Articles

Evidence for Residual Structures in an Unfolded Form of Yeast Phosphoglycerate Kinase†

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Received July 29, 1994; Revised Manuscript Received October 6, 1994®

ABSTRACT: The unfolding−refolding transition of phosphoglycerate kinase followed by steady-state fluorescence has clearly shown the existence of a hyperfluorescent form [Missiakas *et al.* (1990) *Biochemistry 29*, 8683−8689]. In order to determine the contribution of each of the two tryptophans to the fluorescence properties of the enzyme in the equilibrium transition and to characterize the hyperfluorescent form, two single tryptophan mutants in which tryptophans 308 and 333 were replaced by a tyrosine and a phenylalanine, respectively, were constructed. Neither the catalytic nor the physicochemical properties of the enzyme are significantly altered by these mutations. The unfolding−refolding transitions were studied using circular dichroism and tryptophan fluorescence emission. Both tryptophans contribute to the hyperfluorescence observed in the first transition. For guanidine hydrochloride concentrations higher than 0.9 M, it clearly appears that the second transition results from a further unfolding. It is accompanied by a decrease in fluorescence intensity and a 5 nm red shift of the maximum emission wavelength. When the unfolding is induced by urea, the end of the transition corresponds to the hyperfluorescent state. Further addition of guanidine hydrochloride induces complete unfolding. These results suggest the presence of residual microstructures around tryptophan 308 and tryptophan 333 in the hyperfluorescent state. The characterization of these clusters and their contribution as starting structures in the folding process are now under investigation.

The folding pathway of 3-phosphoglycerate kinase has been extensively investigated. The three-dimensional structure of the yeast enzyme (Watson *et al.*, 1982) has shown that the polypeptide chain is folded in two globular domains of approximately the same size. The C-domain contains two tryptophan residues, an external tryptophan at position 308 and a buried tryptophan at position 333 in a well-packed region near the active site. These residues present convenient intrinsic fluorescent probes for the study of the behavior of the C-domain in the unfolding−refolding transition of phosphoglycerate kinase. It has been shown (Nojima *et al.*, 1976) that the fluorescence intensity of the two tryptophans in yeast phosphoglycerate kinase is unusually 2-fold increased during the unfolding process, suggesting that they are quenched by neighboring residues in the native protein. Indeed, the fluorescence quantum yield of native phosphoglycerate kinase is 4.6% (Nojima *et al.*, 1976) whereas it is 20% for a free tryptophan in water at 25 °C (Teale & Weber, 1957). Further information has been obtained (Privat *et al.*, 1980; Dryden & Pain, 1989) showing that, in the native protein, about 60% of the total fluorescence intensity can be assigned to the external, solvent-exposed, tryptophan 308. The equilibrium unfolding−refolding transitions of phosphoglycerate kinase induced by guanidine hydrochloride (Gdn-HCl)[1] as assessed by the variations in fluorescence emission intensity have clearly shown two transitions (Missiakas *et al.*, 1990). The first one corresponds to an increase in fluorescence intensity leading to a state at 0.9 M Gdn-HCl with a fluorescence higher than those of both the native and the completely unfolded states and devoid of secondary structure. The second transition corresponds to a decrease in fluorescence intensity up to 2 M Gdn-HCl. For higher guanidine hydrochloride concentrations, the fluorescence intensity increases linearly with denaturant concentration, indicating a simple solvent effect.

The existence of residual structures in the denatured state of proteins has been recently reported for different proteins such as lysozyme (Evans *et al.*, 1991), staphylococcal nuclease (James *et al.*, 1992), the α-subunit of tryptophan synthase (Saab-Ricón *et al.*, 1993), BPTI (Lumb & Kim, 1994), and FK506 binding protein (Logan *et al.*, 1994). Hydrophobic collapses around tryptophan residues have been observed for several proteins (Evans *et al.*, 1991; Neri *et al.*, 1992). The residual structures observed in the unfolded state of proteins may correspond to nucleation centers in the folding process. In this regard, a regain of interest in the study of the denatured state has recently emerged (Dill &

† This work was supported by the Centre National de la Recherche Scientifique, the Ministère de l'Enseignement Supérieur et de la Recherche, the Fondation pour la Recherche Médicale Française, and by a contract with the Centre National de la Transfusion Sanguine.

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® Abstract published in *Advance ACS Abstracts,* December 15, 1994.

[1] Abbreviations: EDTA, ethylenediaminetetraacetate; Gdn-HCl, guanidine hydrochloride; NATRA, *N*-acetyltryptophanamide; yPGK, yeast phosphoglycerate kinase (EC 2.7.2.3); SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/95/0434-397$09.00/0

FIGURE 1: Structure of yPGK showing the localization of the tryptophan residues.

Shortle, 1991; Dobson *et al.*, 1992; Shortle 1993). The configurational distribution of phosphoglycerate kinase in 4 M Gdn-HCl has been examined using small-angle neutron scattering, statistical mechanical theory, and molecular modeling (Calmettes *et al.*, 1993).

The aim of the present work was first to determine the contribution of each of the two tryptophans to the fluorescent properties of phosphoglycerate kinase in the equilibrium unfolding−refolding transition, especially to characterize the so-called hyperfluorescent state and to study the second transition, which leads to a completely unfolded protein. Thus, two single tryptophan mutants in which tryptophan at positions 308 and 333 was replaced by a tyrosine and a phenylalanine, respectively, were constructed (Figure 1) and studied.

MATERIALS AND METHODS

Site-Directed Mutagenesis and Protein Purification. Two mutants were constructed: W308Y in which tryptophan 308 was replaced by a tyrosine and W333F in which tryptophan 333 was replaced by a phenylalanine. Mutations were introduced by site-directed mutagenesis according to Minard *et al.* (1989a). The same methods of purification (Minard *et al.*, 1989b) were used for the wild-type and mutant proteins. The purity of the mutants and wild-type yPGK was verified after each purification by SDS−PAGE.

