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Unfolding–Refolding Transition of a Hinge Bending Enzyme: Horse Muscle Phosphoglycerate Kinase Induced by Guanidine Hydrochloride†

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ABSTRACT: The unfolding–refolding transition of horse muscle phosphoglycerate kinase induced by guanidine hydrochloride was studied under equilibrium conditions using four different signals: fluorescence intensity at 336 nm, UV difference absorbance at 286 and 292 nm, ellipticity at 220 nm, and enzyme activity. From the following arguments, we found that the process deviates from a two-state model and intermediates are significantly populated even at equilibrum: (1) the noncoincidence of the transition curves and (2) the asymmetry of the transition curve obtained from CD measurements. From these different data and the thermodynamic analysis, it was suggested that the two domains of the horse muscle phosphoglycerate kinase refold independently of one another with different equilibrium constants, the most favorable constant referring to the folding of the C-terminal domain which contains all tryptophans.

Many proteins are folded into several, two or more globular units called domains. Among them, nucleotide binding enzymes have a bilobed structure, each domain being separated by a cleft. Substrate binding induces a conformational change. For hexokinase (Bennett & Steitz 1980a,b) and for phosphoglycerate kinase from yeast (Pickover et al., 1979) and from horse muscle (Banks et al., 1979), an open structure was described for the free enzyme and a closed structure was described or suggested in the presence of specific ligands. The conformational change, which leads to a modification in the relative orientation of the two domains, possibly occurs through a "hinge bending motion" of these domains.

Phosphoglycerate kinase (PGK; EC 2.7.2.3) is the first enzyme of the glycolytic pathway that generates ATP through the high-energy phosphoryl transfer reaction:

1,3-diphospho-D-glycerate + ADP $\overset{Mg^{2+}}{\rightleftharpoons}$ 3-phospho-D-glycerate + ATP

The complete amino acid sequence of the horse muscle enzyme is known; the three-dimensional structure has been determined by high-resolution X-ray analysis (Banks et al., 1979). The two domains of the molecule have almost the same size and correspond to the C-terminal and N-terminal parts of the molecule. The nucleotide substrates bind to the C-terminal domain, and the phosphoglycerate substrates bind to the N-terminal domain.

While there is only a slight and local change in the structure with ATP-Mg and ADP-Mg, 3-phosphoglycerate (3PG) binding induces a conformational change that encompasses the whole enzyme. Moreover, the location of binding sites for

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nucleotides and 3PG on the native form of the enzyme shows a distance larger than that required for the chemical reaction (Banks et al., 1979; Blake & Rice, 1981). These observations led the authors to propose a hinge bending motion of the two domains during the catalytic reaction. Such a hypothesis is supported by X-ray scattering studies of yeast PGK in solution (Pickover et al., 1979).

The existence of a hinge bending motion of the domains implies that each of them behaves like an independent structural unit. In this case, we expect that domains are independent with respect to their folding and stability (Wetlaufer, 1973) as was shown for several proteins, for example, Bence Jones protein (Azuma et al., 1972), serum albumin (Teale & Benjamin 1976a,b, 1977), elastase (Ghélis et al., 1978; Ghélis, 1980), β_2-subunit of tryptophan synthase (Zêtina & Goldberg 1980a,b) [for reviews, see also Wetlaufer (1981), Ghélis & Yon (1982) and Privalov (1982)]. With the aim to verify such a hypothesis, we studied the unfolding–refolding of horse muscle PGK induced by guanidine hydrochloride (Gdn·HCl).[1] A detailed study of the transition is reported in this paper. Four signals were used to follow the equilibrium transition: fluorescence emission, difference spectroscopy, circular dichroism, and enzymatic activity. Horse PGK contains 4 tryptophan, 4 tyrosine, and 16 phenylalanine residues. All tryptophans are located in the C-terminal domain whereas there are tyrosines and phenylalanines in both domains. Difference UV spectrophotometry and fluorescence give information related to different parts of the protein, but mainly to the C-terminal domain since tryptophan is the strongest absorber. Ellipticity at 220 nm reveals the change in ordered structures in the entire molecule. Enzymatic activity gives data that concern the fully native protein involving the last conformational readjustments of the active site. A comparison of the transition curves obtained from different signals can provide useful information about the way of protein folding, especially when deviations from a two-state behavior is indicated by a noncoincidence of these different transition curves.

Material and Methods

Horse muscle PGK was prepared by using the procedure of Scopes (1969) with the modification proposed by Blake (1972). The enzyme preparation presented only one band by electrophoresis in polyacrylamide gel with urea–SDS according to Leammli (1970); the specific activity was 1100 units/mg at 20 °C and pH 7.5.

The enzyme activity was determined by using the procedure of Bücher (1955). Standard assays were run in the following conditions: 200 mM PIPES buffer, pH 7.5, 50 mM K_2HPO_4, 5 mM NH_4Cl, 10 mM 2-mercaptoethanol, 500 μM glyceraldehyde 3-phosphate, 500 μM NAD^+, 1 mM ADP, 20 mM $MgCl_2$, and 500 μM glyceraldehyde-3-phosphate dehydrogenase. The temperature was kept constant at 23 °C. The reaction was initiated by the addition of 10 μL of PGK to 1 mL of the assay solution. NADH absorbance changes were recorded on a Cary 219 spectrophotometer.

