Deamidation of Triosephosphate Isomerase in Reverse Micelles: Effects of Water on Catalysis and Molecular Wear and Tear†

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ABSTRACT: The specific deamidation of asparagine-71 of triosephosphate isomerase increases upon substrate binding and catalysis. This deamidation at the dimer interface initiates subunit dissociation, unfolding, and protein degradation. The apparent connection between catalysis and terminal marking supports the concept of "molecular wear and tear", and raises questions related to the molecular events that lead to deamidation. In order to explore this interaction, triosephosphate isomerase was entrapped in reverse micelles with different water contents that support different catalytic rates. Deamidation was quantified for the free enzyme, the enzyme in the presence of substrates, and the enzyme which had been covalently modified at the catalytic center with the substrate analogue 3-chloroacetol phosphate (CAP). Both in water and in reverse micelles of cetyltrimethylammonium with 3% and 6% water, substrate binding enhanced deamidation. Studies of the extent of deamidation at various water concentrations showed that deamidation per catalytic turnover was about 6 and 17 times higher in 6% and 3% water than in 100% water, respectively. The enzyme was also entrapped in micelles formed with toluene, phospholipids, and Triton X-100 to explore the process at much lower water concentrations (e.g., 0.3%). Under these conditions, catalysis was very low, and hardly any deamidation took place. Deamidation of the CAP-labeled enzyme was also markedly diminished. At these low-water conditions, the enzyme exhibited markedly increased thermostability and resistance to hydrolysis of the amide bonds. The data suggest that the rate of deamidation not only is dependent on the number of catalytic events but also is related to the time that asparagine-71 exists in a conformation or solvent environment more favorable for deamidation.

Triosephosphate isomerase (EC 5.3.1.1, TPI)[1] is a homodimeric enzyme catalyzing the interconversion of D-glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. Its thermodynamics, kinetics, and structural changes which occur during catalysis have been studied in great detail (Albery & Knowles, 1976; Banner et al., 1975; Blacklow et al., 1988; Lolis et al., 1990; Lolis & Petsko, 1990; Knowles, 1991; Sampson & Knowles, 1992; Wierenga et al., 1991, 1992). Upon ligand binding at the catalytic center, a hinged lid (residues 168–177) closes over the catalytic center. This conformational change anchors the substrate into the correct steric position and expels water from the catalytic center, thereby preventing hydrolysis of the reaction intermediate to methylglyoxal and inorganic phosphate (Pompliano et al., 1990). In addition, ligand binding enhances the specific deamidation of Asn71 (Sun et al., 1992a; Yüksel & Gracy, 1986) on each subunit, which appears to be the terminal marking event in the degradation of the enzyme (Yuan et al., 1981). This deamidation is followed by subsequent deamidation of the juxtaposed Asn15 (<0.5 nm away on the neighboring subunit). The introduction of four new negative charges at the subunit interface leads to dissociation, unfolding, and proteolytic degradation (Ahern et al., 1987; Casal et al., 1987; Sun et al., 1992a; Yuan et al., 1981). Hence, studies on the mechanisms involved in deamidation, and the factors which affect protein stability, should shed light on the initial steps of degradation of TPI and other proteins.

The original observations that the rate of deamidation increases as a function of the number of catalytic turnovers (Yüksel & Gracy, 1986) led to the proposal that deamidation may be a consequence of the number of times that the substrate-induced conformational change causes the critical Asn71 to be exposed to the solvent or assume a conformation more likely to deamidate [i.e., "Molecular Wear and Tear" (Sun et al., 1992a; Yüksel & Gracy, 1986)]. Asparagines of certain configurations are especially prone to deamidation (Kossiakoff, 1988). The present study explores the question of whether deamidation depends on the time that critical Asn71 is exposed to the solvent, or exists in a deamidation-favored conformation (i.e., the cumulative effects of the opening and closing of the hinged lid as the odometer of deamidation). To this purpose, deamidation of TPI entrapped in reverse micelles with different water contents was quantified and correlated with the catalytic turnover.

The low-water system was chosen since many studies have shown that the catalytic activity of enzymes (Garza-Ramos et al., 1992a; Luisi & Magid, 1986; Martinek et al., 1986), including TPI (Garza-Ramos et al., 1992b), is markedly influenced by the amount of water in the system. In general, as the water content is decreased, catalytic activity decreases, and this has been ascribed to hindrances in the requisite conformational changes of the protein (including the solvent)

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[1] Abbreviations: CAP, 3-chloroacetol phosphate; TPI, triosephosphate isomerase; W_0, ratio of water molecules to surfactant molecules; TPT, toluene/phospholipid/Triton X-100.

