.

efficiency of about 0.50 remained constant for at least 24 h (Figure 6). However, in reverse micelles recovery of enzyme activity proceeded at a much slower rate and was about 80% after 24 h (Figure 6). This suggests that dimerization in reverse micelles is followed by slower kinetic steps leading to structural rearrangements necessary for enzymatic activity.

DISCUSSION

The mechanism by which a polypeptide chain spontaneously folds into a unique three-dimensional structure constitutes one of the major unsolved problems in biochemistry (Matthews et al., 1993; Creighton, 1993). Fluorescence strategies have been increasingly used to derive information about protein dynamics, structure, distances between defined protein sites, and structure−function relationships (Wu & Brand, 1994; Dos Remedios & Moens, 1995; Martensson et al., 1995; Gazit & Shai, 1995). In particular, fluorescence resonance energy transfer can reveal both absolute distances between specific fluorophores and conformational changes in proteins and nucleic acids (Stryer, 1978; Joshi & Shamboo, 1988; Lakey et al., 1991; Cheung, 1991).

TPI refolding and reactivation occur at a much slower rate in reverse micelles than in aqueous buffer, making these processes more amenable to study. Previous studies have shown that the kinetics of reactivation and intrinsic fluorescence changes of rabbit muscle TPI after GdnHCl dilution in reverse micelles run in parallel (Fernández-Velasco et al., 1995). The dependence of reactivation on protein concentration indicated that dimerization is the rate-limiting step during reactivation. In the course of refolding in reverse micelles, a high intrinsic fluorescence intermediate was detected, and it was proposed that this intermediate could correspond to the partially refolded monomer (Fernández-Velasco et al., 1995).

To more directly monitor the events of monomer folding, dimerization, and dimer structural rearrangements in reverse

micelles, in this work TPI was covalently labeled with a fluorescent energy transfer pair. TPI was modified with either 1,5-IAEDANS or FITC. This donor−acceptor pair has recently been used to investigate subunit dissociation and unfolding of TPI induced by hydrostatic pressure (Rietveld & Ferreira, 1996). Careful characterization of the time course and stoichiometries of labeling allowed choosing conditions where a single site per monomer was modified with either fluorophore. Under these conditions the labeled enzyme retained ∼80% of its activity. Controlled proteolysis, SDS−PAGE, and amino acid sequencing analysis indicated that Cys-217 was modified by 1,5-IAEDANS. Preliminary results of proteolysis with FITC−TPI labeled at a 1:1 molar ratio indicated that this fluorophore binds to one of five lysine residues found in the carboxy terminal of this protein.

Equilibrium studies of the unfolding of TPI by GdnHCl were carried out by measuring both intrinsic fluorescence emission and fluorescence of AEDANS bound to TPI. In aqueous medium, unfolding was accompanied by a significant red shift (23 nm for tryptophan emission and 21 nm for AEDANS−TPI emission) and quenching of the fluorescence (50% for intrinsic fluorescence and 55% for AEDANS emission). These findings indicate a large increase in exposure of tryptophan residues and of AEDANS to water in the unfolded state. Furthermore, the fluorescence anisotropy of AEDANS−TPI decreased from 0.165 in the native state to 0.015 in the GdnHCl unfolded state, indicating a marked increase in mobility of the fluorophore. It is interesting to note that Cys-217, which is labeled by IAEDANS, is located on the external surface of α-helix number 7, in a relatively solvent-exposed domain of TPI. Despite its solvent-exposed location, the fluorescence of AEDANS bound to Cys-217 is a good probe of the folding of TPI. Intrinsic fluorescence or AEDANS fluorescence measurements indicated that unfolding occurred with a midpoint at approximately 0.65 M GdnHCl (Figure 2A).

In aqueous medium, dilution of the denaturant was almost immediately followed (within a couple of minutes) by an increase in fluorescence anisotropy of AEDANS−TPI to 0.111 with a concomitant activity recovery of 80%. Thereafter, both anisotropy and activity slowly (hours) reached the native values. This latter slower phase of renaturation could suggest that, after dimerization, slow conformational rearrangements are required to achieve optimal dimer conformation.