NAD^+, glyceraldehyde 3-phosphate, ADP, and coupling enzyme, glyceraldehyde-3-phosphate dehydrogenase, were purchased from Sigma. Glyceraldehyde 3-phosphate was prepared from the diethyl acetal monobarium salt following the instructions of the supplier. Ultrapure Gdn·HCl was obtained from Pierce. Solutions of Gdn·HCl were prepared daily and adjusted to the desired pH. Denaturant concentrations were determined by refractometry using the table published by Nozaki (1970).

All experiments were done in pH 7.5 100 mM phosphate buffer containing 1 mM EDTA, 10 mM 2-mercaptoethanol, or 1 mM DTT. Final concentrations of PGK ranged between 6 and 13 μM for difference absorbance recordings, 1 and 2 μM for circular dichroism and fluorescence, and 0.1 to 0.7 μM for enzymatic activity.

Stock solution of PGK (50–100 μM) was diluted in the final concentration of denaturant and incubated 24 h at 23 °C for denaturation. Fluorescence was measured on a Perkin-Elmer fluorometer, MPF 44B. Difference spectra were recorded with a Cary 219 spectrophotometer, using double compartment cells, by the procedure of Herskovits & Laskowski (1962). Temperature was kept constant at 23 °C for all experiments. Circular dichroism spectra were recorded with a Mark V dichograph (Jobin and Yvon).

For renaturation experiments, a stock solution of PGK was first diluted in 6 M Gdn·HCl solution and kept for 24 h at 23 °C. Then, this denaturated PGK was diluted to different final concentrations of denaturant; final concentrations of PGK were the same as for denaturation experiments. Measurements were performed after 24 h at 23 °C.

Denaturation as well as renaturation processes were followed by measurement of the enzyme activity using the coupled assay as indicated. Although the titration of the enzyme activity implied an assay under nondenaturing conditions, reactivation was a process slow enough to allow an evaluation of the activity under initial conditions. Indeed, the rate constant of reactivation was $k = 6.7 \times 10^{-3}$ s^{-1} whereas the rate constant for the enzyme reaction was 89 s^{-1} under our experimental conditions (23 °C and pH 6.8).

Results

Transition Followed by Fluorescence Measurements. Figure 1 shows the fluorescence properties of the protein in phosphate buffer and after 24 h in 3 M Gdn·HCl; the excitation wavelength was 292 nm. The main effect of the denaturant was a decrease of about 30% in the fluorescence yield, with a small red shift of the maximum wavelength ($\Delta\lambda_{max}$ = 7 ± 1 nm). This very small shift between the native and denatured protein indicated that most chromophores (i.e., tryptophans) are in a polar environment in the native protein. These results agreed with the X-ray structure which shows three tryptophans totally accessible to the solvent and one partially buried (Trp-335). The emission spectrum of denatured PGK in 3 M Gdn·HCl was nearly the same as the one obtained from a solution of the constitutive chromophores at the same concentration, indicating a total exposure of the chromophores to the solvent at this concentration of denaturant. The small difference observed in 3 M Gdn·HCl reflected differences in the solvent effect.

The variation of fluorescence intensity at 336 nm was studied for Gdn-HCl concentrations ranging from 0 to 6 M. After 24 h, the equilibrium was complete. Under these conditions, the transition curve displayed a decrease in fluorescence intensity for denaturant concentrations ranging from 1 to 2.5 M Gdn·HCl (Figure 2). For concentrations of Gdn·HCl higher than 2.5 M there was a linear dependency of the fluorescence intensity with regard to the denaturant concentration. This was due to the solvent effect. Taking into account the solvent effect in the transition zone, we obtained a normalized transition curve (insert) which represented the unfolding–refolding transition of the protein. There was a good coincidence between the unfolding and refolding transition

[1] Abbreviations: Gdn·HCl, guanidine hydrochloride; SDS, sodium dodecyl sulfate; PIPES, 1,4-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol.

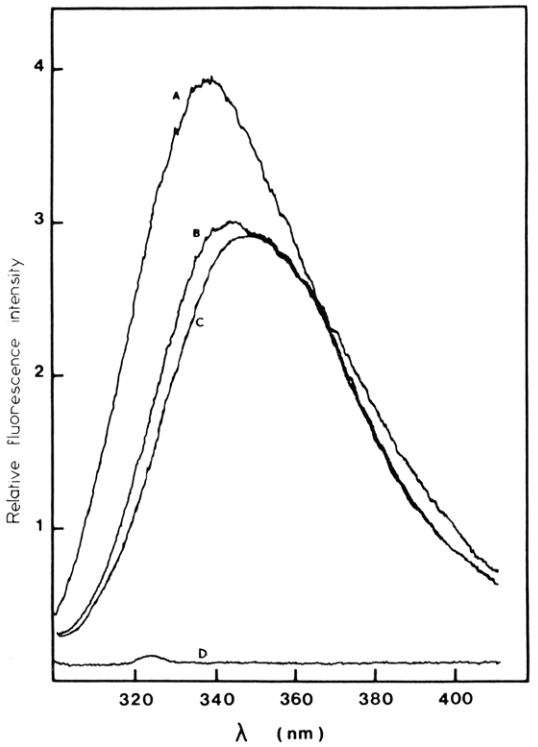

FIGURE 1: Fluorescence emission spectra of native and denatured PGK. (A) PGK (2 μM) in a 0.1 M phosphate buffer, 5 mM 2-mercaptoethanol, and 1 mM EDTA, pH 7.5; (B) PGK (2 μM) in 3 M Gdn·HCl in the same buffer; (C) emission spectra of a equimolar mixture of 8 μM *N*-acetyl-tyrosinamide and *N*-acetyltryptophanamide in 3 M Gdn·HCl phosphate buffer; (D) fluorescence base line of the phosphate buffer.