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during a catalytic cycle (Garza-Ramos et al., 1992a; Zaks & Klibanov, 1984). Moreover, the steps of a catalytic cycle have a distinct water requirement (Barrabin et al., 1993), which suggests that by varying the amount of water in contact with the enzyme it is possible to trap catalytic cycle intermediates (Escamilla et al., 1989). With respect to TPI, it was reasoned, that if at relatively low water concentrations the catalytic cycle could be induced to function in "slow motion", there would be a longer dwell time for Asn71 in the deamidation-favorable conformation (or exposure to the solvent) and an opportunity to examine the relationship between catalysis and deamidation.

The studies described here show that as the rate of catalysis is diminished by low water, deamidation per catalytic cycle is increased. However, at very low water concentrations in which catalysis is hardly detectable, deamidation is largely prevented. At these low water concentrations, TPI exhibits a high thermostability due to a decrease in protein flexibility (Garza-Ramos et al., 1989, 1990) and resistance to covalent modification.

MATERIALS AND METHODS

TPI (5.3.1.1) from rabbit muscle (lyophilized powder), as well as cetyltrimethylammonium bromide, *n*-octane, hexanol, soybean phospholipids (asolectin), and Triton X-100, was obtained from Sigma; toluene was from Merck.

Deamidation of Triosephosphate Isomerase in Reverse Micelles. TPI dissolved in 50 mM triethanolamine, pH 9.6, was entrapped in either of two types of micelles. One type was composed of a mixture of 200 mM cetyltrimethylammonium bromide in *n*-octane to hexanol in a ratio of 8.7:1 (by volume) (Hilhorst et al., 1984; Tyrakowska et al., 1990). Reverse micelles were formed by adding aqueous 50 mM triethanolamine, pH 9.6, with or without 2.0 mM glyceraldehyde 3-phosphate. After micelles were formed, TPI at the desired concentration was introduced. Depending on the experiment, the final concentration of water was either 3 or 6% by volume; these correspond to W_0 values (ratio of water to surfactant molecules) of 8.2 and 16.5, respectively. TPI was also transferred to reverse micelles formed with toluene/Triton X-100 (85:15 by volume) that contained 10 mg of soybean phospholipids/mL (Garza-Ramos et al., 1990). The amount of water in this system was either 0.3 or 3.8% (by volume); these values correspond to W_0 values (ratio of water molecules to molecules of Triton X-100 + phospholipids) of 0.7 and 8.9, respectively. At the lower water concentrations, micelles were formed by injection of a water solution (50 mM triethanolamine, pH 9.6, with or without 2.0 mM glyceraldehyde 3-phosphate adjusted to pH 9.6) that contained TPI. In some cases, TPI entrapped in TPT-type reverse micelles was incubated for up to 10 days; the reverse micelle system was stable for this length of time. The water solubility diagrams of the two types of micelles have been previously described (Garza-Ramos et al., 1992c).

The transfer of TPI to reverse micelles was made at room temperature. Prior to its transfer, the freshly dissolved enzyme was maintained in an ice bath (not more than 15 min). After transfer, the enzyme was incubated at the temperatures and for the times indicated under Results. At different times of incubation, TPI entrapped in reverse micelles was transferred back to water. When the enzyme was entrapped in micelles of the cetyltrimethylammonium bromide type, the enzyme was recovered by carefully layering 50 μL of reverse micelles over 1 mL of 40 mM triethanolamine, 10 mM ethylenediaminetetraacetate, and 1 mM dithiothreitol, pH 7.4, and centrifuged at 105000*g* for 60 min at 20 °C. Afterward, the water phase was collected. The same procedure was followed to recover TPI from reverse micelles formed with phospholipids and Triton X-100, except that centrifugation was carried out at 15000*g*. Generally, the water phase from four to eight identical tubes was collected. The water phases were pooled, and remaining surfactant that remained in the mixture was extracted with isoamyl alcohol (Ramirez et al., 1983). The water extracts were kept at 4 °C. As calculated from the total TPI activity in the water phase, between 70 and 90% of the enzyme was recovered.

