Water Requirements in Monomer Folding and Dimerization of Triosephosphate Isomerase in Reverse Micelles. Intrinsic Fluorescence of Conformers Related to Reactivation†

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ABSTRACT: The possibility of using reverse micelles to stabilize monomers prior to formation of dimeric triosephosphate isomerase (TPI) from rabbit muscle was studied. TPI denatured with guanidine hydrochloride undergoes reactivation in reverse micelles formed with *n*-octane, hexanol, cetyltrimethylammonium bromide, and water. Reactivation of around 80% is observed at TPI concentrations of about 2 μg/mL of reverse micelles and water concentrations above 4.0%. With 3.0% water, reactivation is about 10%. If denatured TPI is incubated for a few seconds in reverse micelles with 5.0% water (or higher) followed by incubation in 3.0% water, reactivation is between 35% and 50%. That is, a brief exposure of denatured TPI to reverse micelles with a relatively high water concentration yielded a significant amount of structures competent for formation of catalytically active dimers. As evidenced by kinetic data, these structures correspond to monomers of TPI [Garza-Ramos, G. Tuena de Gómez-Puyou, M., Gómez-Puyou, A., & Gracy R. W. (1992) *Eur. J. Biochem. 208*, 389−395]. After a 5−2.0% water transition, competent monomers were stabilized for at least 30 min; a subsequent rise in water concentration led to dimerization and appearance of activity. By changes in the amount of water, it was possible to determine in reverse micelles the amount of water required for monomer folding and dimerization; i.e., less water was required in the dimerization step. Experiments with a model system, trypsin and the soybean inhibitor, showed that, in reverse micelles with 2.0% water, protein−protein interactions readily take place. Hence, the lack of dimerization of TPI monomers in micelles with this water content suggests that, after collision, interacting monomers must undergo conformational changes that result in a stable catalytically active dimer. The intrinsic fluorescence spectra of native and denatured TPI entrapped in reverse micelles with 6.0% water were clearly different. Both differed from that of monomers competent for reactivation. With time, the intrinsic fluorescence of the latter changed in parallel to the appearence of catalytic activity. The intrinsic fluorescence of competent and incompetent conformers was sensitive to water concentration. At equal water concentrations no obvious difference was observed between the two. TPI at various states of denaturation produced by various concentrations of guanidine hydrochloride in standard aqueous media could also be stabilized in reverse micelles with 2.0% water, an increase in water concentration induced reactivation. The lack of interconversion between folded monomers and active dimers in reverse micelles with low water content suggests that in these conditions there is a kinetic barrier that prevents formation of active dimers from folded monomers.

Kauzmann (1959) proposed that water is a predominant component in protein folding, mainly as consequence of the unfavorable entropy of water molecules near nonpolar residues. More recent studies on the role of water on protein folding explored the role of hydration of polar and nonpolar amino acids on protein stability (Privalov & Gill, 1988; Makhatadze & Privalov, 1993; Privalov & Makhatadze, 1993). These and other studies (Rupley & Careri, 1991; Jaenicke, 1987) have led to the concept that water is fundamental in the function and stability of proteins. However, the precise contribution of water, and particularly how much water is needed for protein folding and/or formation of oligomeric proteins, is not known. As continuation of studies concerning the contribution of water in specific events of enzyme mobility (Barrabin et al., 1993), this work explores the water requirements in monomer folding and dimerization of homodimeric triosephosphate isomerase (TPI).[1]

TPI is formed by two identical eight strand β/α subunits of around 26 000 Da. It catalyzes the interconversion of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate

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through well established mechanisms (Albery & Knowles, 1976, 1977; Knowles, 1991). As a side reaction, TPI catalyzes the formation of methylglyoxal phosphate (Richard, 1991), and in repeated catalytic cycles, it undergoes specific covalent modifications (Tang et al., 1990). The crystal structure of TPI from several sources (Banner et al., 1975; Alber et al., 1981; Lolis et al., 1990, Wierenga et al., 1991, 1992; Noble et al., 1993; Mande et al., 1994) has been determined at high resolution.

From studies of TPI reactivation from unfolded monomers at various protein concentrations in standard aqueous media (Waley, 1973; Zabori et al., 1980), and in reverse micelles (Garza-Ramos et al., 1992b), the following simplified reaction sequence was derived:

$$\begin{array}{l} M_u \overset{I}{\rightleftharpoons} M_f \overset{II}{\rightarrow} D \\ \downarrow \\ M_x \end{array} \qquad (1)$$

M_u is an unfolded monomer that is first transformed into M_f. M_f stands for a monomer competent for dimerization with a structure different from that in the catalytically active dimer (D). M_x is a broad term that indicates structures that do not form the active dimer. In both standard aqueous media, and reverse micelles, at relatively low protein concentrations, the rate limiting step is dimerization (step II). However, the process is orders of magnitude slower in reverse micelles (Garza-Ramos et al., 1992b).

The latter observation, and previously reported data (Rupley & Careri, 1991; Zaks & Klibanov, 1985; Ramírez-Silva et al., 1993), suggested that systems with a low water content may be used to ascertain important features of enzyme action. In fact, in such systems it was possible to trap different enzyme conformers (Russell & Klibanov, 1988) and intermediates of catalytic cycles (Escamilla et al., 1989). Thus, it was thought that reverse micelles, in addition to current approaches (Hughson et al., 1990; Baker et al., 1992), could be applied to trap intermediates of the formation of dimeric TPI. If so, the system could be used to explore if the different reactions that lead to TPI dimerization have distinct water requirements, to determine the structural characteristics of conformers related to the formation of the TPI dimer, and to determine the modifications that they undergo in the transition from a catalytically inactive monomer to a quaternary structure that is catalytically active.