In reverse micelles with 6.0% water, the fluorescence anisotropy of AEDANS−TPI was lower (0.12) than in aqueous medium, probably indicating that the fluorophore's binding site has higher flexibility in micelles than in all-aqueous medium. This suggests that the micellar environment induces structural alterations of TPI relative to the protein in aqueous solution.

Although renaturation in reverse micelles containing 6% water allowed full recovery of enzyme activity, some of the characteristics of refolding were different from refolding in aqueous medium. Dilution of the denaturant in this system yields an intermediate whose intrinsic fluorescence is higher than that of both the native and the denatured enzyme (Fernández-Velasco et al., 1995). In micelles, the fluorescence emission of renatured AEDANS−TPI was approximately 10% higher than for native labeled enzyme, and its fluorescence anisotropy remained low (∼0.08) throughout the renaturation process. This anisotropy value is close to that of the trapped monomer obtained in reverse micelles containing 1 M GdnHCl (0.076). Considering that full recovery of TPI activity is achieved, the incomplete recovery of fluorescence anisotropy during renaturation may result from constraints imposed by the micellar environment on dimerization. Apparently, in micelles the AEDANS binding domain of the renatured enzyme remains in a more mobile condition than in the native labeled dimer.

In order to directly assay dimer formation, fluorescence energy transfer was measured between TPI monomers labeled with FITC and AEDANS. AEDANS binds to Cys-217 of TPI and FITC to one of five lysine residues in its carboxy terminal (from amino acid 176 to 248). Using the crystal information from chicken muscle TPI (Banner et al., 1975; Alber et al., 1981), the structure of the rabbit enzyme was modeled, and from it the calculated distances between Cys-217 and each one of these five lysines was found to be in the range from 57 to 69 Å. In all-aqueous medium TPI renaturation occurs in very short times (Waley, 1973; Fernández-Velasco et al., 1995). Efficient energy transfer within heterodimers was observed 2−3 min after dilution of the denaturant and remained approximately constant for 24 h (data not shown). The distances between FITC and AEDANS in a hetero-TPI dimer calculated from the FRET results shown in Table 2 yielded an average distance of 48 ± 3 Å, in reasonable agreement with the distance predicted from the TPI structure.

In reverse micelles, the appearance of enzyme activity during renaturation correlated with a highly fluorescent intermediate state, which has been thought of as being a partially folded monomer (Fernández-Velasco et al., 1995). However, the kinetics of energy transfer observed when AEDANS- and FITC-labeled monomers were mixed under renaturing conditions were much faster than the changes in TPI intrinsic fluorescence (Figure 6). FRET was observed as soon as GdnHCl was diluted ($E = 0.36$), and an increase (to $E \sim 0.50$) occurred up to 60 min of refolding. After 24 h, when 80% of the enzyme activity was recovered, the efficiency of FRET was 0.50. It is thus possible that an inactive dimer could be formed in reverse micelles within the first minutes after denaturant dilution, consistent with the high initial FRET, with a subsequent transition of the inactive (or less active) dimer to a fully active conformation. If this explanation is correct, the intermediate with high intrinsic fluorescence (Fernández-Velasco et al., 1995) is not a monomer but the inactive dimer. Alternatively, the high rate of FRET observed initially could result from the fact that monomer−monomer interactions in reverse micelles may last long enough for energy transfer to occur without stable dimer formation. The exchange of micellar content has been measured to be in the order of milliseconds in some systems (Fletcher, 1988). However, the exchange rate of micelles containing proteins having affinity for each other is not known. The slower increase in FRET could reflect stabilization of the dimer and correlates better with the appearance of activity. The interaction between trypsin and the soybean trypsin inhibitor occurs within minutes in reverse micelles (Fernández-Velasco et al., 1995), indicating that subunits of the size of TPI can bind in the micellar system with faster kinetics than the recovery of TPI activity.

To the best of our knowledge this is the first fluorescence energy transfer study of protein folding and subunit interac-

tions in reverse micelles. This strategy can be further utilized to improve our understanding of how proteins interact in this low-water system. Reverse micelles offer the possibility of trapping intermediates and studying how water affects folding and subunit interactions. Regarding TPI in reverse micelles, the work presented here indicates that, after monomer refolding, an inactive dimer is generated which has to undergo further conformational changes to yield the functional dimer.

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