The water extracts were concentrated in Centricon filters by centrifugation at 5–10 °C to volumes of 30–50 μL. These were assayed for the presence of deamidated isoforms by analytical gel electrophoresis under nondenaturing conditions as described by Maizel (1971). The gels were stained for both TPI activity (Snapka et al., 1974) and protein (Coomassie blue). In some experiments, the Coomassie blue stained gels were quantified by scanning densitometry on an LKB Ultrascan XL densitometer using the software integrated into the instrument.

The activity of TPI in all water mixtures or in reverse micelles was measured as described previously (Garza-Ramos et al., 1992b) in the presence of glyceraldehyde 3-phosphate, NADH, and α-glycerophosphate dehydrogenase. The concentration of TPI was calculated from its absorbance at 280 nm (Lu et al., 1984).

Circular Dichroism Spectroscopy. Circular dichroic analyses of TPI in 100% water or when entrapped in reverse micelles of the cetyltrimethylammonium type were carried out with an Aviv Model 62 HDS spectropolarimeter in 0.1-cm quartz cuvettes at 25 °C. In these studies, TPI was at a concentration of 0.052 mg/mL per milliliter of either 100% water or reverse micelles of 3 or 6% water. The samples were scanned from 300 to 215 nm. Due to the high background of reverse micelles (without protein), it was not possible to carry out analysis at lower wavelengths. The ellipticity data at 222 nm were obtained by graphic interpolation, and the mean residue ellipticity (Sun et al., 1992b) was calculated from $[\theta] = [\theta_{obs}]/10(MRC)l$, where (MRC) = (496 amino acid residues)(0.981×10^{-6} M) and l = 0.1 cm.

Fluorescence Spectroscopy. The fluorescence emission spectra of TPI excited at 295 nm were recorded between 305 and 450 nm on a Shimadzu RF 5000U spectrofluorometer. The bandwidths of excitation and emission wavelengths were set at 5 nm. The enzyme concentration was 0.2 mg/3.0 mL of either 50 mM triethanolamine, pH 9.6, or reverse micelles of the cetyltrimethylammonium type formed with the same buffer at a concentration of 3% or 6% water (by volume). The fluorescence spectra of the blanks (identical samples, except for the enzyme) were subtracted from those that contained the enzyme.

RESULTS

Enzyme Turnover and Deamidation in Low-Water Systems. Ligand-induced deamidation of mammalian TPI readily occurs at 37 °C at mildly alkaline pH (Sun et al., 1992a; Yuan et al., 1981; Yüksel & Gracy, 1986), and, thus, deamidation studies in low water were also carried out at pH 9.6 and at 37 °C. In order to determine if deamidation of TPI in reverse micelles was related to catalytic turnover, experiments were conducted in reverse micelles formed with cetyltrimethylammonium bromide, *n*-octane, hexanol, and 3% or 6% water. We have recently demonstrated that the catalytic activity of TPI is significantly different in 3, 6, and 100% water (Garza-

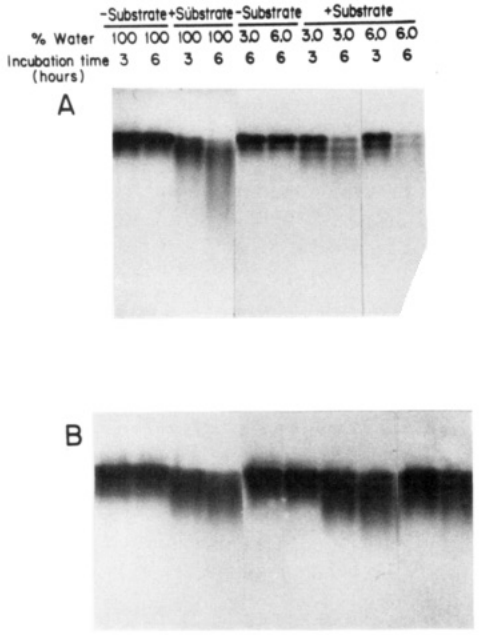

FIGURE 1: Deamidation of TPI in reverse micelles. In all-aqueous medium (100% water), TPI (2.8 mg/mL) was incubated in 50 mM triethanolamine, pH 9.6, with or without 2.0 mM glyceraldehyde 3-phosphate. For the experiments in 3% and 6% water, TPI dissolved in the same buffer was transferred to reverse micelles of the cetyltrimethylammonium bromide type, yielding a final water concentration of 3% and 6%, with or without 2.0 mM glyceraldehyde 3-phosphate (as indicated). The final enzyme concentration was 68 μg/mL of reverse micelle system. The samples were incubated at 37 °C. At the indicated times, aliquots were withdrawn, and the enzyme was transferred back to water for electrophoretic analysis under nondenaturing conditions. Native TPI and its isoforms were visualized by Coomassie blue (A) and activity staining (B).