Enzyme Activity and Molar Extinction Coefficient. The enzyme activity was measured as described by Bücher (1955) with a 0.2 nM protein concentration. The mutant concentrations were checked by absorbance using molar extinction coefficients (ϵ) of 0.45 and 0.48 mL·mg^{-1}·cm^{-1} for mutants W308Y and W333F, respectively. These molar extinction coefficients were determined by bicinchoninic acid assay, using wild-type yPGK as a standard.

Unfolding−Refolding Studies. All spectroscopic measurements were performed in a 20 mM Tris buffer, pH 7.5, containing 0.5 mM EDTA and 1 mM 2-mercaptoethanol at 22 °C. Measurements were carried out with a 2 μM protein concentration after 12 h incubation at 22 °C in different concentrations of Gdn-HCl or urea. Refolding transitions were performed on proteins previously incubated in 4 M Gdn-HCl during 12 h and then diluted in different Gdn-HCl concentrations for 12 h more incubation.

To avoid the possible presence of cyanates (Stark, 1965), the urea was previously deionized on an Amberlite column and used within 1 day. Urea and ultrapure Gdn-HCl were obtained from Pierce. The denaturant concentration was checked by refractometry, using the relationship provided by Nozaki (1970).

In a set of experiments, the unfolding was induced by Gdn-HCl until the end of the first transition and then urea from 0 to 6 M was added. Protein solutions (2 μM final concentration) were incubated for 4 h in the presence of Gdn-HCl and 12 h more after the addition of urea. Conversely, in another set of experiments, the protein was first unfolded in urea, and then Gdn-HCl (from 0 to 6 M) was added. In this set, the final concentrations of urea were 2.7, 2.3, and 2.6 M for wild-type yPGK and mutants W308Y and W333F, respectively.

Steady-state fluorescence emission spectra were recorded between 300 and 400 nm after excitation at 295 nm with a SLM 8000C Aminco spectrofluorometer and a 10 mm light-path cell. Transition curves were constructed by plotting either the variations in fluorescence intensity at 350 nm or the shift in the maximum emission wavelength upon denaturant concentration.

Circular dichroic spectra were recorded between 200 and 260 nm for each Gdn-HCl concentration using a Mark V dichrograph (Jobin et Yvon) and a 2 mm light-path cell. Transition curves were constructed by plotting the mean residue ellipticity at 222 nm.

Analysis of the Transition Curves. Transition curves obtained by CD and enzymatic measurements were analyzed by using the linear dependency of ΔG_x upon denaturant concentration, x, according to Pace (1986):

$$\Delta G_x = \Delta G_0 - mx \quad (1)$$

ΔG_0 being the variation of free energy in the absence of denaturant.

In order to take into account the solvent effect on signals, the following equation was derived from eq 1:

$$y_x = y_n + s_n x + \left\{\frac{e^{(\Delta G_0 - mx)/RT}}{1 + e^{(\Delta G_0 - mx)/RT}}\right\}[A + (s_d - s_n)x] \quad (2)$$

where y_x is the experimental signal in the presence of x molar Gdn-HCl, y_n is the signal of the native form, s_n and s_d are the solvent effects on the native and denatured signals, respectively, and A is the amplitude of the transition.

The complex transition curves obtained by fluorescence measurements were fitted to an equation in which both the base lines and the transition regions of the curve are fitted simultaneously. The transition region was described by a linear combination of two single transition curves with opposite amplitudes, following equations derived from the denaturant binding model, as described by Missiakas *et al.* (1990).

All data were fitted by using a simplex procedure based on the Nelder and Mead algorithm (Press *et al.*, 1986).

RESULTS

Effect of the Substitutions on Protein Stability and Activity. Tryptophans 308 and 333 were respectively substituted by a tyrosine and a phenylalanine according to their own particular environment. As shown in Table 1, for mutant W308Y, the enzyme activity value is slightly altered, and the free energy of unfolding in the absence of denaturant ($\Delta G°$) and the midpoints of the unfolding transition (C_m) followed by circular dichroism and activity are slightly

Table 1: Activities and Thermodynamic Stabilities of Wild-Type Yeast Phosphoglycerate Kinase, Mutant W308Y, and Mutant W333F

signal	parameter	wild-type yPGK[a]	W308Y	W333F
activity	percentage	100	91	49
	C_m (M Gdn-HCl)	0.70 ± 0.02	0.58 ± 0.02	0.60 ± 0.01
	$\Delta G°$ (kcal/mol)	nd	5.8 ± 0.5	9 ± 3
	m (kcal/mol^2)	nd	9.9 ± 0.6	16.2 ± 1.1
circular dichroism	C_m (M Gdn-HCl)	0.80 ± 0.02	0.55 ± 0.20	0.64 ± 0.06
	$\Delta G°$ (kcal/mol)	7.8 ± 0.5	4.2 ± 2.5	10.0 ± 2.8
	m (kcal/mol^2)	9.7 ± 1.3	8 ± 1	16 ± 2

[a] From Missiakas *et al.* (1990).

different from those of wild-type yPGK. These values are close to those obtained for wild-type yPGK, suggesting that the substitution of tryptophan 308 by a tyrosine does not strongly perturb the activity and stability of the protein.

On the other hand, $\Delta G°$ is slightly higher for mutant W333F than for wild-type yPGK, even though the C_m values determined from activity and circular dichroism transition curves are slightly smaller and the activity is reduced by half. The increased stability can be explained by the substitution of a tryptophan in a very hydrophobic environment by a more hydrophobic residue (Wolfenden *et al.*, 1979), and the decrease in activity is probably due to the position of the mutation, which is very close to the active site. Nevertheless, the activities and thermodynamic stabilities are sufficiently maintained to allow further studies of the unfolding pathway of yPGK with these mutants.