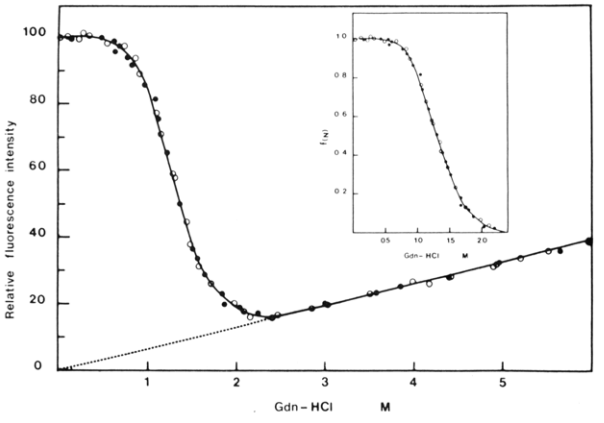

FIGURE 2: Gdn·HCl induced transition studied by emission fluorescence intensity at 336 nm (excitation at 292 nm). Filled symbols are for the renaturation process; open symbols are for denaturation. The insert shows the normalized curve obtained by using the relation $f(N) = (F - F_D)/(F_N - F_D)$, where F_N and F_D are the fluorescence intensity of the native and unfolded PGK respectively and f(N) is the fraction of native protein.

curves allowing to conclude that the transition studied by fluorescence emission is a fully reversible process. The midpoint of the transition indicated a c_m of 1.30 ± 0.05 M Gdn·HCl.

Transition Followed by Difference Spectrophotometry. After 24-h incubation in 3 M Gdn·HCl, the denatured PGK showed a difference spectrum with native PGK as indicated in Figure 3. This difference spectrum was characterized as follows: (1) two main minima were observed, the one at 292 nm corresponding to tryptophan and the other at 286 nm representing tyrosine and tryptophan contributions; (2) an elbow at 280 nm reflected the tyrosine absorbance; (3) three

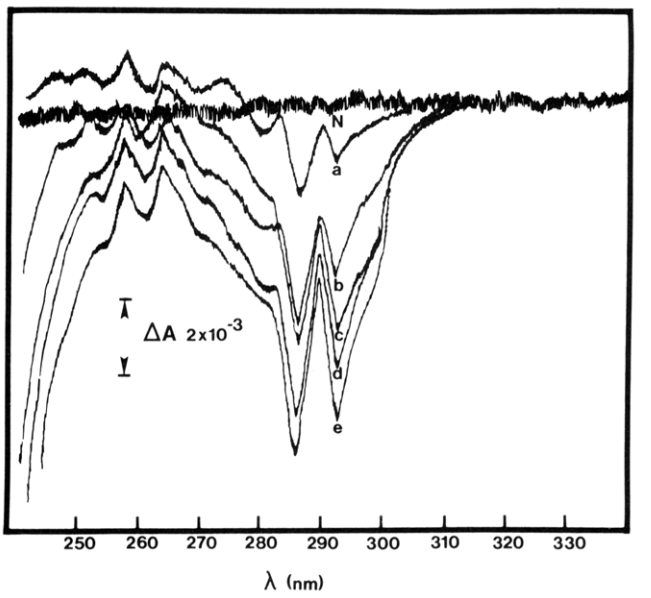

FIGURE 3: Gdn-HCl-induced difference UV absorption spectra of PGK (concentration 6 μM in the same buffer as in Figure 1): (N) base-line native PGK; (a) 0.8 M Gdn·HCl; (b) 1 M Gdn·HCl; (c) 2.5 M Gdn·HCl; (d) 4.3 M Gdn·HCl; (e) 6 M Gdn·HCl.

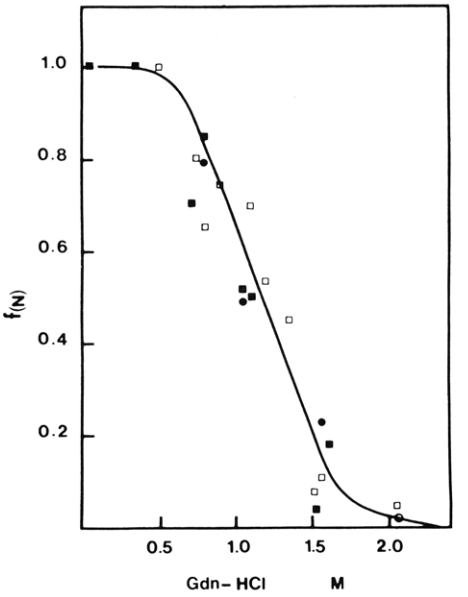

FIGURE 4: Gdn·HCl-induced transition studied by UV difference spectrophotometry: (□, ■) data obtained from measurements at 292 nm; (○, ●) data obtained from measurements at 286 nm. Open symbols are for the denaturation process, and filled symbols are for the renaturation process. $f(N)$ is defined as in Figure 2.

other well-resolved secondary maxima between 255 and 262 nm reflected the contribution of phenylalanines.