Ramos et al., 1992b). Here, TPI was incubated with and without substrate in all aqueous media, and in reverse micelles containing 3% and 6% water. After 3 and 6 h of incubation, the enzymes were recovered into all aqueous media and analyzed. Native gel electrophoresis showed that in the absence of substrate and independent of water concentration, the amount of native TPI remained constant, indicating no deamidation was taking place (Figure 1). The presence of glyceraldehyde-3-phosphate, however, induced a time-dependent decrease in the amount of the native enzyme band with the simultaneous formation of acidic deamidated isoforms (Figure 1). Table 1 shows the extent of substrate-induced deamidation as the loss of native protein. The catalytic activities of these samples assayed under the same conditions they were incubated are also shown (Table 1). At any given time, the relative amount of deamidated protein was about 20% higher in 100% water than in 3.0% or 6.0% water. However, the activity of TPI in 3% and 6% water was about 25-fold and 9-fold lower than in 100% water, respectively. Since the extent of deamidation depends on the number of catalytic cycles that the enzyme undergoes, the data indicate that deamidation per catalytic turnover increased as the rate of catalysis diminished by limiting the amount of water in contact with the enzyme. In fact, one can calculate that deamidation per catalytic activity is approximately 6 and 17 times higher in 6% and 3% water than in 100% water, respectively (Table 1, last column). This in turn suggests that the extent of deamidation is not solely dependent on the number of catalytic turnovers.

To eliminate the possibility of gross structural changes of TPI in reverse micelles, CD and fluorescence spectra of the enzyme were recorded under these conditions (Figure 2). Either spectra recorded at the three water concentrations were superimposable. Quantitatively, the minor differences in molar ellipticity at 222 nm were not significant (Figure 2A). The intrinsic tryptophan fluorescence of TPI recorded at the three water concentrations peaked at 330 nm, and the magnitude of fluorescence was also markedly similar (Figure 2B). Hence, it is obvious that there are no gross changes in the structure of TPI at the three water concentration in which substrate-induced deamidation was studied.

Very Low-Water Systems. After it was found that TPI entrapped in two different types of low-water micelles underwent substrate-induced deamidation, studies were conducted to explore if the process was modified by further decreasing the water concentration to much lower levels. Thus, the phospholipid/Triton X-100 type (TPT) micelles were used whereby enzymes can be transferred to reverse micelles with water concentrations as low as 0.3%; this corresponds to W_0 = 0.7 (Garza-Ramos et al., 1990). Moreover, this system is stable at temperatures greater than 90 °C, and allows thermostability studies at much higher temperatures. With TPI entrapped in micelles with 0.3% water, with or without substrate, there was no deamidation. It is important to note that the catalytic activity of enzymes placed in reverse micelles of the TPT type ($[H_2O]$ <0.5%) is extremely low (Garza-Ramos et al., 1990).

Since deamidation of TPI is enhanced by catalysis and/or substrate or analog binding (Sun et al., 1992a; Yüksel & Gracy, 1986), it was necessary to determine if the absence of deamidation in 0.3% water was due to lack of catalysis or substrate binding. In this respect, the use of chloroacetol phosphate (CAP) instead of natural substrates presented two advantages: (a) it binds covalently to the catalytic site of the enzyme (Hartman, 1971; Norton & Hartman, 1972) and thereby induces deamidation (Sun et al., 1992a); (b) CAP–TPI can be easily identified by native gel electrophoresis because of the shift in mobility toward the anode (Sun et al., 1992a; see Figure 3, lanes 1 and 2). The enzyme was first transferred to the TPT system at pH 7.4, followed by the introduction of CAP at ratios of 1.6 and 10 CAP per monomer (the final water concentration was 0.3%). After 24 h, the enzyme was transferred back to water, concentrated, and analyzed by native gel electrophoresis. Two protein bands with a mobility faster than that of native TPI were clearly evident (Figure 3). The isoform with intermediate migration was more clearly observed in samples that had the lower CAP:TPI ratio, and represents dimeric TPI derivatized at only one catalytic site. Similar to all-water media, after prolonged incubation, the derivatized enzyme "lost" a fraction of bound CAP (lanes 7 and 8, Figure 3). This has been observed previously and shown to be due to progressive hydrolysis of the CAP Glu165 ester (Sun et al., 1992a). The faster migrating band (observed with the highest CAP:TPI ratio) corresponded to that of dimeric TPI derivatized at both of the catalytic sites. Thus, it is evident that the entire population of TPI entrapped in reverse micelles formed with 0.3% water has the capacity to bind CAP. Moreover, depending on the CAP:TPI ratio, it is possible to derivatize one or two of the catalytic sites of dimeric TPI.