Studies of the Unfolding−Refolding Transitions Induced by Gdn-HCl. For both mutants W308Y and W333F, unfolding−refolding transitions followed by fluorescence intensity are similar to that of wild-type yPGK and completely reversible. They present a first transition in which the fluorescence intensity increases, with a C_m value for the mutants a little lower than for wild-type yPGK and a second transition, corresponding to a decrease in fluorescence intensity. As shown in Figure 2, the areas of the spectra of both mutants are lower in 2 M Gdn-HCl than in 0.7 or 0.9 M Gdn-HCl.

Unexpectedly, the fact that this transition is observed for both mutants indicates that both tryptophans are involved in the hyperfluorescence. However, when the two unfolding transitions are superimposed (Figure 3), the second transitions appear to have the same amplitude for both mutants and thus seem to be independent of the tryptophan three-dimensional environment in the native protein. In contrast, the first transition has a smaller amplitude for mutant W333F. This is due to a higher native state fluorescence of tryptophan 308 in mutant W333F relatively to the native state fluorescence of tryptophan 333 in mutant W308Y. Incidentally, it is interesting to note that the changes in the native structure occurring upon W333F mutation lead to an increase in the intrinsic fluorescence of tryptophan 308. Indeed, this mutant has an intrinsic fluorescence intensity similar to that of the wild-type enzyme which contains two tryptophan residues.

The hyperfluorescent form was investigated through various physicochemical measurements. We thereby investigated many signals such as enzyme activity, circular dichroism values at 222 nm, fluorescence maximum wavelength, and fluorescence intensity of tryptophans. These provide information on the overall protein conformation, the content in secondary structure, the tryptophan environment polarity, and the local protein conformation around the tryptophans, respectively. As shown in Table 2, the C_m values corresponding to the unfolding−refolding transition of the mutants are slightly lower than those obtained for wild-type yPGK.

The C_m values determined by all four methods are similar within experimental error. Furthermore, transition curves obtained by monitoring the variation in ellipticity at different wavelengths are superimposable (data not shown). This allows us to conclude that, at the Gdn-HCl concentration where the fluorescence intensity is maximum, wild-type yPGK and the mutants are devoid of residual activity and secondary structures. In addition, the polar environment of the tryptophan residues implies that this state is also devoid of tertiary structure. The unfolding transitions as assessed by the variation in the maximum emission fluorescence wavelength are shown in Figure 4. A small increase in this maximum is observed from the denaturant concentrations corresponding to the hyperfluorescent form to those corresponding to the completely unfolded protein. Even though this shift of 5 nm does not allow us to conclude that there is a second transition, it has to be taken into account and may imply changes in the environment of the tryptophans.

Guanidine Hydrochloride- and Urea-Induced Unfolding Transitions. Residual structures in denatured states are frequently observed in several proteins and increasingly studied. From recent studies, it emerges that these structures are present particularly in urea-induced unfolded states (Neri *et al.*, 1992; Saab-Rincón *et al.*, 1993). Since urea is generally considered to be a less potent denaturant than Gdn-HCl (Tanford, 1968), the presence of residual structures in the urea-induced unfolded state of yPGK was investigated.

The unfolding equilibrium transitions induced by Gdn-HCl and urea were compared using the fluorescence emission intensity at 350 nm as a conformational probe. The slopes of the linear dependencies of NATRA and unfolded protein fluorescence intensity upon denaturant were also compared for each denaturant, so that these effects could be taken into account. The results are illustrated in Figure 5. For mutant W333F, the fluorescence intensity of the Gdn-HCl-induced hyperfluorescent state is close to that of the urea-unfolded state. Such a similarity does not appear clearly for mutant W308Y. Furthermore, the slope of the linear dependencies of the fluorescence intensity of NATRA upon urea concentration is 1.6-fold higher than that of the unfolded protein for mutant W333F, whereas it is approximately similar, within the experimental error, for mutant W308Y. Thus, tryptophan 308 in the urea-induced unfolded protein is not in a completely solvent-accessible environment, indicating the presence of residual structures. Such residual structures appear to be more floppy around tryptophan 333.

Evidence for Residual Structures around Tryptophan 308 and Tryptophan 333. As shown in Figure 6, the addition of Gdn-HCl to the urea-induced partially unfolded mutant W333F is accompanied by a decrease in fluorescence intensity until 5 M total denaturant concentration and, for higher Gdn-HCl concentration, the fluorescence intensity increases linearly with the total denaturant concentration. Conversely, the addition of urea to the Gdn-HCl-induced hyperfluorescent form does not modify the exposure of tryptophan 308 to the solvent.