The results on the reactivation of TPI in reverse micelles here described show that by changes of their water content, it was possible to arrest, or initiate, the formation of active dimers. This allowed the determination of the amount of water required for monomer folding and dimerization. Moreover, since the monomers could be trapped for a substantial time, it was possible to determine their intrinsic fluorescence and the changes that accompany formation of the catalytically active dimer.

MATERIAL AND METHODS

Triosephosphate isomerase from rabbit muscle, trypsin, 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. Soybean trypsin inhibitor was from Worthington. The activity of TPI in all aqueous mixtures, measured as described elsewhere (Rozacky et al., 1971), was around 4 000 μmol/(min·mg) with glyceraldehyde 3-phosphate as substrate. TPI concentration was determined by its absorbance at 280 nm, $E^{1\%}_{1cm} = 12.9$ (Rozacky et al., 1971).

Reverse Micelles. The basic system for reverse micelles contained 200 mM cetyltrimethylammonium bromide suspended in *n*-octane and hexanol in a ratio of 8.7:1 (v/v). Reverse micelles were formed by adding a water solution of 40 mM triethanolamine, 10 mM EDTA, and 1 mM dithiothreitol, pH 7.4, to the desired water concentration (1.5−6.0% v/v; $W_0 = 4.1-16.6$, respectively). This was followed by vigorous stirring. The reverse micelle system with and without TPI was stable for at least a week. The water solubility diagrams of this type of reverse micelles have been described (Hilhorst et al., 1984; Fernández-Velasco et al., 1992). Under these conditions, the concentration of reverse micelles is between 1 and 4 mM (Laane et al., 1987).

Assay of TPI Activity in Reverse Micelles. In all cases activity was measured at 24 °C in reverse micelles that contained 6.0% water (v/v); this water phase contained 40 mM triethanolamine, pH 7.4, 1 mM dithiothreitol, 10 mM EDTA, 3.3 mM glyceraldehyde 3-phosphate, 0.2 mM NADH (overall concentration), and 5−7 μg of α-glycerophosphate dehydrogenase. The latter enzyme was introduced after the micelles had been formed with the aforementioned components; previously, ammonium sulfate in the enzyme was removed (Garza-Ramos et al., 1992b) by filtration−centrifugation in Sephadex G-50 columns equilibrated with 40 mM triethanolamine, 10 mM EDTA, and 1 mM dithiothreitol, pH 7.4. For the the assay of TPI activity, an aliquot (generally less than 20 μL) of reverse micelles that contained native or TPI at various levels of reactivation was added. The decrease in optical density at 340 nm was used as measure of activity. Activity was linear with time until NADH concentration became limiting. The activity of TPI in reverse micelles with 6.0% water was around 420 μmol/(min·mg).

Denaturation of TPI in All Aqueous Media and Its Reactivation in Reverse Micelles. TPI was denatured in 4−6 M GdnHCl of a standard aqueous media containing 40 mM triethanolamine, 10 mM EDTA, and 1 mM dithiothreitol, pH 7.4, for 1 h at 24 °C. In the experiments in which only reactivation was determined, the concentration of protein was 3−6 mg/mL. In the fluorescence experiments the protein concentration was 25−30 mg/mL. Reactivation was started by injection of 1 μL of the denatured enzyme into 3.0 mL of reverse micelles formed at the desired water concentration with a solution of 40 mM triethanolamine, 10 mM EDTA, and 1 mM dithiothreitol, pH 7.4 . The transfer of denatured TPI to reverse micelles resulted in at least a 75-fold dilution of GdnHCl (maximal concentration of GdnHCl in the water phase was 80 mM). The concentration of protein ranged between 1 and 8 μg/mL of reverse micelles system (40−320 nM monomer, overall concentration). As the concentration of micelles is 1−4 mM (Laane et al., 1987), on a

[1] Abbreviations: BAEE, N^{α}-benzoyl-L-arginine ethyl ester; EDTA, ethylenediaminetetraacetate; GdnHCl, guanidine hydrochloride; SCM, spectral center of mass; TPI, triosephosphate isomerase; W_0, ratio of water to surfactant molecules.

statistical basis, not more than one monomer will be localized in a single micelle. After transfer, the system was incubated at 24 °C. To follow reactivation of TPI, aliquots were withdrawn at various times and their activity assayed. As noted, activity was always measured in reverse micelles that contained 6.0% water (v/v); $W_0 = 16.6$. No reactivation took place during the activity measurements, i.e., activity was linear with time.

In several experiments, the concentration of water in the system was varied during the course of TPI reactivation. When a decrease in the amount of water was desired, the reverse micelle system was diluted with a mixture of dry cetyltrimethylammonium bromide, *n*-octane, and hexanol, or reverse micelles that had the desired water concentration. When a rise in water concentration was needed, an aliquot of an aqueous solution of 40 mM triethanolamine, 10 mM EDTA, and 1 mM dithiothreitol, pH 7.4, was injected. Controls were made in all conditions; these comprised native TPI transferred to reverse micelles that had water at the concentration in which reactivation of TPI was studied. When changes in water concentration were made during reactivation, the same changes were made in control TPI. Reactivation is expressed as percent of the activity of a control that had not been exposed to GdnHCl but that had undergone identical changes in water concentrations. It is noted that the stability of the native enzyme was not affected by such changes; i.e., enzyme activity of these samples remained constant for at least 48 h irrespective of the water content of the micelles.

