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Unfolding–Refolding of the Domains in Yeast Phosphoglycerate Kinase: Comparison with the Isolated Engineered Domains†

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ABSTRACT: The role of domains as folding units was investigated with a two-domain protein, yeast phosphoglycerate kinase. Each of the domains was produced independently by site-directed mutagenesis. It has been previously demonstrated by several criteria that these domains are able to fold in vivo into a quasi-native structure [Minard et al. (1989a) *Protein Eng. 3*, 55–60; Fairbrother et al. (1989) *Protein Eng. 3*, 5–11]. In the present study, the reversibility of the unfolding–refolding process induced by guanidine hydrochloride was investigated for the intact protein and the isolated domains. The transitions were followed by circular dichroism for both domains and the intact protein and by the variations in enzyme activity for the intact protein. Tryptophan residues were used as intrinsic conformational probes of the C-domain. An extrinsic fluorescent probe, *N*-[[(iodoacetyl)amino]ethyl]-8-naphthylamine-1-sulfonic acid (IAEDANS), was bound to the unique cysteinyl residue Cys97 to observe the conformational events in the N-domain. The unfolding–refolding transitions of each domain in the intact protein and in the isolated domains prepared by site-directed mutagenesis were compared. It was shown that the two domains are able to refold in a fully reversible process. A hyperfluorescent intermediate was detected during the folding of both the isolated C-domain and the intact yeast phosphoglycerate kinase. The stability of each isolated domain was found to be similar, the free energy of unfolding being approximately half that of the intact molecule.

The molecular mechanisms by which a polypeptide chain folds into its biologically active three-dimensional structure have been widely investigated by using several different proteins as models. The protein folding process has often been described as a hierarchical succession of structural events (Rose, 1979). At present, the generally accepted model consists of an early stage during which short-range interactions lead to the formation of nucleation centers; then, some stretches of ordered structures are formed that interact to produce new, higher levels of structure, which merge into domains. Finally, the domains associate and interact to generate the native protein (Kim & Baldwin, 1982). It has been proposed that domains are structural entities that are capable of folding independently (Wetlaufer, 1973). Domains, therefore, would constitute the elementary folding units of proteins. Many attempts to demonstrate experimentally the role of domains as kinetic intermediates in the folding pathway of multidomain proteins have been reported. Until now, the only possible approach to studying the behavior of domains during the refolding process has required the isolation of the domains following limited proteolysis or chemical cleavage [see Wetlaufer (1981), Ghélis and Yon (1982), and Jaenicke (1987) for reviews].

Phosphoglycerate kinase is a good model for the study of domain folding. The three-dimensional structures of horse muscle (Banks et al., 1979) and yeast (Watson et al., 1982) phosphoglycerate kinases, both monomeric enzymes, have revealed two globular units corresponding to the amino- and carboxyl-terminal halves of the molecules called the N- and C-domains, respectively. The C-domain, which binds nucleotide substrates, contains a Rossmann fold. The study of the unfolding–refolding transition at equilibrium of the horse muscle phosphoglycerate kinase, using different conformational probes (Betton et al., 1984), has suggested that the domains refold independently and that the C-domain is more stable. Kinetic data have confirmed the presence of an intermediate in which the C-domain is partially or totally folded (Betton et al., 1985). Limited proteolysis of this enzyme, using conditions under which the folding intermediates are more populated, has given a stable fragment identified as the C-terminal part of the protein (Betton et al., 1989). This fragment has been found to be partially folded, supporting the conclusion that the C-domain might be a folding unit. Adams et al. (1985) have obtained a fragment corresponding to a part of

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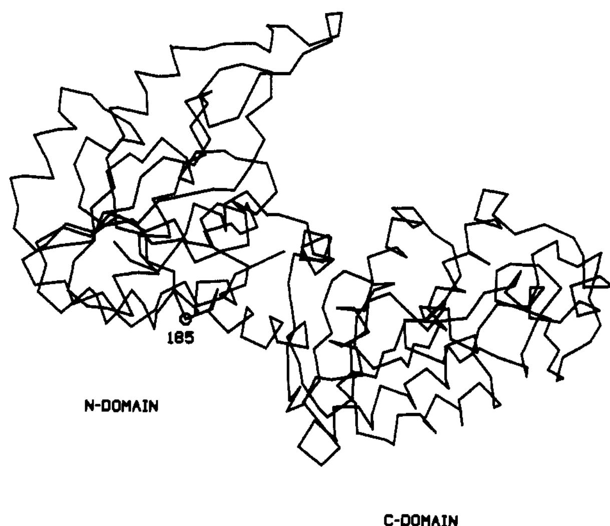


FIGURE 1: Three-dimensional structure of yeast PGK (Watson et al., 1982). F185, indicated by the circle, was chosen as the structural limit of the two domains (Minard et al., 1989a).

the N-domain of the yeast enzyme after cleavage of the protein by cyanogen bromide. This fragment has been found to have some degree of structure, but its dimerization has prevented further investigation. Conclusive evidence that domains have evolved as modular folding units have been provided by the construction of a chimeric phosphoglycerate kinase (Mas et al., 1986). The engineered protein, combining domains from the human and yeast enzymes, has been found to exhibit full activity.