Variations in the difference of absorbance at 292 nm and 286 nm were studied under the same conditions as above. Similarly to the transition obtained by fluorescence recording, equilibrium was completely reached after 24 h, and full reversibility was obtained. When the solvent effect was taken account, a transition curve was deduced (Figure 4). The results obtained at two wavelengths, 286 and 292 nm, did not differ significantly. The midpoint of the transition gave a c_m value of 1.2 ± 0.1 M Gdn·HCl not significantly different from the c_m deduced from fluorescence measurements. At 292 nm, we observed only the signal of tryptophan. Both tyrosine and tryptophan contribute to the signal at 286 nm, but since the contribution of tryptophans is larger than the contribution of

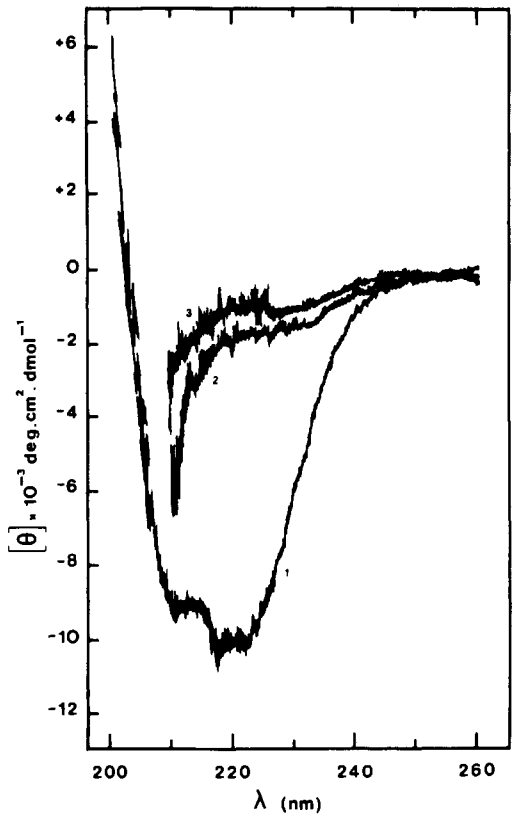

FIGURE 5: CD spectra of native and denatured PGK: (1) 2 μM PGK in 0.1 M phosphate buffer, 500 mM DTT, and 1 mM EDTA, pH 7.5; (2) 2 μM PGK in 3 M Gdn·HCl, in the same buffer; (3) 2 μM PGK in 6 M Gdn·HCl, in the same buffer.

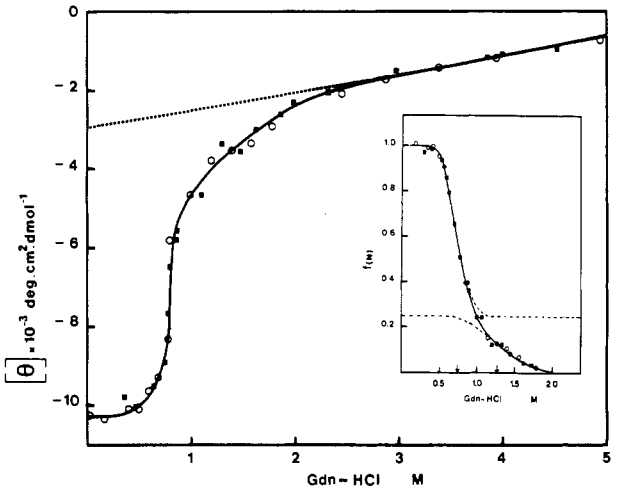

FIGURE 6: Gdn·HCl-induced transition studied by variation of ellipticity at 220 nm. Open symbols are for denaturation; filled symbols are for renaturation. In the insert are represented the normalized data. The fraction of native protein f(N) was obtained by using the same relation as in Figure 2. Data points were analyzed by using eq 5 (see text).

tyrosines, all these observations reflected mainly variations in tryptophan environment.

Transition Followed by Circular Dichroism. Figure 5 shows typical spectra of native and denatured PGK between 200 (or 210 nm) and 260 nm, i.e., in a range which reflects the variations in ordered structure content. Denatured protein exhibited a typical monotonic curve, whereas native protein had a minimum at 220 nm reflecting the secondary structure of the protein.

Figure 6 shows the transition obtained from variations in ellipticity at 220 nm. Figure 6 is the experimental curve

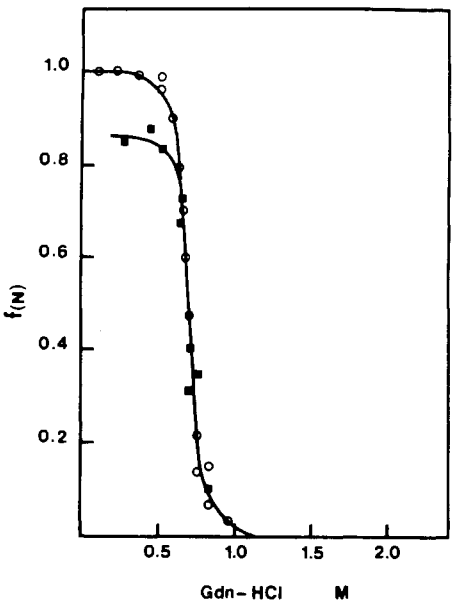

FIGURE 7: Gdn·HCl-induced transition studied by variation of enzymatic activity. Open symbols are for denaturation; filled symbols are for renaturation.

indicating linear dependency of the ellipticity of the denatured protein with the denaturant concentration. The normalized data are presented in insert. This curve was asymmetrical, the apparent c_m being 0.8 M Gdn·HCl.