Intrinsic Fluorescence. The intrinsic fluorescence emission spectra of TPI in reverse micelles were determined in an SLM AMINCO 8000 spectrofluorometer with 2 nm slits in both excitation and emission. At an excitation wavelength of 286 nm, emission spectra were recorded between 290 and 430 nm. The fluorometer cell was thermostated at 24 °C. The spectral center of mass (SCM) was calculated according to the following equation (Bismuto et al., 1987):

$$\lambda_{av} = \frac{\int \lambda I(\lambda)\, d\lambda}{\int I(\lambda)\, d\lambda}$$

where λ and $I(\lambda)$ are wavelength (in nanometers) and fluorescence intensity at a given wavelength, respectively. In all cases, emission fluorescence spectra of identical samples (without protein) were recorded. These were substracted from the experimental samples.

Assay of Trypsin Activity in Reverse Micelles. Trypsin activity in reverse micelles was assayed as described by Bru and García-Carmona (1991) with *N*-α-benzoyl-L-arginine ethyl ester as substrate.

RESULTS

Denatured TPI transferred to a reverse micelle system that contains water concentrations above 5.0% reactivates by at least 80%. When the system contains 3.0% water ($W_0 = 8.3$) or less, reactivation is around 10%; if the water concentration of the latter sample is raised to 5.0%, reactivation remains at about 10% (Garza-Ramos et al., 1992b). This indicated that the transfer of the denatured enzyme to reverse micelles with a relatively low water content produces structures that fail to form a catalytically active dimer. Here we explored if the transfer of denatured TPI monomers to reverse micelles with high water concentrations (>5.0%; $W_0 > 13.9$) could yield structures capable of following the pathway to an active dimer at lower water concentrations.

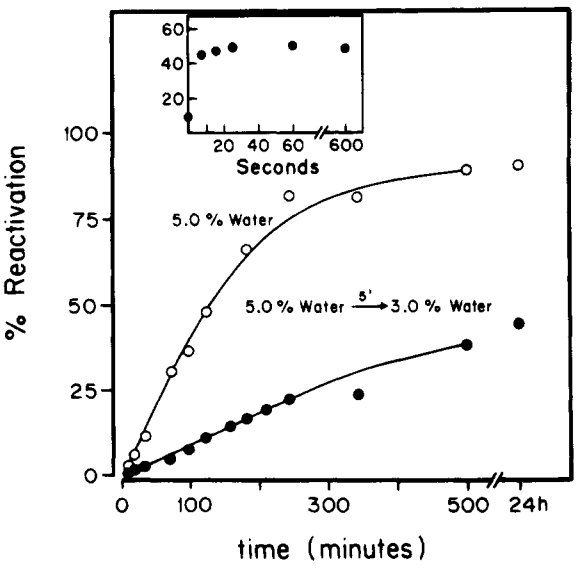

FIGURE 1: Kinetics of TPI reactivation in reverse micelles after a 5% to 3% water transition. TPI denatured in 6.0 M GdnHCl was transferred to reverse micelles that contained 5.0% water, yielding a concentration of 3.7 μg of protein/mL of reverse micelles. After 5 min, the water content was decreased to 3.0% by mixing with the dry basic mixture for formation of reverse micelles (●), or with an equivalent volume of reverse micelles with 5.0% water (○). At the indicated times, activity was measured. The inset shows the time at which reverse micelles with 5.0% water were mixed with dry basic mixture; activity was measured after 24 h of the water jump.

Figure 1 shows the results of such an experiment. Denatured TPI was transferred to reverse micelles with 5.0% water; after 5 min of incubation, the water content of the mixture was diminished to 3.0%. At this time, TPI activity was hardly detectable; however, it progressively appeared with the time of incubation (Figure 1). After 24 h, activity was around 40% of that of the native enzyme. The time course of reactivation of denatured TPI in reverse micelles in which water was always at a concentration of 5.0% is also shown; in this case, reactivation was close to 100%. As noted above, denatured TPI transferred *directly* to micelles with 3.0% water underwent reactivation of about 10%.

The findings of Figure 1 illustrate that a brief incubation of denatured TPI monomers in reverse micelles with a water content of 5.0% produced a substantial amount of conformers competent for formation of active dimers in 3.0% water. An attempt was made to determine the minimal time that the denatured monomer had to be exposed to 5.0% water for acquisition of such a conformation (inset, Figure 1). The shortest time that could be assayed was 8 s. This sufficed to produce a population of conformers competent for undergoing dimerization; longer times of incubation in 5.0% water (up to 10 min) did not result in higher reactivation (inset of Figure 1). The kinetics of reactivation at various protein concentrations (Garza-Ramos et al., 1992b) show that the rate limiting step for formation of the active dimer from unfolded monomers is the bimolecular step. Hence, in comparison to reactivation in micelles with 5.0% water, the lower reactivation that is observed after a 5.0−3.0% water transition may indicate that, at the low water concentration, there is an equilibrium between M_u and competent monomers