In a second experiment, TPI derivatized in all-water media with two CAP per dimer was transferred to reverse micelles at pH 9.6, incubated for up to 10 days, and analyzed for deamidation (Figure 3, lanes 6 and 9, respectively). After 10 days of incubation, only slight deamidation of the CAP–TPI complex was observed (Figure 3, lane 9, and compare to the pattern at pH 7.4). These data indicate that ligand-induced

Table 1: Activity, Deamidation, and Stability of TPI as a Function of Water Concentration[a]

water content (%)	catalytic activity (units/mg)[b] –G3P 3 h	–G3P 6 h	+G3P 3 h	+G3P 6 h	deamidated protein (%)[c] –G3P 6 h	+G3P 3 h	+G3P 6 h	deamidation per catalysis (% units^{-1} mg^{-1})[d]
100	4190 ± 434	4030 ± 541	3300 ± 237	2380 ± 346	15 ± 3.5	61 ± 3	86 ± 5	0.036
6	478 ± 47	452 ± 54	408 ± 72	286 ± 80	20 ± 1.5	46 ± 7	63 ± 11	0.220
3	138 ± 18	143 ± 33	139 ± 53	113 ± 36	23 ± 1.3	53 ± 19	69 ± 8	0.610

[a] The experiments ($n \geq 3$) were carried out as described in Figure 1, except the TPI concentration was 50–70 μg/mL of reverse micelles. [b] Activity of the enzyme incubated at 37 °C for the indicated time periods at the respective water concentrations. In all cases, the activity at time zero was virtually identical to the activity after 3-h incubation in the absence of substrate. [c] The relative protein concentrations were calculated from densitometric scans of Coomassie blue stained gels as shown in Figure 1. The disappearance of the native (nondeamidated) band was taken as the extent of deamidation. Gel scans of stock TPI that had not undergone any treatment exhibited isoforms; these comprised 15% of the total protein. [d] Deamidation per catalysis was calculated by dividing the amount of deamidated protein (%) by the catalytic activity of the enzyme which had been incubated for 6 h in the presence of G3P.