Transition Followed by Measurements of the Enzyme Activity. The recovery of activity of the enzyme completely denatured in 6 M Gdn·HCl vs. time was described by only one exponential, and the initial rate (given by the slope of the tangent at time zero) was nil. Thus, it was possible to study the transition by the signal of enzyme activity. A very cooperative curve was obtained for the denaturation process (Figure 7). In the regeneration mixture, the maximum renaturation yield as measured by enzyme activity was 85% whereas 100% of optical signals were recovered. This reactivation yield was obtained for 0.17 μM PGK. Similar yield of renaturation (82%) was reported for yeast PGK (Conroy et al., 1981). A better yield was obtained (92%) when the denatured protein was transitorily incubated for 24 h in low and nondenaturing Gdn·HCl concentrations before final incubation in the regeneration mixture. For higher concentration of protein a partial irreversibility of denaturation was observed, the critical concentration of denaturant being near the end of inactivation transition (J.-M. Betton et al., unpublished results).

A c_m value of 0.70 (±0.05) M Gdn·HCl was determined for the transition obtained following the enzyme activity, significantly smaller than the c_m obtained from optical signals (Figure 7).

Thermodynamic Analysis of the Transition Curves. In the classical treatment of a two-state mechanism between native (N) and denatured (D) protein

$$N \underset{}{\overset{K_D}{\rightleftharpoons}} D$$

the equilibrium constant is related to the observable parameter y according to the following relationship:

$$K_D = \frac{D}{N} = \frac{f_D}{1-f_D} = \frac{(y_N - y)}{(y - y_D)} = \exp[-\Delta G/(RT)] \quad (1)$$

where f_D is the fraction of denatured protein and y_N and y_D are the values of the observable parameter for the native and the denatured forms, respectively. When intermediates are

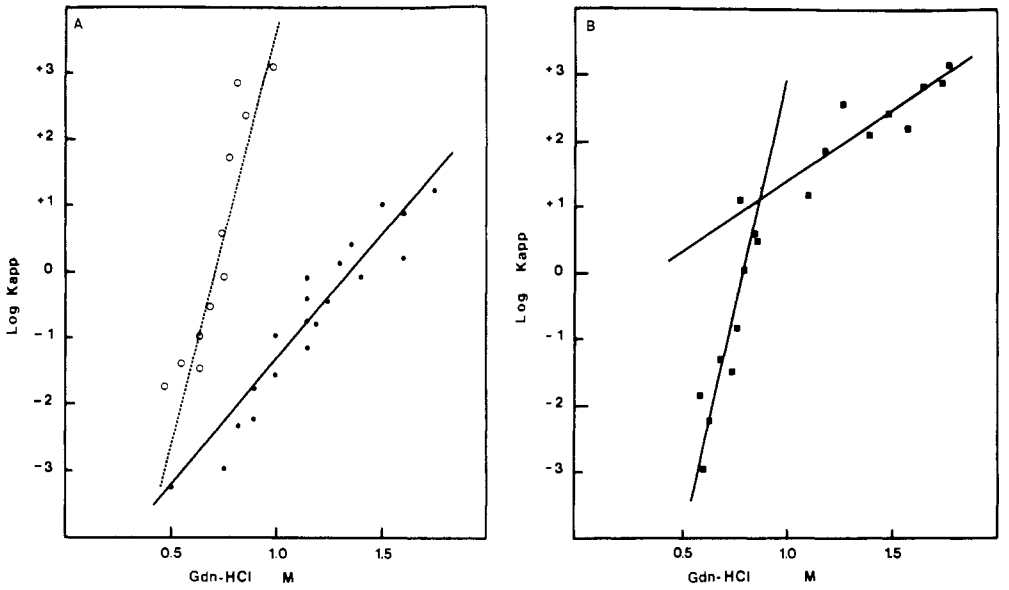

FIGURE 8: Gdn·HCl dependence of K_{app}. (A) Data obtained at equilibrium from fluorescence measurements (●) and activity (○). (B) Data obtained at equilibrium from CD measurements.

significantly present under equilibrium conditions, an apparent constant of denaturation can be defined as follows:

$$K_{app} = K_D[1 + \sum_i z_i(K_i/K_D)]/[1 + \sum_i (1 - z_i)K_i] \quad (2)$$

with $K_i = f_i/f_N$, f_i being the fraction of intermediates, and z_i is equal to $(y_i - y_N)/(y_D - y_N)$, with y_i the value of the observable for the intermediate i. Thus, even in this last case an empirical analysis of the transition can be made. In this treatment, it was implicitely assumed that $0 \leq z_i \leq 1$; i.e., the physical parameter referring to the intermediate species has an amplitude between that of the native and denatured proteins.

Several estimates of the parameters obtained from transitions induced by denaturants such as Gdn·HCl or urea have been proposed (Tanford 1968, 1970; Aune & Tanford, 1969; Greene & Pace, 1974; Pace, 1975; Schellman, 1978). A phenomenological relationship applying only for the transition range was used by Tanford (1968) and Schellman (1975):

$$K_D = \frac{f_D}{1 - f_D} = K_0 c^n \quad (3)$$

with K_D being the denaturation constant, K_0 being this constant in the absence of denaturant, c being the denaturant concentration, and n being a cooperativity index. From the log K_{app} vs. log c plot, we determined a cooperativity index for the different transition curves. Table I summarizes the data. A greater cooperativity was observed for the transition followed by the enzyme activity ($n = 12 \pm 2$), whereas n was only 6.5 ± 0.5 when the transition was followed by variations of the optical signals.