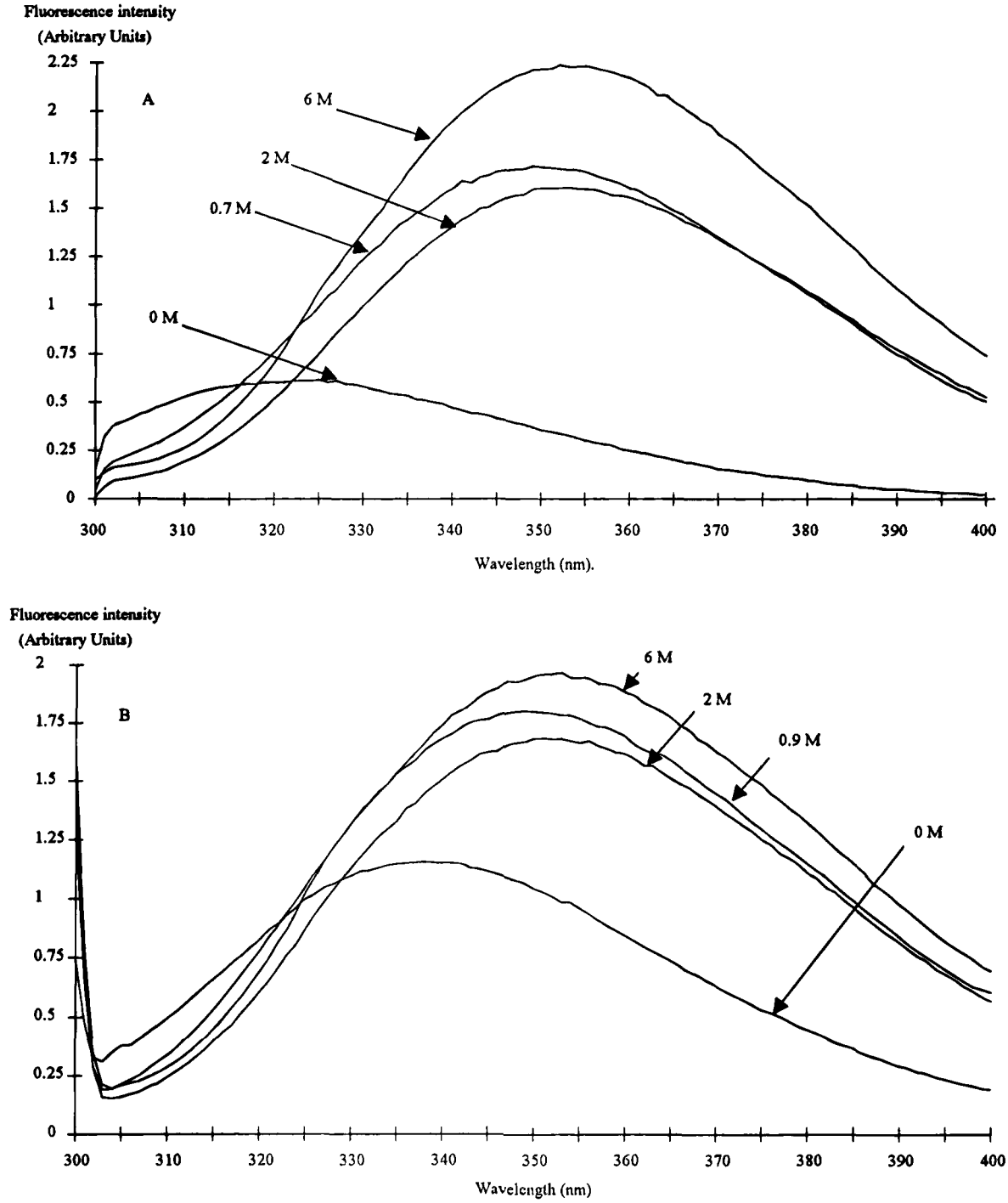

FIGURE 2: Fluorescence emission spectra in several Gdn-HCl concentrations (excitation at 295 nm). (A) Mutant W308Y. (B) Mutant W333F.

The addition of Gdn-HCl to the urea-induced unfolded mutant W308Y does not give rise to a second transition when the fluorescence intensity is monitored at 350 nm (Figure 7). Conversely, the addition of urea to the Gdn-HCl-induced hyperfluorescent state leads to the same result. When fluorescence intensity is monitored at 340 nm, the addition of urea to the Gdn-HCl-induced hyperfluorescent form gives rise to a second transition whereas addition of Gdn-HCl to the urea-induced unfolded mutant W308Y leads to a linear dependency. The difference in transition curves according to the wavelength of fluorescence intensity monitoring is due to a slight red shift induced by addition of urea.

Urea thus appears to be potent enough to reduce the residual structures contained around tryptophan 333 in the Gdn-HCl-induced hyperfluorescent state and to lead to an almost unfolded state which contains residual structures less stable than those observed around tryptophan 308.

Moreover, as shown in Table 3, the urea-induced unfolding transitions followed by circular dichroism, variations in the maximum emission wavelength, and fluorescence intensity do not present the same C_m values and give rise to significantly different $\Delta G°$ values. This indicates the existence of an intermediate. In particular, C_m values indicate that, at 1.7 M urea, tryptophan 333 is already located in a

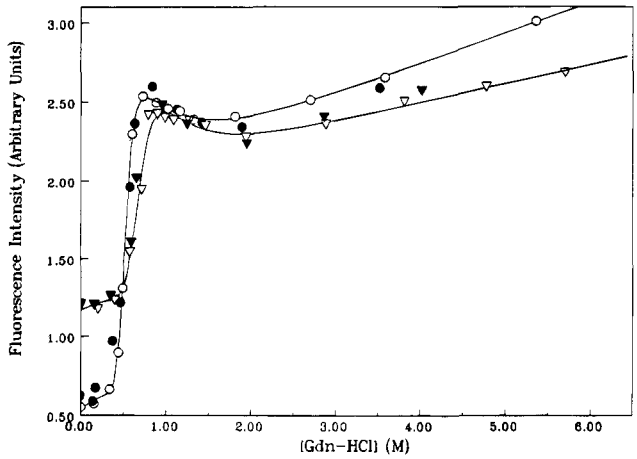

FIGURE 3: Superimposed unfolding–refolding transitions as assessed by variation in the fluorescence intensity at 350 nm (excitation at 295 nm) upon denaturation by Gdn-HCl. Unfolding: (○) mutant W308Y; (▽) mutant W333F. Refolding: (●) mutant W308Y; (▼) mutant W333F. The fluorescence intensity is expressed in arbitrary units. Experimental data were fitted according to eq 2.