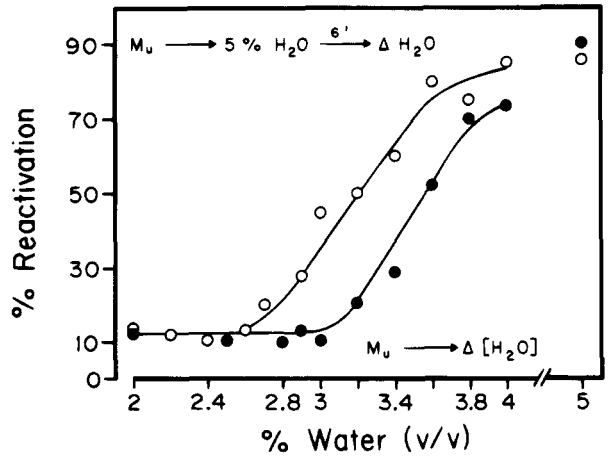

FIGURE 2: Amount of water required for monomer folding and dimerization in reverse micelles. The protocol was as in Figure 1. In the traces depicted with (●), TPI denatured with 6.0 M GdnHCl was transferred to reverse micelles that had the indicated water concentrations and incubated for 24 h. In the trace depicted with (○), denatured TPI was transferred to reverse micelles with 5.0% water; after 6 min, the water concentration of various aliquots was adjusted to the concentrations shown by mixing with different volumes of dry basic mixture, or by adding aqueous buffer so as to yield the water concentrations shown. The samples were incubated for 24 h and activity was measured.

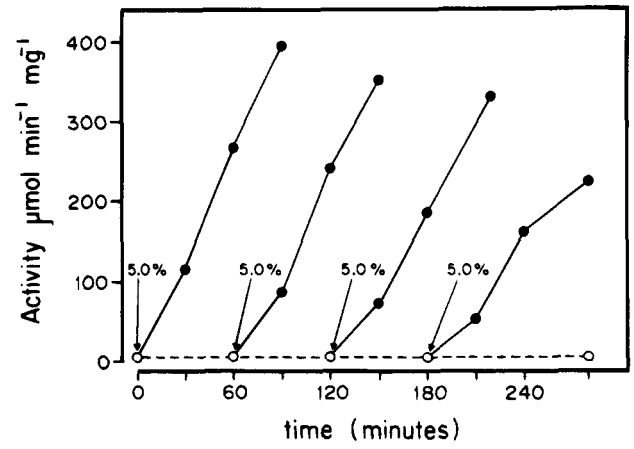

FIGURE 3: Reactivation of denatured TPI after 5.0% to 2.0% to 5.0% water transitions. The protocol was as in Figure 1, except that 5 min after transfer of TPI denatured in 6.0 M GdnHCl to reverse micelles with 5.0% water, the water content of the system was decreased to 2.0%; this is time zero in the figure. At the times shown by the arrows, the water content of the samples was brought back again to 5.0% by addition of aqueous buffer; activity was measured at the times shown (●). The trace with the arrow at time zero indicates denatured TPI transferred to reverse micelles with 5.0% water that was diluted after 5 min with reverse micelles with 5.0% water.

(M_f) that are able to undergo dimerization and/or a reduction in the number of productive collisions due to a lower concentration of competent monomers.

The data in Figure 1 also show that, at equal protein concentrations, the reactivation rate was higher in micelles with 5.0% than with 3.0% water; i.e., the half-lives for maximal reactivation were 110 and 260 min, respectively. This suggests that, in addition to protein concentration, the rate of active dimer formation is controlled by the water content of the micelles.

Water Requirements for Monomer Folding and Dimerization of Denatured TPI in Reverse Micelles. To estimate the amount of water required in the two general steps of the pathway (monomer folding and dimerization; see reaction sequence 1 in the introduction), reactivation was measured under two conditions. In one, the enzyme was transferred directly to reverse micelles with different water contents (trace depicted by closed circles in Figure 2). In a second condition, the denatured enzyme was first transferred to 5.0% water; after 6 min, the water content of the system was adjusted to various levels (open circles in Figure 2). In both cases, activity was measured after 24 h. In the two conditions, the curves of the extent of reactivation versus the amount of water appeared sigmoidal.

In the experiment of Figure 2, reactivation of denatured TPI transferred directly to different water concentrations reflected water requirements in the overall process of active dimer formation. In the samples in which the denatured protein was first incubated in 5.0% water to allow monomer folding and subsequently exposed to different water concentrations, the level of reactivation indicated the amount of water required for dimerization. From the curves, it is evident that more water is needed for monomer folding than for dimerization, i.e., 3.6% and 3.2% for half-maximal reactivation.

Stabilization of Competent Monomers in the Formation of TPI Dimers. When denatured TPI was transferred to reverse micelles with 5.0% water, the enzyme reactivated to values of 80% or more. In contrast, when denatured TPI was transferred to micelles with 5.0% water, followed by a reduction in water concentration to 3.0%, reactivation ranged between 35% and 50%. The lower reactivation after the 5.0−3.0% water transition indicated that, at the latter water concentration, the population of M_u and M_f formed in 5.0% water had sufficient mobility to follow the pathway toward the active dimer or to an incorrect structure (M_x in reaction sequence 1). As protein mobility depends on the amount of water in contact with the protein (Rupley & Careri, 1991; Zaks & Klibanov, 1984; Affleck et al., 1992; Garza-Ramos et al, 1992a), it was asked if, at water concentrations lower than 3.0%, the conformation of the competent monomeric structure could be stabilized.

Denatured TPI was transferred to reverse micelles with 5.0% water to induce formation of the folded monomer; after 5 min, the amount of water was reduced to 2.0%. Hardly any reactivation took place (open circles in Figure 3). However, if the concentration of water was brought back to 5.0%, reactivation was restored (closed circles, Figure 3). It is noted that, after 30 min in 2.0% water, a rise in water concentration produced a rate of reactivation that was close to that of the enzyme that throughout the experiment remained in 5.0% water. At longer times of incubation in 2.0% water, reactivation was slower. These results indicated that by decreasing the amount of water in the micelles to values of 2.0%, it was possible to stabilize competent monomers for a substantial length of time.