The thermodynamic parameter ΔG_0 was evaluated from linear extrapolation of ln K_{app} vs. c (Figure 8). The experimental slope was obtained from the following equation:

$$\frac{\delta \ln K_{app}}{\delta c} = \Delta b^\circ_{2,3}$$

according to Schellman (1978). It was determined and compared with the predicted value calculated from the free energy of transfer of amino acids and peptide bonds to a solution of Gdn·HCl:

$$\Delta G_{Gdn\cdot HCl} = \Delta G_0 + \sum_i \alpha_i n_i \delta g_{i,tr}$$

$\delta g_{i,tr}$ is the free energy of transfer of group i from water to a given concentration of denaturant, n_i is the number of these groups, and α_i is an empirical coefficient corresponding to the fractional change of the exposure of amino acid side chains; we used the averaged value $\bar{\alpha} = 0.35$ as given by Tanford (1970).

Table I: Thermodynamic Parameters for the Unfolding–Folding Transition of Horse Muscle PGK Induced by Gdn·HCl

observable	n	c_m (M)	$\Delta b^\circ_{2,3}$ (M^{-1})	ΔG_0 (kcal/M)
enzyme activity	12 ± 3	0.7 ± 0.05	12 ± 2	−6.4
fluorescence	6.5 ± 0.5	1.3 ± 0.02	4 ± 1	−3
differential absorbance	6.5 ± 0.5	1.2 ± 0.1	4 ± 1	−3
circular dichroism	$3 < n < 12$	0.8 (app)	$3 < \Delta b^\circ_{2,3} < 14$	
		0.7		
		1.2		
theoretical value			12	

The values of free energy of transfer $\delta g_{i,tr}$ used were those determined by Nozaki & Tanford (1970) from amino acid and peptide solubilities in water and in various solutions of Gdn·HCl. By extrapolation, we obtained an approximate value of ΔG_0, the free energy of denaturation in the absence of denaturant. All these values are given in Table I. For transitions induced by Gdn·HCl, the $\Delta b^\circ_{2,3}$ value was equal to the calculated one when the transition was followed by the enzyme activity and less than half when the transition was studied by optical signals. For transitions studied by circular dichroism, ln K_{app} vs. c gave a broken line as expected from the asymmetry of the transition curve. The slopes were 14 ± 2 and 3.0 ± 0.5 M^{-1}.

Discussion

Four experimental parameters were used to follow the reversible unfolding of horse muscle PGK induced by Gdn·HCl: fluorescence emission at 336 nm, difference UV absorbance, ellipticity, and enzyme activity. From the following arguments it can be inferred that the process deviates from a two-state model and intermediates are significantly populated even at equilibrium: (1) the noncoincidence of the transition curves and (2) the asymmetry of the transition curve obtained by CD measurement at 220 nm.

As appears clearly from our data, the transition curves obtained from the four signals were significantly different. The transition followed by the loss of enzyme activity occurred between 0.5 and 0.90 M Gdn·HCl. In this range, optical properties (fluorescence and difference absorbance) were still those of the native protein. The transition observed by variations in fluorescence was shifted by 0.6 M Gdn·HCl toward higher concentration of denaturant (shift of the c_m value). The transition curve obtained from ellipticity measurements was intermediate between the two previous ones (see Table I).

Noncoincidence of such transition curves was reported for several proteins: carbonic anhydrase (Wong & Tanford, 1973), penicillinase (Robson & Pain, 1973; Adams et al., 1978), α-lactalbumin (Nitta & Sugai, 1972; Kuwajima, 1977), cytochrome *c* (Knapp & Pace, 1974), T4 phage lysozyme (Desmadril & Yon, 1984), and Bence Jones protein (Azuma et al., 1972). Except for lactalbumin and cytochrome *c*, all these proteins are built up of at least two domains. Similarly, asymmetry in transition curves obtained by CD were observed for multidomain proteins: T4 lysozyme (Desmadril & Yon, 1984), β_2-subunit of trytophan synthase (Zetina & Goldberg 1970), penicillinase (Robson & Pain, 1973), elastase (E. Zilber and G. Ghélis, unpublished results). Small proteins such as ribonuclease (Ahmad & Bigelow, 1979) have symmetrical transition curves.

The profile obtained by circular dichroism can be analyzed as the sum of two transition curves (insert, Figure 6). The phenomenological relationship (eq 3) proposed by Tanford (1968) and Schellman (1975) can be written as

$$f_D = K_0 c^n/(1 + K_0 c^n) \quad (4)$$

and also as

$$f_D = c^n/(K^n + c^n) \qquad K^n = 1/K_0$$

This equation represents a symmetrical transition curve of sigmoidal shape. Assuming that the asymmetrical transition curve was a linear combination of two single transition curves, it was described by the following equation:

$$f_D = \alpha\frac{c^{n1}}{K^{n1} + c^{n1}} + (1 - \alpha)\frac{c^{n2}}{K^{n2} + c^{n2}} \quad (5)$$

where α represents the contribution of the first transition and $1 - \alpha$ that of the second transition. The data points obtained by CD were analyzed following eq 5 by using the simplex procedure according to Nelder & Mead (1965). The parameters thus obtained are given in Table I. They indicated that the transition curve observed by variations in ellipticity was the sum of two transitions, the one equivalent to that observed following enzyme activity and the other similar to the fluorescence transition curve. The first transition with a c_m of 0.7 M Gdn·HCl, identical with the one determined following the enzyme activity, represented about 70% of the total amplitude. The other with a c_m of 1.2 M Gdn·HCl identical with the one determined from optical signals (fluorescence and UV difference spectra) corresponded to 30% of the amplitude. The extreme values of $\Delta b^\circ_{2,3}$ (see Table I) are also consistent with this decomposition.