Table 2: Gdn-HCl-Induced Unfolding Transition Midpoints of Wild-Type Yeast Phosphoglycerate Kinase, Mutant W308Y, and Mutant W333F (M Gdn-HCl)

signal	wild-type yPGK[a]	W308Y	W333F
activity	0.70 ± 0.02	0.58 ± 0.02	0.60 ± 0.01
circular dichroism	0.80 ± 0.02	0.55 ± 0.20	0.64 ± 0.06
$\Delta\lambda_{max}$	0.73 ± 0.02	0.49 ± 0.02	0.62 ± 0.01
fluorescence intensity at 350 nm[b]	0.73 ± 0.02	0.53 ± 0.01	0.63 ± 0.01

[a] From Missiakas *et al.* (1990). [b] Data for the first transition.

polar environment. It is still quenched by neighboring residues (as it is in the native structure), and the protein still possesses secondary structures. Thus, mutant W308Y unfolds in urea through an intermediate containing secondary structures but in which tryptophan 333 is located in a polar environment. These results clearly indicate that the residual structures located around tryptophan 308 and tryptophan 333 do not involve the same kind of interactions, these interactions being more resistant to the denaturant around tryptophan 308.

DISCUSSION

In order to investigate the contribution of the environment of each of the two tryptophan residues to the fluorescence of yPGK during the unfolding process of this enzyme, single tryptophan containing proteins were prepared by site-directed mutagenesis. The tryptophans at positions 308 and 333 were replaced by a tyrosine and a phenylalanine, respectively.

It was first verified that these mutations did not significantly alter the catalytic and physicochemical properties of yPGK. The unfolding–refolding transitions of both mutants were studied using two spectroscopic methods: circular dichroism and tryptophan fluorescence emission. By both methods, the transitions were found to be completely reversible. A simple transition was observed by circular dichroism for both mutants as well as for wild-type PGK. Similar C_m values were found for both mutants, slightly lower than that corresponding to the wild-type protein. In contrast, when the same process was studied by variation in the

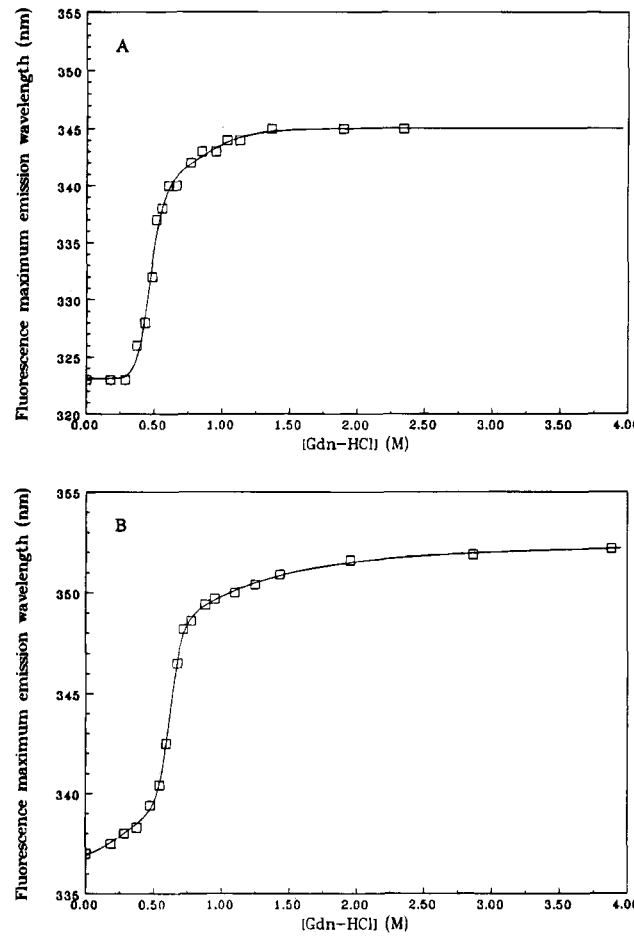

FIGURE 4: Unfolding transitions as assessed by variation in the fluorescence maximum wavelength (excitation at 295 nm) upon denaturation by Gdn-HCl. (A) Mutant W308Y; (B) mutant W333F.

fluorescence emission intensity at 350 nm, two transitions were clearly detected. The first one is accompanied by an increase in fluorescence intensity and leads to a hyperfluorescent form. For both mutants, this first transition coincides with the simple one observed by circular dichroism, showing that this state is devoid of secondary structures. The second transition corresponds to a decrease in fluorescence intensity; both single tryptophan mutants behave as the wild-type protein. Furthermore, this second transition appears to have approximately the same amplitude for both mutants. Previous unfolding studies of yPGK (Nojima *et al.*, 1977; Adams *et al.*, 1985; Szpikowska *et al.*, 1994) have been interpreted as a unique two-state transition, the decrease in fluorescence intensity being attributed to a linear dependency between 0.9 and 2.0 M Gdn-HCl. In contrast, previously published studies (Endo *et al.*, 1983) have shown the existence of intermediate species characterized by an elution volume and a fluorescence intensity different from those of the native and the denatured proteins. More recently, unfolding studies of a homologous protein, PGK from *Bacillus stearothermophilus*, have clearly shown the existence of this second transition (Staniforth *et al.*, 1993). When higher denaturant conditions are taken into account for transition curve analysis, it clearly appears that this second transition results from a further unfolding process.

The nature of this second transition was studied by fluorescence spectroscopy. This second transition is accompanied by a 5 nm red shift of the maximum fluorescence emission wavelength for both mutants, indicating that the