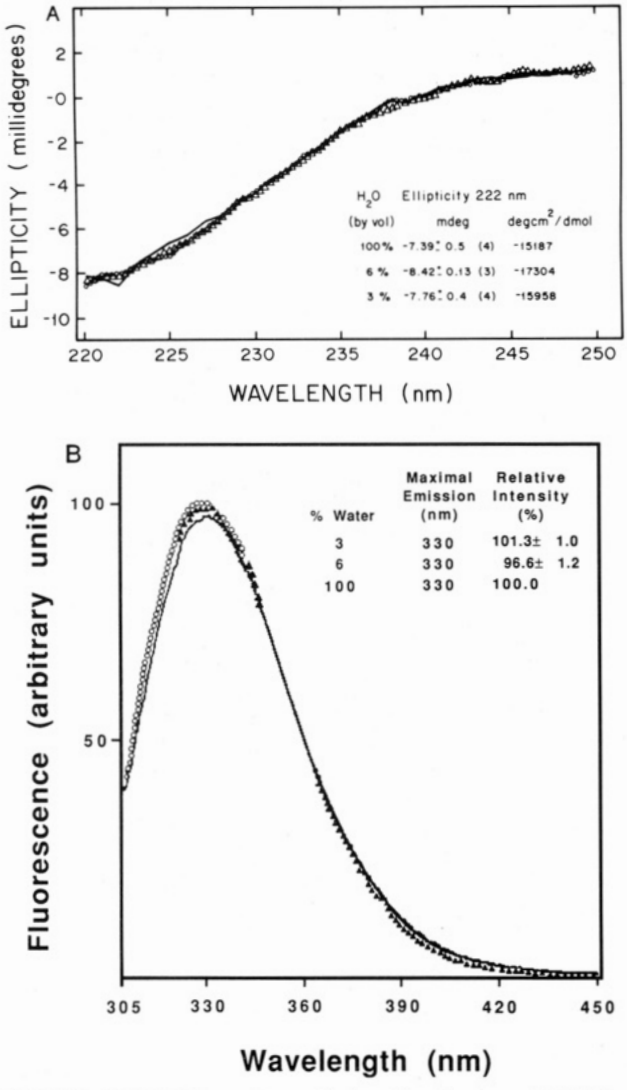

FIGURE 2: Effect of the water content on the structure of TPI. To assess any structural changes of TPI upon entrapment in reverse micelles, CD (A) and fluorescence (B) spectra were recorded as described under Materials and Methods in 3% (O), 6% (—), and 100% water (Δ). The insets show the averages ± standard deviations of ellipticity at 222 nm, relative emission intensity, and wavelength of maximal fluorescence.

deamidation of TPI is markedly decreased when the enzyme is entrapped in reverse micelles with 0.3% water, but not with 3% or higher water concentrations (see above).

Stability in Low Water. In light of the preceding data, it became of interest to determine if at low water concentrations impairments in the hydrolysis of side chain amide bonds of Asn were accompanied by an increase in resistance of the

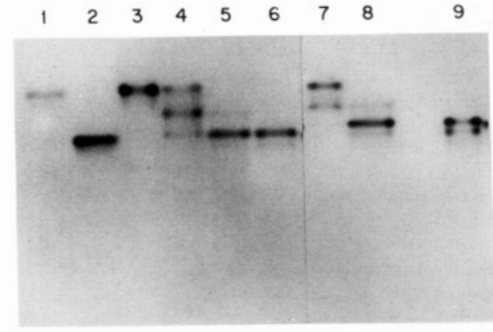

FIGURE 3: Formation of the CAP–TPI complex in reverse micelles and deamidation of the complex in reverse micelles with 0.3% water. Micelles formed with toluene, phospholipids, and Triton X-100 with a final concentration of 0.3% water were used. The concentration of TPI or derivatized CAP–TPI was 48 μg/mL of micelle system. Lane 1 shows the electrophoretic migration in native gels of native TPI, whereas lane 2 shows the migration of TPI derivatized with CAP in all-water media. Lane 3 shows TPI transferred to reverse micelles and back to water. In lanes 4 and 5, TPI dissolved at pH 7.4 in 50 mM triethanolamine was transferred to reverse micelles followed by the addition of CAP (ratios of 1.6 and 10 CAP per monomer, respectively); after 24 h of incubation at 37 °C, the enzyme was transferred back to water. Lanes 7 and 8 correspond to the same samples after 10 days of incubation. Lanes 6 and 9 correspond to TPI derivatized in water and transferred to reverse micelles at pH 9.6; after 24 h and 10 days of incubation, respectively, the enzymes were brought back to water. The figure shows Coomassie blue stain of the samples.

enzyme to thermal denaturation. The incubation of TPI in all-water mixtures at temperatures of 57 and 75 °C brought about its rapid irreversible inactivation, and the inactivation rate was increased by substrate (Figure 4). The time for half-maximal inactivation at 57 °C was approximately 5 and 10 min with and without substrate, respectively. At 75 °C, with or without substrate, most of the activity was lost within 5 min of incubation [(*) and (+) in Figure 4, respectively]. TPI entrapped in reverse micelles with 0.3% water was much more stable than in all-water media. For example, after 2 h at 75 °C, only 30% of the activity was lost, and at 57 °C, 50% of the activity remained after 24 h of incubation (data not shown). Analysis by native gel electrophoresis showed, that during the incubation at elevated temperature, the enzyme in all-water media underwent drastic changes (Figure 5A). In addition, it is apparent that in the presence of substrate, these changes occurred more readily. On the other hand, notwithstanding the presence of substrate, essentially no changes were observed in TPI that had been incubated in 0.3% water reverse micelles for extended periods (7 and 24 h) at the elevated temperatures (Figure 5B). These findings indicate, that when placed in a low-water environment, the catalytic capacity and structure of TPI are largely preserved even at elevated