Stabilization of monomers by a 5.0−2.0% water transition was also observed at different extents of reactivation in micelles with 5.0% water. In the experiment of Figure 4, reactivation of TPI in 5.0% water was allowed to proceed for various times. At this state, a reduction of the water content to 2.0% arrested reactivation. Note that, after the decrease in water concentration, the activity of the previously formed dimers was maintained. When water concentration was brought back again to 5.0%, reactivation reappeared;

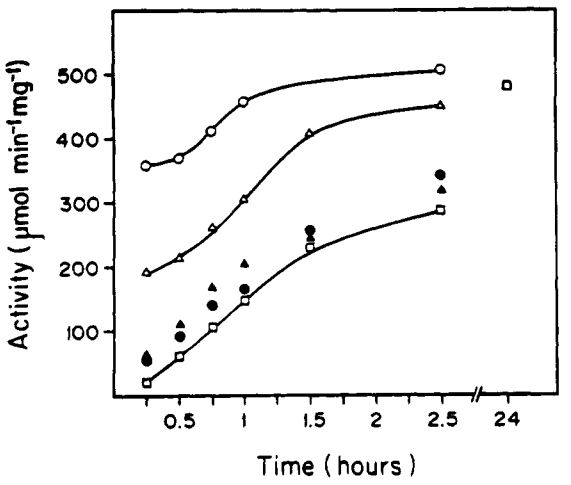

FIGURE 4: Arrest and reinitiation of TPI reactivation. Denatured TPI was allowed to reactivate in reverse micelles with 5.0% water. The time course is illustrated by □. At different times of reactivation, the following protocol was followed, where /•/ indicates a water transition: (●) 5.0% /•/ 2.0% (24 h) → activity; (▲) 5.0% /•/ 2.0% (48 h) → activity; (○) 5.0% /•/ 2.0% (1 h) /•/ 5.0% (24 h) → activity; (△) 5.0% /•/ 2.0% (24 h) /•/ 5.0% (24 h) → activity.

hence, in 2.0% water, both the active and the inactive structures were stabilized. It is pointed out that as the time of incubation in 2.0% water was prolonged, the extent of reactivation was lower, but even after standing for a day at 2.0% water, at least 40% of the monomers were able to dimerize after a rise in water concentration (Figure 4).

Intrinsic Fluorescence of TPI during Reactivation in Reverse Micelles. In reverse micelles, reactivation of TPI occurs in times much larger than in conventional water systems. Thus, in this system, it is relatively easy to study the structural changes that accompany reactivation. Accordingly, the changes in intrinsic fluorescence that TPI undergoes during reactivation were determined (Figure 5). Prior to these studies, the spectra of native TPI in reverse micelles that contained 2.0%, 3.0%, and 6.0% water, and that of denatured TPI transferred to reverse micelles formed with equivalent concentrations of 4.0 M GdnHCl, were determined (Figure 5). In reference to native TPI, the denatured enzyme exhibited a spectra with a higher fluorescence emission and a higher spectral center of mass (SCM).

To follow the possible changes in intrinsic fluorescence that TPI could undergo during reactivation, the enzyme was denatured for 1 h in standard aqueous mixtures with 4.0 M GdnHCl. At this time a 1 μL aliquot was transferred to 3.0 mL of reverse micelles that contained 2.0%, 3.0%, and 6.0% water. The fluorescence spectra of the samples were recorded within 5 min after transfer (at this time, hardly any catalytic activity was detected). In the samples with 2.0%, 3.0%, and 6.0% water, the intrinsic fluorescence of the protein was higher than that of native or denatured TPI entrapped in reverse micelles. It was also observed that the magnitude of fluorescence emission was highest with 2.0% and lowest with 6.0% water. After 24 h of incubation, the intrinsic fluorescence of the three samples diminished, but the decrease was larger in the sample with 6.0% water. This was accompanied by a shift in the SCM of about 7 nm (from 349 to 342 nm), thus approaching the spectra of the native enzyme. The reactivation of the sample in 6.0% water was 83%, whereas in reverse micelles with 2% and 3% water, reactivation was 5% and 12%, respectively. It is noted that for fluorescence measurements protein concentration in the range of 8−10 μg/mL of reverse micelles had to be employed. In this concentration range, the rate of reactivation was still dependent on protein concentration (not shown).

The changes in fluorescence and activity that denatured TPI underwent in reverse micelles formed with 6.0% water were also determined as a function of time. There was a strong parallelism in the two processes (Figure 6). The time for half-maximal changes in intrinsic fluorescence and activity were almost equal.

Fluorescence Properties of Competent and Incompetent TPI Monomers in Reverse Micelles. Depending on the water concentration of the micelles, it is possible to trap monomers that are either competent or incompetent for dimerization. For example, competent monomers may be stabilized by transfer of denatured TPI to micelles with 5.0% water, followed by a diminution of water to 2.0% (Figure 3). Incompetent monomers may be trapped by transferring denatured TPI directly to micelles with 2.0% water. Thus, the system allows a comparison of the intrinsic fluorescence of both types of monomers, and how the amount of water affects their intrinsic fluorescence. The SCM and the relative fluorescence intensity of both types of monomers at various water concentrations are shown in Table 1. The intrinsic fluorescence of the competent monomers in 5.0% water decreased when water concentration was diminished to 2.0%; the SCM was affected, but slightly. At the latter water concentration, the spectrum of the competent monomer was strikingly similar to that of denatured TPI transferred directly to reverse micelles with 2.0% water (incompetent monomer). It was also observed that the fluorescence spectra of the incompetent monomers decreased when water concentration was raised to 5.0%, yielding a spectrum similar to that of the competent monomer (Table 1). These findings showed that the amount of water is central to the fluorescence properties of correct and incorrect monomers and that there are no obvious differences in their intrinsic fluorescence.