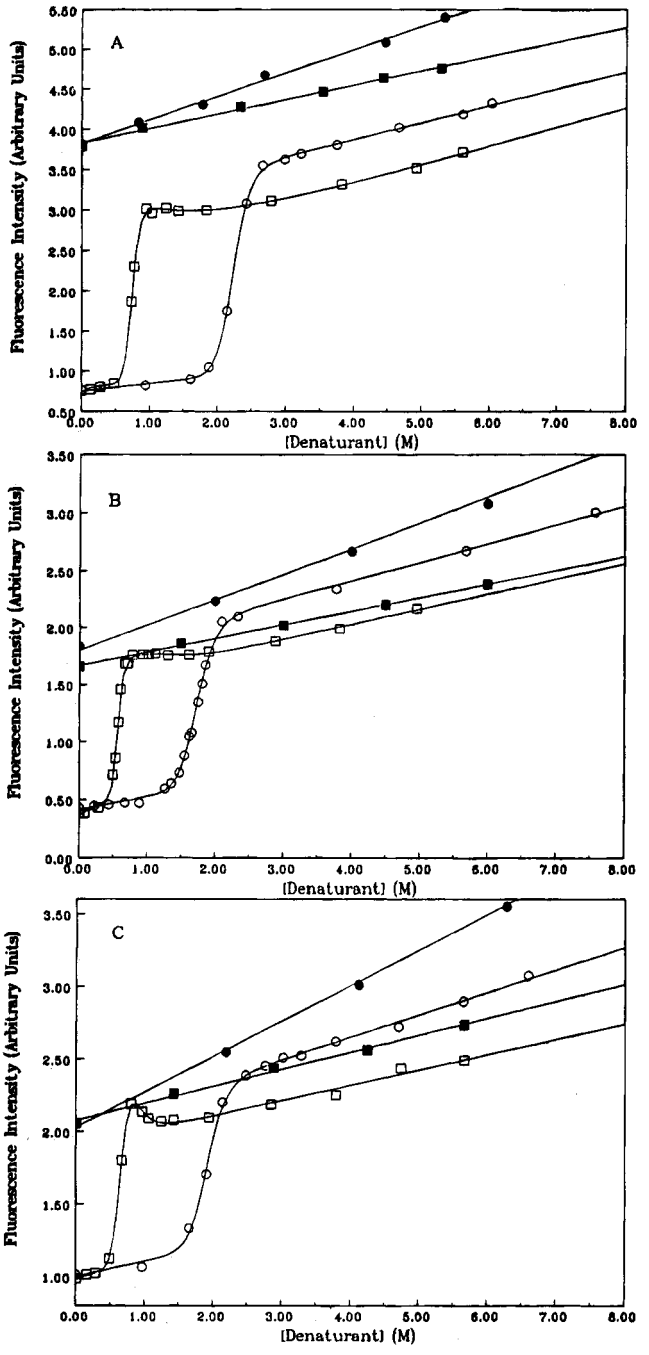

FIGURE 5: Unfolding transitions of wild-type yPGK (A), mutant W308Y (B), and mutant W333F (C) as assessed by the fluorescence intensity at 350 nm (excitation at 295 nm). For each protein, curves represent the effect of Gdn-HCl on the protein (□) and on NATRA (■) and the effect of urea on the protein (○) and on NATRA (●).

tryptophan environment is modified by the addition of Gdn-HCl to the hyperfluorescent form. This could reflect the persistence of residual structures around each tryptophan residue. This hypothesis was strengthened by the effect of Gdn-HCl on the urea-unfolded protein.

The unique tryptophan in each mutant provides a probe of the local events during the unfolding−refolding of phosphoglycerate kinase. The increase in fluorescence intensity upon denaturation is due to the unusually low fluorescence quantum yield of the native protein. This low intensity is partly due to the presence of a negative charge quenching the fluorescence of tryptophan 308 (Dryden & Pain, 1989). From the three-dimensional structure, the best

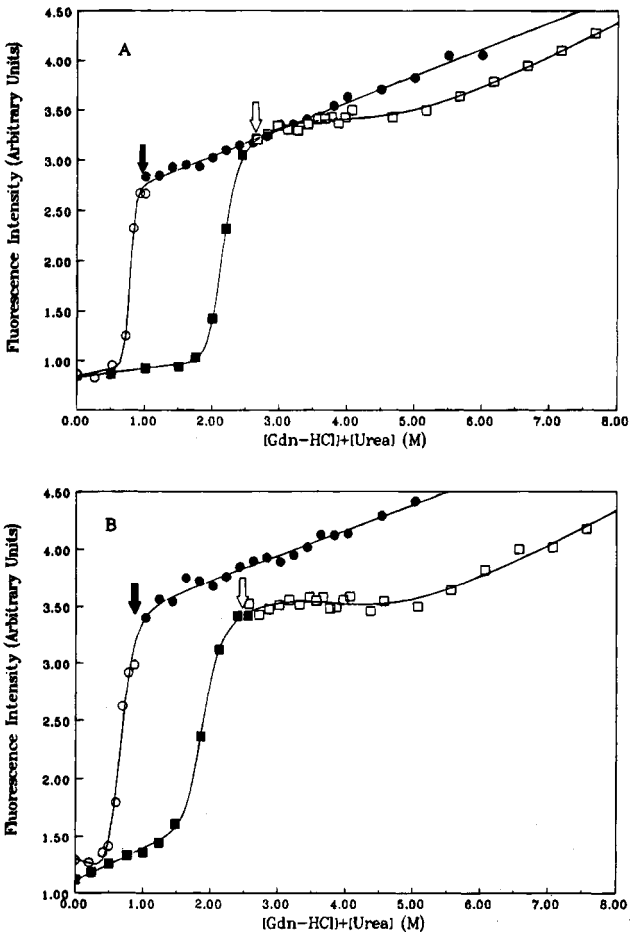

FIGURE 6: Unfolding transitions of wild-type yPGK (A) and mutant W333F (B) as assessed by the fluorescence intensity at 350 nm (excitation at 295 nm). The first transition was obtained by dilution of the Gdn-HCl-induced hyperfluorescent state (○) or of the urea-unfolded state (■). The white arrow represents the urea concentrations of the solutions to which concentrations of Gdn-HCl from 0 up to 6 M were added (□), i.e., 2.7 and 2.6 M for wild-type yPGK and mutant W333F, respectively. The black arrow represents the Gdn-HCl concentrations of the solutions to which the urea concentrations from 0 up to 6 M were added (●), i.e., 1 and 0.9 M Gdn-HCl for wild-type yPGK and mutant W333F, respectively. The abscissa axis represents the sum of the denaturant concentrations.

possible quencher appears to be Asp 287, located about 4 Å from the indole ring of Trp 308. The first transition, which corresponds to an increase in fluorescence intensity, probably reflects the removal of Asp 287 far from Trp 308 and therefore the suppression of quenching. In the hyperfluorescent state, some residual structures appear to be present around Trp 308. They persist in the urea-induced unfolding transition of mutant W333F, plausibly forming a hydrophobic cluster which can be destroyed by further addition of Gdn-HCl.