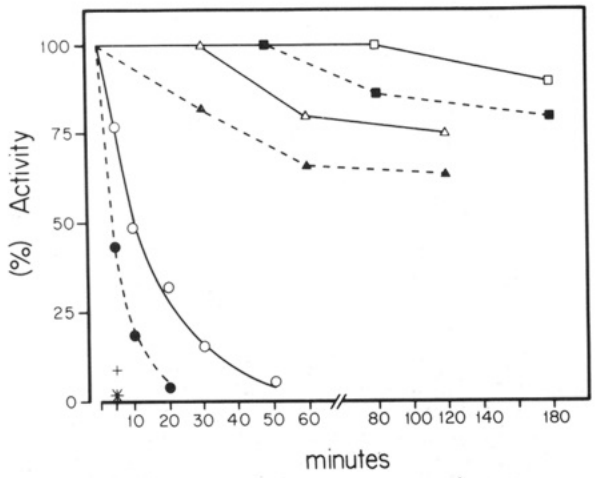

FIGURE 4: Inactivation of TPI in all-water media and in reverse micelles with 0.3% water. TPI was dissolved in 50 mM triethanolamine, pH 9.6, with (●) or without (○) 2 mM glyceraldehyde 3-phosphate at a concentration of 0.83 mg/mL (100% water). At time zero, the mixtures were placed in a water bath at 57 °C. (* and + indicate the activity of TPI incubated at 75 °C with and without substrate, respectively.) At the times shown, aliquots were withdrawn and placed in an ice bath. TPI dissolved in 50 mM triethanolamine, pH 9.6, with (■, ▲) or without (□, △) 2 mM glyceraldehyde 3-phosphate was transferred to reverse micelles of the toluene, phospholipid, and Triton X-100 type, yielding a final water concentration of 0.3% (by volume). At time zero, the tubes were placed in water baths at 57 °C (□, ■) and 75 °C (△, ▲). At different times, aliquots were cooled in an ice bath and subsequently transferred to all-water media. The activity of the water phases gave the values indicated in percent of the activity of TPI without thermal treatment (3200 μmol min^{-1} mg^{-1}).

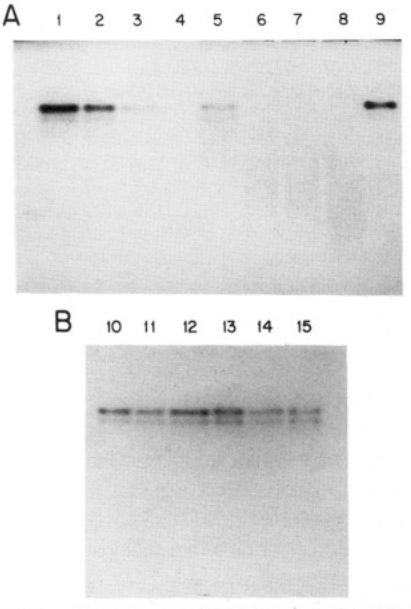

FIGURE 5: Native gel patterns of TPI incubated at 57 and 75 °C in all-water media (A) and in reverse micelles (B). The protocol was as in Figure 4, except that the samples were concentrated and applied to native gels and stained for protein. (A) 100% water without (lanes 1–4) and with (lanes 5–8) 2 mM glyceraldehyde 3-phosphate at 57 °C for 10, 20, 30, and 50 min, respectively. Lane 9 shows the gel pattern of native TPI. In (B), TPI was transferred to reverse micelles without (lanes 10, 12, and 14) and with substrate (lanes 11, 13, and 15) and incubated at 57 °C for 7 h (lanes 10, 11) and 24 h (lanes 12, 13), or at 75 °C for 2 h (lanes 14, 15).

temperatures. It should also be noted that, under these conditions, no deamidated isoforms were detected.

Taken together, the results in water and in reverse micelles show, that by diminishing the amount of water, it is possible to achieve conditions in which the probability of deamidation per turnover number is higher. However, if water is further decreased, deamidation is prevented. The latter probably reflects the water requirements of the deamidation reaction per se.