Trapping in Reverse Micelles of Conformers Produced by GdnHCl in Conventional Aqueous Media. Concerning the stabilization of monomers in reverse micelles, another question addressed was if the conformers that exist at various concentrations of denaturant in conventional aqueous systems may also be trapped by transferring them to reverse micelles. TPI was incubated with various concentrations of GdnHCl in standard water mixtures for 2 h; at this time, aliquots of the various mixtures were transferred to reverse micelles with 2.0% water. Immediately after transfer, the activity of the various mixtures was measured (open circles, Figure 7). The observed values are considered to reflect the amount of active dimers existing at the time of transfer. After 24 h of incubation, activity was again measured. In all samples a relatively low level of reactivation was observed, i.e., 10% or less (not shown).

In another part of the experiment of Figure 7, after a 15 min incubation in 2.0% water, the water content of the various reverse micelles mixtures was raised to 5.0%. Their activity was measured after 24 h (closed circles, Figure 7). Significant activation was observed in the samples that had been exposed to GdnHCl concentrations between 0.7 and 1.0 M. In this concentration range, GdnHCl produces TPI monomerization (Sawyer & Gracy, 1975). Hence, it would appear that monomers of TPI formed in conventional water systems can be successfully trapped in reverse micelles with

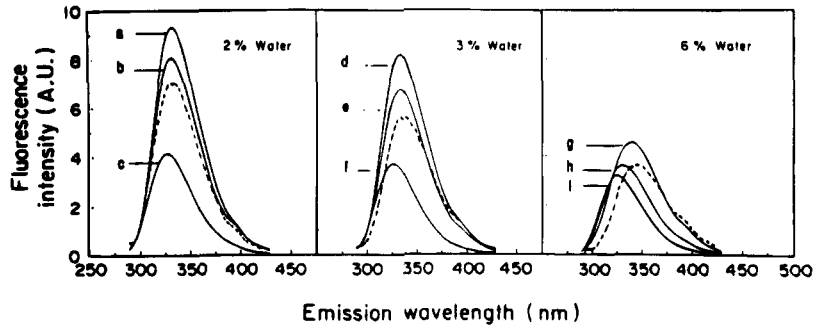

FIGURE 5: Intrinsic fuorescence emission spectra of native, denatured, and reactivated TPI in reverse micelles. Native TPI and TPI denatured in 4.0 M GdnHCl were transferred to reverse micelles formed with aqueous buffer or a solution of 4.0 M GdnHCl, respectively; the concentration of these solutions in the reverse micelles was 2.0%, 3.0%, or 6.0% (as shown). Traces c, f, and i show the spectra of native TPI in micelles formed with 2.0%, 3.0%, and 6.0% buffer; the dashed lines depict the spectra of the denatured enzyme in reverse micelles formed with 4.0 M GdnHCl. Denatured TPI was also transferred to reverse micelles formed with aqueous buffer at a concentration of 2.0%, 3.0% and 6.0% water. Traces a, d, and g show spectra taken 5 min after transfer. Traces b, e, and h show the spectra of the latter samples after 24 h. The concentration of GdnHCl in the aqueous phase of all samples (except those shown by the dashed lines) was adjusted to 66 mM.

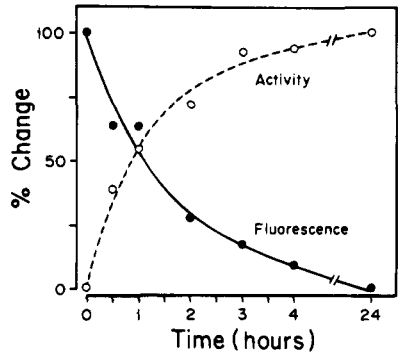

FIGURE 6: Fluorescence and activity of TPI during reactivation. TPI was denatured in 4.0 M GdnHCl and transferred to reverse micelles with 6.0% water. At the indicated times, emission fluorescence spectrum and activity were determined. The results are plotted as percent of the maximal changes in activity and fluorescence (intensity at the spectral center of mass) that the enzyme underwent in 24 h.

Table 1: Intrinsic Fluorescence of Competent and Noncompetent Monomers in Reverse Micelles with 2.0% and 5.0% Water[a]

condition	SCM	rel fluorescence intensity
5.0% water	343	3577
5.0% /•/ 2.0% water	344	6241
5.0% /•/ 2.0% /•/ 5.0% water	344	3686
2.0% water	343	6653
2.0% /•/ 5.0% water	344	4674

[a] TPI was denatured in 4.0 M GdnHCl for 1 h and thereafter transferred to reverse micelles that had the indicated water concentrations. /•/ denotes that water concentration in reverse micelles was changed to the level indicated. Five minutes after transfer of TPI to reverse micelles with 2.0% or 5.0% water, fluorescence emission spectra were recorded. After these traces were obtained, water was changed to the concentration shown, and the spectra were again recorded.

2.0% water in a state competent for subsequent formation of the active dimer.