The Gdn-HCl-induced unfolding of mutant W308Y as assessed by variations in the fluorescence intensity also displays a biphasic transition like that of the W333F mutant. Thus, Trp 333, buried in the native structure of PGK in a hydrophobic environment, contributes to the hyperfluorescence observed in the unfolding process. However, the microstructures observed around Trp 333 in the hyperfluorescent form differ slightly from those around Trp 308. Indeed, when the unfolding of mutant W308Y was induced by urea, further addition of Gdn-HCl did not produce any

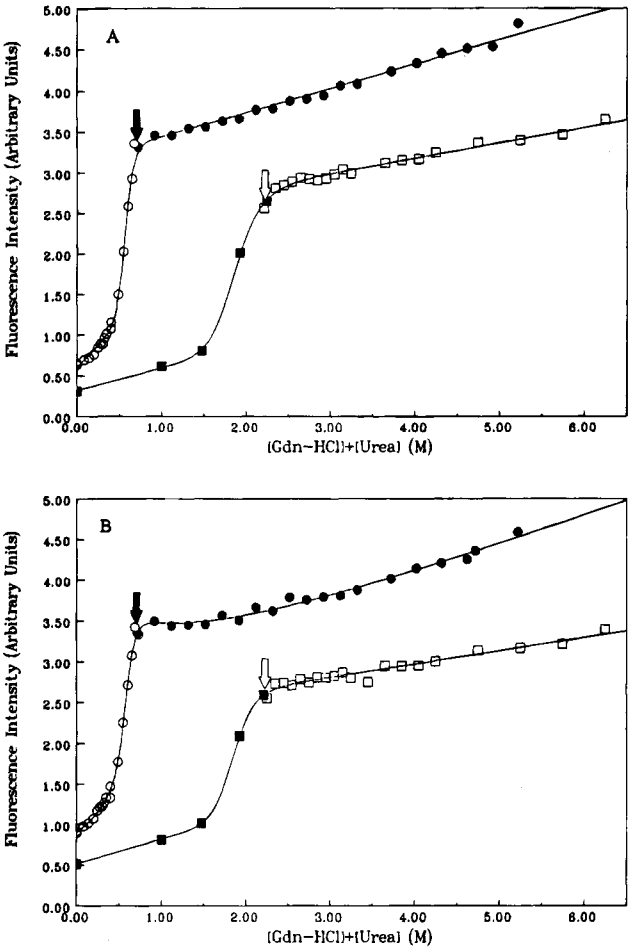

FIGURE 7: Unfolding transition of mutant W308Y as assessed by variation in the fluorescence intensity at 350 (A) and 340 nm (B) after excitation at 295 nm. The first transition was obtained by dilution of the Gdn-HCl-induced hyperfluorescent state (○) or of the urea-unfolded state (■). The white arrow represents the urea concentrations of the solutions to which concentrations of Gdn-HCl from 0 up to 6 M are added (□), i.e., 2.3 M. The black arrow represents the Gdn-HCl concentrations of the solutions to which urea concentrations from 0 up to 6 M are added (●), i.e., 0.7 M Gdn-HCl. The abscissa axis represents the sum of the denaturant concentrations.

Table 3: Thermodynamic Parameters of the Urea-Induced Unfolding Transition for Wild-Type Yeast Phosphoglycerate Kinase, Mutant W308Y, and Mutant W333F

parameter	signal	wild-type yPGK	W308Y	W333F
C_m (M urea)	CD	2.22 ± 0.07	1.80 ± 0.08	2.01 ± 0.04
	$\Delta\lambda_{max}$	2.24 ± 0.03	1.55 ± 0.02	1.89 ± 0.03
	fluorescence intensity	2.28 ± 0.03	1.80 ± 0.02	1.86 ± 0.04
ΔG° (kcal/mol)	CD	7.43 ± 0.27	7.3 ± 0.6	8.3 ± 0.5
	$\Delta\lambda_{max}$	7.85 ± 0.12	5.3 ± 0.1	8.1 ± 0.2
	fluorescence intensity	8.25 ± 0.02	9.0 ± 0.2	7.59 ± 0.27
m (kcal/mol^2)	CD	3.39 ± 0.12	4.1 ± 0.3	4.2 ± 0.2
	$\Delta\lambda_{max}$	3.53 ± 0.05	3.43 ± 0.05	4.28 ± 0.11
	fluorescence intensity	3.63 ± 0.01	5.14 ± 0.12	4.08 ± 0.14

decrease in the fluorescence emission intensity at 350 nm. Conversely, the addition of urea to the hyperfluorescent state induced the second transition when the fluorescence emission was observed at 340 nm. These results suggest that the interactions involved in the microstructures around Trp 333 are different from those around Trp 308. Moreover, contrary to that observed for the Gdn-HCl-induced transition, tryptophan 333 becomes accessible to urea under conditions where some secondary structures still persist, as shown bythe difference in the C_m value obtained from the transition observed by circular dichroism and the shift of the maximum emission fluorescence wavelength.

The existence of this hyperfluorescent form is attributed to local structures around two residues distant in the sequence. This could suggest that these two tryptophans are associated with distinct local structures. It should be noted that the first transition in yPGK corresponds to an increase in fluorescence intensity, although in many proteins, unfolding is associated with a decrease in fluorescence intensity. It is clear that the second transition would be much more difficult to observe in this latter case as one can see by comparing the unfolding transitions of different mutant PGK from *Bacillus stearothermophilus* (Staniforth *et al.*, 1993). The hyperfluorescent form observed for yPGK is particularly apparent because of this first unusual transition. Therefore, it does not seem unlikely that this state is present in other proteins although within a less apparent phenomenon. It seems appropriate to note, here, that a recent observation (Karlin *et al.*, 1994) of the distance distribution around different type of residues has suggested a model in which tryptophan and tyrosine residues are associated with local structures in proteins.