DISCUSSION

Crystallographic data of TPI from chicken, yeast, and trypanosomes indicate that substrate binding and catalysis result in the movement of the hinged lid over the active site (Lolis & Petsko, 1990; Wierenga et al., 1991, 1992). As evidenced by the enhanced rate of deamidation of Asn71 in the presence of substrate or covalently bound substrate analogs (e.g., CAP), this conformational change must be transmitted to the "interdigitating loop" at the subunit interface (Sun et al., 1992a; Yuan et al., 1981; Yüksel & Gracy, 1986). Regarding the mechanism(s) of this ligand-induced deamidation, it has been instructive to compare the process in water and in low-water environments. In all-water media, it is well documented that as the number of catalytic turnovers is increased, the rate of deamidation is increased (Sun et al., 1992a; Yüksel & Gracy, 1986). However, as water concentrations are decreased and catalysis slowed down, a different picture emerges (Table 1). For example, deamidation of the enzyme in reverse micelles with 3% and 6% water was roughly the same, but the rates of catalysis were 3 times higher in 6% water (Figure 1). This implies that, per catalytic cycle, deamidation is higher at the low water concentration. Since the circular dichroism and fluorescence spectra of the enzyme were similar in micelles with 3%, 6%, and 100% water, the differences in the rates of deamidation do not seem to be due to grossly different conformations. Hence, with respect to the relationship between catalytic rates and deamidation, there are clear differences between all-water and low-water systems. These observations point out the advantage of utilizing low-water model systems to explore reaction mechanisms in which water is clearly a participant.

The catalytic activity of enzymes entrapped in reverse micelles decreases as the amount of water is diminished (Garza-Ramos et al., 1990, 1992a,b,c; Luisi & Magid, 1986; Martinek et al., 1986). This is thought to be due to impairments in the interactions between the solvent and protein that are necessary for catalysis (Garza-Ramos et al., 1989, 1990, 1992a,b,c). Thus, under conditions of limited water, the steps of the catalytic cycles that involve water–protein interactions are slower than in all-water media. As the rate of catalysis is diminished, the time in which the critical asparagines are prone to deamidation would be longer. As a consequence, there would be a higher probability of deamidation per catalytic turnover number. It is clear (Figure 1) that deamidation per enzyme turnover is higher in the micellar system. After 7 h of incubation, similar deamidation levels were observed in 100% water and in 6% water; albeit the catalytic activity was about 10 times higher in all-water media.

Altogether, the data suggest that in all-water media, deamidation is a statistical event that depends on the number of times that TPI acquires "the conformation" in which the Asn side chain amide bond is cleaved. The data in reverse micelles illustrate that the time that TPI remains in this conformation is also a factor (i.e., the longer the time of exposure to the solvent, the greater the probability of deamidation).

Ahern et al. (1987) and Casal et al. (1987) showed that TPI undergoes deamidation at high temperatures and concluded that the specific asparagine residues at the subunit interface constitute the "weak links" in the maintenance of

the native structure. Asparagines have also been shown as weak points in the thermal denaturation of other enzymes. At 37 °C in 100% water or in reverse micelles with water concentrations above 3%, substrate-induced deamidation of TPI was observed prior to the appearance of changes in the structure of TPI (e.g., as evidenced by inactivation and smearing of the protein in gels) and corroborates previous studies (Sun et al., 1992a; Yuan et al., 1981; Yüksel & Gracy, 1986). These findings further indicate, that at physiological temperature and in the presence of substrate, the initial event of degradation is deamidation of Asn71. However, are these asparagines the weakest points in thermal inactivation of TPI, and are they affected by the amount of water? In all-water media and at a temperature of 57 °C, changes that lead to irreversible thermal inactivation of TPI take place. In the presence of glyceraldehyde 3-phosphate, inactivation and multiple alterations of TPI occurred more readily than in the absence of substrate (Figure 5A). In aqueous media, substrate-induced deamidation of Asn71, or the overall conformation that the enzyme acquires in the presence of substrate, confers to TPI an increased sensitivity to thermal inactivation. Various groups have observed that in a restricted water environment, enzymes exhibit increased thermostability, and this has been ascribed to the greater rigidity that proteins acquire in such conditions (Barrabin et al., 1993; Escamilla et al., 1989; Zaks & Klibanov, 1984). However, we have now shown that, notwithstanding the presence of substrate, cleavage of protein covalent bonds (deamidation) is also reduced when the enzyme is placed in a limited water space. Hence, it would appear that both the increased rigidity of the enzyme and the resistance to hydrolysis of covalent bonds account for the increased thermal resistance of protein stability in low-water conditions.

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