Causes for Lack of Dimerization in Reverse Micelles with 2.0% Water. In reverse micelles with 2.0% water, competent monomers do not form an active dimer. This finding was central for the trapping of monomers that upon a rise in water concentration follow the pathway toward active dimers. Thus, the question arised as to the causes that prevent dimerization in reverse micelles with 2.0% water. Among several possibilities, this could be due to hindrances in the transfer of two monomers to a single micelle. This alternative was

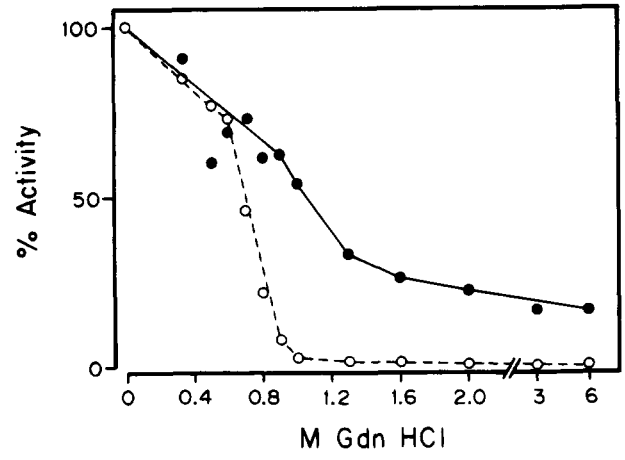

FIGURE 7: Reactivation in reverse micelles of TPI that had been exposed to various GdnHCl concentrations. TPI was incubated for 2 h in the indicated concentrations of GdnHCl. At that time it was transferred to reverse micelles with 2.0% water, and its activity was measured (○). After 15 min, the water concentration was raised to 5.0% and after 24 h activity was measured (●).

examined with a model system. Trypsin and the trypsin inhibitor from soybean have a M_w of around 23 800 and 21 500, respectively; these are relatively close to that of the TPI monomer (M_w = 26 000). Trypsin entrapped in reverse micelles with 2.0% water was catalytically active. Thus the time course for the inhibition of trypsin activity by the inhibitor was determined in reverse micelles with 2.0% water (Figure 8). At concentrations of trypsin and trypsin inhibitor similar to those at which reactivation of TPI was measured, half-maximal inhibition of trypsin activity took place in a time range of 5 min; albeit inhibition was about 80% whereas in all aqueous media, it was 100% (the cause of the lower inhibition in reverse micelles has not been studied). Nevertheless, the observation that trypsin and its inhibitor readily interact in reverse micelles with 2.0% water suggests that the lack of formation of active TPI dimers was not due to impairments in the transfer of two monomers to a single micelle. Instead, it would appear that, in order to attain catalytically active TPI dimers, the monomers must undergo internal structural arrangements that stabilize the dimeric structure and that relatively high water concentrations are needed to support such arrangements.

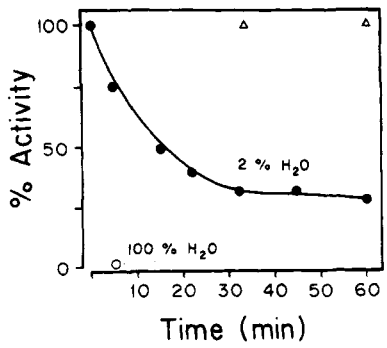

FIGURE 8: Inhibition of trypsin activity by the soybean trypsin inhibitor in reverse micelles with 2.0% water. Reverse micelles were formed at a concentration of 2.0% water that contained 100 mM Tris-HCl, 5 mM $CaCl_2$, and either 0.06 μM trypsin or 0.06 μM soybean trypsin inhibitor at pH 8.0. At time zero, equal volumes of the two types of micelles were mixed. At the indicated times aliquots were withdrawn and their activity was assayed in reverse micelles that contained 0.25 mM BAEE (overall concentration). (●) shows activity at the indicated preincubation times. (△) indicates activity of trypsin preincubated without inhibitor. (○) shows the activity of trypsin preincubated in aqueous media with inhibitor for 5 min.

DISCUSSION

Intermediates of the folding pathway of monomeric proteins have been trapped under mild denaturing conditions, where neither the folded nor the unfolded states are stable (Hughson et al., 1990). Baker et al. (1992) have also trapped an intermediate in the folding of α-lytic protease using a protein that lacks its pro region. In oligomeric proteins, the stability of intersubunit contact has been altered by high hydrostatic pressure (Silva & Weber, 1993), ammonium sulfate (Girg et al., 1983), and site-directed mutagenesis (Ahern et al., 1987; Casal et al., 1987; Borchert et al., 1993). Hagen et al. (1990a,b) studied the renaturation of monomeric proteins in reverse micelles. Here it is described that by decreasing the water content of reverse micelles, it is possible to stabilize monomers competent for formation of dimeric TPI, as well as monomers formed in aqueous media. In both conditions, the trapped monomers remain in such state for substantial lengths of time; a rise in water concentration destabilizes the monomer and dimerization ensues. Thus, it was possible to determine that less water is required for dimerization than for monomer folding.

Intrinsic Fluorescence of Conformers Involved in the Formation of Active Dimers. The slow time course of TPI reactivation in reverse micelles allowed studies of the structural changes that accompany reactivation. Upon transfer of denatured TPI to reverse micelles with a relatively high water content, the protein acquires fluorescence characteristics that differ from those of the native and unfolded enzyme. Its intrinsic fluorescence is higher, and its spectral center of mass is intermediate between that of denatured and native enzymes. With time, catalytic activity progressively appears, concomitant to changes in emission fluorescence. When activity and fluorescence no longer change, the enzyme exhibits a fluorescence pattern that approaches that of the native enzyme. It was also observed that lowering the amount of water in contact with competent monomers stopped reactivation and changes in fluorescence; a subsequent rise in the water concentration restored reactivation and related fluorescence changes. Hence, the highly fluorescent conformers that appear after transfer of denatured TPI to reverse micelles represent an intermediate step in the pathway of an unfolded monomer to an active dimer. The data also show that dimerization involves a final arrangement of aromatic residues.