Since the transitions are completely reversible, the persisting local microstructures have implications in the understanding of the early events which direct protein folding. They can provide nucleation centers that originate the process. Neri *et al.* (1992) have shown that the 63 amino-terminal residues of the 434 repressor form a hydrophobic cluster that corresponds to a localized nucleation center that initiates the formation of an α-helix in a following step. When FK506 binding protein is unfolded in urea and Gdn-HCl, some regions of secondary structures are present; the residues involved in these structures in the unfolded protein are found in the same type of structure in the folded protein (Logan *et al.*, 1994).

Detailed structural characterization of such residual structures is not easy. Logan *et al.* (1994) have used heteronuclear multidimensional NMR experiments to characterize these structures in FK506 binding protein. Lumb and Kim (1994) have used peptide models corresponding to residues 17−24 of BPTI and observed the interresidue nuclear Overhauser effect. Site-directed mutagenesis could provide a useful tool to determine the amino acids involved in the persistence of residual microstructures. In the present study, fluorescence emission indicated the presence of local residual microstructures around Trp 308 and 333 in the unfolding process of yPGK. Further experiments are now necessary to determine the other amino acids involved in these clusters and their contribution as starting structures in the folding process. It would be particularly interesting to determine whether these hydrophobic clusters reflect the existence of microdomains according to the diffusion−collision model proposed by Karplus and Weaver (1976, 1994).

REFERENCES

Adams, B., Burgess, R. J., & Pain, R. H. (1985) *Eur. J. Biochem. 152*, 715−720.

Bücher, T. (1955) *Methods Enzymol. 1*, 415−422.
Calmettes, P., Roux, B., Durand, D., Desmadril, M., & Smith, J. C. (1993) *J. Mol. Biol. 231*, 840−848.
Dill, K. A., & Shortle, D. (1991) *Annu. Rev. Biochem. 60*, 795−825.
Dobson, C. M. (1992) *Curr. Opin. Struct. Biol. 2*, 6−12.
Dryden, D. T. F., & Pain, R. H. (1989) *Biochim. Biophys. Acta 997*, 313−321.
Endo, S., Saito, Y., & Wada, A. (1983) *Anal. Biochem. 131*, 108−120.
Evans, P. A., Topping, K. D., Woolfson, D. N., & Dobson, C. M. (1991) *Proteins: Struct., Funct., Genet. 9*, 248−266.
James, E., Wu, P. G., Stites, W., & Brand, L. (1992) *Biochemistry 31*, 10217−10225.
Karlin, S., Zucker, M., & Brocchieri, L. (1994) *J. Mol. Biol. 239*, 227−248.
Karplus, M., & Weaver, D. L. (1994) *Protein Sci. 3*, 650−668.
Logan, T. M., Thériault, Y., & Fesik, S. W. (1994) *J. Mol. Biol. 236*, 637−648.
Lumb, K. J., & Kim, P. S. (1994) *J. Mol. Biol. 236*, 412−420.
Minard, P., Hall, L., Betton, J. M., Missiakas, D., & Yon, J. M. (1989a) *Protein Eng. 3*, 55−60.
Minard, P., Desmadril, M., Ballery, N., Perahia, D., Mouawad, L., Hall, L., & Yon, J. M. (1989b) *Eur. J. Biochem. 185*, 419−423.
Missiakas, D., Betton, J. M., Minard, P., & Yon, J. M. (1990) *Biochemistry 29*, 8683−8689.
Neri, D., Billeter, M., Wider, G., & Wüthrich, K. (1992) *Science 257*, 1559−1563.
Nojima, H., Ikai, A., & Noda, H. (1976) *Biochim. Biophys. Acta 427*, 20−27.
Nojima, H., Ikai, A., Oshima, T., & Noda, H. (1977) *J. Mol. Biol. 116*, 429−442.
Nozaki, Y. (1970) *Methods Enzymol. 26*, 43−50.
Pace, C. N. (1986) *Methods Enzymol. 131*, 266−280.
Press, W. H., Flannery, B. P., Teukolsky, S. K., & Vetterling, W. T. (1986) in *Numerical recipes*, Cambridge University Press, Cambridge, U.K.
Privat, J. P., Wahl, P., Auchet, J. C., & Pain, R. H. (1980) *Biophys. Chem. 11*, 239−248.
Saab-Rincón, G., Frœbe, C. L., & Matthews, C. R. (1993) *Biochemistry 32*, 13981−13990.
Shortle, D. (1993) *Curr. Opin. Struct. Biol. 3*, 66−74.
Staniforth, R. A., Burston, S. G., Smith, C. J., Jackson, G. S., Badcoe, I. G., Atkinson, T., Holbrook, J. J., & Clarke, A. R. (1993) *Biochemistry 32*, 3842−3851.
Stark, G. R. (1965) *Biochemistry 4*, 1030−1036.
Szpikowska, B. K., Beechem, J. M., Sherman, M. A., & Mas, M. T. (1994) *Biochemistry 33*, 2217−2225.
Teale, F. W. J., & Weber, G. (1957) *Biochem. J. 65*, 476−482.
Watson, H. C., Walker, N. P. C., Shaw, P. J., Bryant, T. N., Wendell, P. L., Fothergill, L. A., Perkins, R. E., Conroy, S. C., Dobson, M. J., Tuite, M. F., Kingsman, A. J., & Kingsman, S. M. (1982) *EMBO J. 1*, 1635−1640.
Wolfenden, R. V., Cullis, P. M., & Southgate, C. C. F. (1979) *Science 206*, 575−577.

BI941740L