All these data clearly indicate the existence of stable intermediates in the folding pathway of PGK, but they cannot give information about the nature of these intermediates. However, much information can be obtained through consideration of the different transition curves.

If a stable intermediate I is sufficiently populated in the transition followed by variations in ellipticity such as

$$N \rightleftharpoons I \rightleftharpoons D$$

Table II: z_i Values Estimated from Relation 6[a]

[Gdn·HCl] (M)	K_{app}	K_D	K_i	z_i
0.62	0.09	0.037	0.21	0.31
0.65	0.11	0.028	0.36	0.30
0.70	0.15	0.082	0.56	0.24
0.73	0.23	0.136	0.113	0.86
0.75	0.242	0.12	0.88	0.31
0.77	0.63	0.5	5.64	0.40
0.82	1.1	1.33	14.3	0.52
0.85	1.7	1.75	9.75	0.63
0.98	16	5.25	18.5	0.98

[a] K_{app} was evaluated from the experimental transition curve obtained from CD. f_D, the fraction of totally denatured protein, was evaluated from the experimental transition curve obtained by fluorescence measurements. f_N, the fraction of native protein, was evaluated from the transition curve obtained by activity measurements. The fraction of intermediate was $f_i = 1 - f_N - F_D$; K_D and K_i are defined in the text.

we can apply eq 2 for determining z_i, assuming (1) that z_i is close to zero when the observable is enzyme activity and (2) that it tends toward 1 when the observable is fluorescence or UV difference spectra.

With these assumptions and by use of the experimental values of f_N (enzyme activity), f_D (fluorescence), and K_{app} (circular dichroism) for Gdn·HCl concentrations included in the transition range (and not extreme values), it was posible to give an evaluation of z_i. Equation 2 simplifies into

$$z_i = \frac{K_{app}(1 + K_i) - K_D}{K_i(1 + K_{app})} \quad (6)$$

Table II gives the estimated values of z_i. They were not constant in the concentration range of Gdn·HCl corresponding to the three transition curves. This result indicated that either the data were not consistent with a simple folding pathway involving only one intermediate or that assumptions 1 and 2 concerning fluorescence and enzyme activity of intermediate I were oversimplified. It was plausible to admit $z_i = 0$ for the enzyme activity since this property is very sensible to any, even very small, change. But we had no indication concerning the z_i value for fluorescence; we admitted $z_i = 1$ for the sake of simplicity. Nevertheless, these results, associated with the thermodynamic analysis of the unfolding–refolding of PGK, indicated that the assumption of only one intermediate is not sufficient to account for all the data.

Considerations of thermodynamic parameters for the reversible unfolding of PGK by Gdn·HCl indicate a $\Delta b^\circ_{2,3}$ value of 4 M^{-1} when the transition is observed by fluorescence or absorbance and a value slightly more than the double of the previous one when the transition was followed by the enzyme activity (13 ± 2 M^{-1}). According to Rowe & Tanford (1972), a twofold difference would signify an independent unfolding–refolding of the two domains with identical equilibrium constants; a factor greater than 2 indicates an independent unfolding–refolding but with different equilibrium constants.

The loss and recovery of enzyme activity followed an apparent two-state behavior implying the entire molecule. The value of ΔG_0 obtained from this parameters supported this view. ΔG_0 of −6.4 kcal M^{-1} (i.e., −26.9 kJ M^{-1}) referred to the entire molecule while ΔG_0 of −3 kcal M^{-1} (i.e., −12.6 kJ M^{-1}) would correspond to the stabilization energy of the C-terminal domain only, since it was obtained from fluorescence measurements.

However, extrapolation to zero from linear variations of ln K_{app} vs. denaturant concentration in the transition zone is not very accurate, because of the narrowness of the transition in a range of concentrations of denaturant higher than those

required for denaturation by Gdn·HCl. As pointed out by Schellman & Hawkes (1978), the "demonstration of linearity of ΔG_u in urea or Gdn·HCl would cover a wide range of denaturant concentration, below the transition zone, that is usually difficult to investigate".

By contrast, for the reversible unfolding induced by Gdn·HCl of both yeast and B. *Thermus thermophilus* PGK a good coincidence in the transition curves observed by fluorescence and molar ellipticity at 225 nm, with a c_m of 0.62 M at 25 °C, was reported by Nojima et al. (1977). In their study of the same transition for yeast PGK followed by circular dichroism at 222 and 278 nm, Adams et al. (1978) reported an unique, very cooperative and apparently symmetrical transition curve with a $c_m = 0.735$ M. But the authors found an experimental slope $\Delta b^\circ_{2,3}$ slightly smaller than half the calculated one and suggested an independent refolding of domains with different equilibrium constants. Furthermore, in our case, the results suggest a more favorable refolding constant for the C-terminal domain, since it unfolds for higher Gdn·HCl concentrations. Indeed, the transition curves obtained from fluorescence at 336 nm (excitation at 292 nm) and difference in absorbance at 292 nm and even at 286 nm mainly revealed variations in the environment of tryptophans. The four Trp are localized in the C-terminal domain, three exposed to the solvent and Trp-339 localized on the β_j segment partially or totally buried. This part of the molecule seems more resistant to the denaturant. By contrast, enzyme activity gave a very cooperative transition in a lower range of Gdn·HCl concentration. The end of the transition was reached for Gdn·HCl values where PGK still had the fluorescence of the native protein. Since the expression of the activity reflects the conformation of the active site which implies very small conformational readjustments, this part of the molecule is particularly sensitive to denaturation. This aspect was particularly emphasized by Ghélis & Yon (1978, 1982).