So far, we have failed to detect this fluorescent intermediate during TPI reactivation in all aqueous mixtures; i.e., spectra of TPI obtained 2−3 min after GdnHCl dilution were similar to those of the native protein (not shown). Since dimerization and reactivation of TPI in all aqueous media are rapid processes (Waley, 1973; Zabori et al., 1980), the failure to detect a high fluorescent intermediate in standard aqueous media may be due to technical limitations. However, it may be that the fluorescent intermediate with high fluorescence exists only in reverse micelles. That is, the fluorescence enhancement of the monomer in reverse micelles may result from folding to a monomer with high fluorescence, but alternatively, it may correspond to a monomer whose fluorescence is perturbed by the medium in the micellar core, and/or its interaction with the charged micellar wall. Indeed, there is evidence indicating that the fluorescence (Desfosses et al., 1991) and phosphorescence (Strambini & Gonelli, 1988) of protein tryptophans are modified by micellar surfaces.

Although the existence of the highly fluorescent monomer was not visualized in all aqueous media, in reverse micelles the intermediate species could be trapped for a substantial length of time. Moreover, in reverse micelles incompetent TPI monomers could also be locked in a state amenable to studies of their intrinsic fluorescence. It is noteworthy that the intrinsic fluorescence spectra of competent and incompetent monomers were markedly similar. This suggests that structural differences between the two types of monomers may be rather subtle, and that other approaches must be used to ascertain differences between the two.

Monomer−Dimer Interconversion in Reverse Micelles. It has been reported that dissociation of multimeric proteins in reverse micelles is favored by decreasing the size of the micellar water pool (Kabanov et al., 1991). Thus, dimerization in reverse micelles involves the association constant between monomers and the restrictions of the water space imposed by the physical limits of the micelle. The experiments with a model system (trypsin and the soy bean trypsin inhibitor) indicated that, at low water concentrations, proteins can interact. In consequence, it would appear that 2.0% water in reverse micelles does not suffice to support the final conformational arrangements that lead to the formation of the active dimer. In this context, it has been described that, after collision, complexes of multisubunit proteins undergo further transformations (Rudolph et al., 1986; Le Bras et al., 1989; Jaenicke & Buchner, 1993).

Regarding the barriers that hinder dimerization in reverse micelles, it is relevant that, with 2.0% water, the activity measurements indicated that after prolonged incubation times (48 h) formation of active dimers from competent monomers was less than 10%. On the other hand, when native TPI was transferred to reverse micelles with 2.0% water, the population of active dimers remained constant (100%). Hence, in 2.0% water there is no interconversion between competent monomers and active dimers. Near-equilibrium levels between the two conformers could only be reached at high water concentrations. Therefore, at low, but not at high water concentration, there is a kinetic barrier that hinders monomer−dimer interconversion.

Advantages and Disadvantages in the Use of Reverse Micelles for Studying Protein Folding and Dimerization. Enzymes entrapped in reverse micelles exhibit characteristics that may be used to study events related to protein mobility. Events such as catalysis (Garza-Ramos et al., 1994) and dimerization occur in "slow motion". For instance, in conventional aqueous media, formation of active TPI dimers from denatured monomers occurs in seconds, whereas in reverse micelles, the time for half-maximal reactivation is more than 1 h when 1−4 μg of protein/mL of reverse micelles is used. Moreover, in reverse micelles, it is possible to arrest or reinitiate the process by changes in water concentration. In this regard, reverse micelles have clear advantages over conventional aqueous media.

However, reverse micelles systems have disadvantages. These are principally due to their physicochemical properties, and in particular to the ill-defined characteristics of protein-filled micelles. Rahaman and Hatton (1991) recently reviewed the effects that a protein exerts on the host micelle, and on the system as a whole. From the available data, it is not yet clear to what extent micellar structure and size are affected by the protein. Likewise, it is possible that the charged micellar surface might prevent free mobility of the protein and induce distortions of the intermediates that occur in the "normal" pathway of folding. If these distorsions take place, it is clear that, at least for TPI, the perturbations do not affect formation of the competent monomers and catalytically active dimers. However, one of the main problems in the use of reverse micelles concerns the amount of water that is needed for a given process. For instance, with denatured TPI transferred to reverse micelles with 3.0% water ($W_0 = 8.3$), there is a reactivation of around 10%. At this water concentration, there are about 1700 water molecules per micelle. Following Rupley and Careri (1991), about 1100 water molecules are required to surround TPI with a monolayer. Thus, on these grounds, it would appear that more than a monolayer of water molecules is needed to support formation of the active dimer. However, it is stressed that these figures do not necessarily indicate that this is the amount of water that is in contact with the protein in a protein-filled micelle. The latter is of importance, since very likely these water molecules are the ones that support or arrest structural arrangements during protein folding and dimerization (Zaks, 1992). In this respect, it is of interest that NMR analysis of water protons in reverse micelles of the type used here revealed that, up to 4.0%, water exists as "bound" water; above this concentration, water protons exhibit the properties of "bulk" water (Kernen et al., 1993). Hence, in the evaluation of the amount of water that is needed to support folding and dimerization of TPI, both the amount and the state of the solvent should be taken into consideration. Nevertheless, the present results illustrate that, in addition to other approaches (Udgaonkar & Baldwin, 1988; Roder et al., 1988; Serrano et al., 1992; Radford et al., 1992), water may be used to probe events involved in protein folding and dimerization. This is because water is an essential component of such processes.

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