Circular dichroism reflects the content in ordered structures. Seventy percent of ellipticity was lost in the first part of the transition, the one corresponding to the enzyme activity. We tried to determine the variations in α helices and in β structures along the transition. With this aim, we compared the decomposition obtained using two different methods of estimation: that of Chen et al. (1972) and that of Brahms & Brahms (1980), with the secondary structures determined from X-ray data. For the native protein, the first estimation gives the most realistic results. However, in the transition region, the absorbance of Gdn·HCl imposes severe restrictions in the recording of the spectrum which becomes possible only from 210 nm. Thus, in the absence of informations around 190 nm, it was not possible to obtain accurate estimations of the variations in helices and β strands along the transition region. However, in the second part of the CD transition curve, the one which corresponds to the transition curve observed by fluorescence measurements and therefore referring mainly to the C-terminal domain, the residual ordered structures corresponding to 30% of the total ellipticity seem mostly to consist in β strands.

PGK is an α/β protein with helical segments localized near the surface of the protein; they seemed more sensitive to denaturation than β structures which are at the interior of the molecule and therefore less accessible. Thus, it is plausible to assume that most of the secondary structures which are unfolded in the transition which occur at lower concentrations of Gdn-HCl are mainly helices. The transition obtained for higher Gdn·HCl concentration accompanied by the unfolding of the remaining structures (30% of ellipticity) corresponds to the change in the environment of tryptophans and may be related to the C-terminal domain. This lobe is more resistant to the denaturant than the N-terminal domain.

The experimental results obtained from equilibrium studies of the reversible unfolding of PGK by Gdn·HCl suggest that the two domains of the horse muscle enzyme refold independently of one another with different equilibrium constants, the most favorable refolding constant referring to the folding of the C-terminal domain. Kinetic studies under way in our laboratory agree with this suggestion.

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Registry No. PGK, 9001-83-6; Gdn·HCl, 50-01-1.

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Effects of Urea and Guanidine Hydrochloride on Peptide and Nonpolar Groups†

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ABSTRACT: The free energy transfer of several *N*-acetyl(glycine)$_n$ ethyl esters (n = 1–3) and side chain derivatives (Ala, Val, Nva, Leu, Nle, and Phe) from water to urea and guanidine hydrochloride solutions has been determined from the solubility and distribution coefficients of these compounds between aqueous and nonaqueous phases. These uncharged model peptides, unlike the amino acids used for a similar study, avoid complication due to charge effects for the transfer process. The compounds with an increase in the number of glycyl groups show additivity of the group free energy toward the transfer from water to urea solution but not to guanidine hydrochloride solution. The derivatives with a side chain show that the principle of group additivity does not hold true for the aliphatic side chains for the transfer to either urea or guanidine hydrochloride solutions. In fact, the free energy of transfer of the side chains, viz., aliphatic ones, is found to be energetically unfavorable in moderately high denaturant concentration. Phenylalanyl, the only aromatic side chain studied here, showed a favorable free energy of transfer to the denaturant solutions. In addition, the values of the favorable free energy obtained in this study are much smaller than the values obtained from the study of the amino acids. The transfer of the glycyl group to the denaturant solutions is exothermic whereas the transfer of the side chains is endothermic in nature. The interaction of these solutes with urea and guanidine hydrochloride solutions has been explained on the basis of the hypothesis of Roseman & Jencks [Roseman, M., & Jencks, W. P. (1975) *J. Am. Chem. Soc. 97*, 631–640], which states that the driving force for the favorable interaction effect of cosolvents with solutes arises from a more favorable sum of the free energies of cavity formation and nonpolar interaction in the presence of cosolvents (denaturants). With polar solutes, the cosolvent must also be able to form hydrogen bonds to make the overall free energy of transfer favorable.

A number of studies have been made of the interaction of denaturants urea and Gdn·HCl[1] with model compounds to understand the mechanism of denaturation of proteins in these solutions (Tanford, 1968, 1970; Pace, 1975; Eagland, 1975; Franks & Eagland, 1976). Small molecules, viz., amino acids and peptides, have been used mostly to understand the thermodynamic nature of interaction of urea and Gdn·HCl with the amino acid residues in proteins. The results have been generally obtained from solubility and calorimetric measurements (Nozaki & Tanford, 1963, 1970; Robinson & Jencks, 1965a; Wetlaufer et al., 1964; Lapanje et al., 1978; Schonert & Stroth, 1981; Cesaro et al., 1982). From the solubility studies of a large number of amino acids in urea and Gdn·HCl, Tanford and co-workers (Nozaki & Tanford, 1963, 1970; Tanford, 1970) observed that the free energies of transfer, Δg_{tr}, of the contributing groups in the amino acids from water to denaturant solutions are additive and independent of the nature of the molecule (Wetlaufer et al., 1964).[2] The Δg_{tr} values for individual groups can then be used to calculate the stability of the protein molecule in denaturant solution from its amino

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[1] Abbreviations: Ag_1E, Ag_2E, Ag_3E, and Ag_4E, acetylmonoglycine, -diglycine, -triglycine, and -tetraglycine ethyl esters, respectively; AAE, AVE, AnVE, ALE, AnLE, and APE, acetylalanine, -valine, -norvaline, -leucine, -norleucine, and -phenylalanine ethyl esters; Gdn·HCl, guanidine hydrochloride.

[2] The principal of additivity is based on the consideration that the free energies of transfer of the constituent groups in the compound would yield the total free energy of transfer of the compound.

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