A new powerful method for producing polypeptide fragments corresponding to structural domains involves site-directed mutagenesis. We have used this technique to prepare the two domains of yeast phosphoglycerate kinase (Figure 1). It has been demonstrated by several criteria that these domains are able to fold, in vivo, to give a quasi-native structure (Minard et al., 1989a; Fairbrother et al., 1989). We describe here the unfolding–refolding transitions of the engineered domains and compare them to those of the intact protein. This study was undertaken to answer the following questions: Is the refolding–refolding of the domains a reversible and independent process in vitro and, if so, to what extent can the stability of the domains be estimated? Finally, do detectable intermediates exist during the formation of each domain? The transitions induced by guanidine hydrochloride under equilibrium conditions were followed by using different signals. Since this study was carried out with the isolated and the integrated domains, the presence of probes reflecting the conformational events in each domain was required. Tryptophan residues (Trp308 and Trp333), both in the C-domain, provided a valuable signal from this region. An extrinsic fluorescent label, *N*-[[(iodoacetyl)amino]ethyl]-8-naphthylamine-1-sulfonic acid (1,8-IAEDANS)[1] was bound to the unique cysteinyl residue (Cys97) and, thus, reflected the conformational events in the N-domain.

Materials and Methods

Protein Purifications. Recombinant phosphoglycerate kinase (PGK; EC 2.7.2.3) was prepared as described by Minard et al. (1989b). The purification and characterization of the individual domains have been previously reported (Minard et al., 1989a).

Labeling of PGK and the N-Domain with 1,8-IAEDANS. The unique cysteinyl residue, Cys97, located in the N-domain was chemically modified with 1,8-IAEDANS PGK (1 mL, 200 μM) and treated at 21 °C for 2.5 h with a 20-fold molar excess of 1,8-IAEDANS in a 50 mM Tris buffer, pH 7.50, containing 500 μM EDTA and 4 M Gdn·HCl. The N-domain (2 mL, 200 μM) was treated at 21 °C for 2.5 h with a 40-fold molar excess of 1,8-IAEDANS in the same buffer free of denaturant. The excess of reagent was removed by gel filtration on a Sephacryl 100HR (Pharmacia LKB Biotechnology) column (2 × 10 cm) equilibrated with the same buffer. The labeled PGK was renatured by 20-fold dilution followed by dialysis at 4 °C for 12 h in a 20 mM Tris buffer, pH 7.50, containing 500 μM EDTA. The concentrations of the modified proteins were determined according to the method of Lowry et al. (1951). The extent of the modification was then determined by the absorbance of AEDANS at 341 nm using $\epsilon = 6300\ M^{-1}\ cm^{-1}$ (Hudson & Weber, 1973).

Unfolding–Refolding Studies. All the unfolding–refolding experiments were carried out in a 20 mM Tris buffer, pH 7.50, containing 500 μM EDTA, 1 mM β-mercaptoethanol, or 1 mM DTT at 21 °C. The concentrations of protein during incubation in Gdn·HCl were 2 μM for the transition followed by measurement of the enzyme activity, 2 μM for the circular dichroism studies, and 3.5 μM for the fluorescence experiments. For the denaturation experiments, the protein solutions were incubated in increasing concentrations of Gdn·HCl. For the renaturation experiments, a stock solution of protein (about 100 μM) was incubated in a buffer containing 6 M Gdn·HCl before dilution to the desired final concentration of denaturant. Measurements were performed after 12 h of incubation.

Ultrapure Gdn·HCl was obtained from Pierce; the denaturant concentrations were checked by refractometry (Nozaki, 1970).

Determination of Enzyme Activity. The transition of PGK was followed by measurement of enzyme activity using the coupled assay with glyceraldehyde-3-phosphate dehydrogenase as described by Bücher (1955). The final concentration of PGK in the assay was 200 pM. Under these conditions, the renaturation rate of the unfolded protein fraction was slow enough to allow an accurate measurement of the remaining enzyme activity.

Fluorescence and Circular Dichroism. The circular dichroic (CD) spectra were recorded with a Mark V dichrograph (Jobin et Yvon) using a 2-mm light-path cell.

Fluorescence spectra were recorded with a MPF 44B fluorometer (Perkin-Elmer) using a 10-mm light-path cell.

Analysis of the Transition Curves. The experimental data were analyzed by using the simplex procedure (Press et al., 1986), following equations derived from the denaturant binding model (Aune & Tanford, 1969), as described by Betton et al. (1984):

$$f_d = C^n/(K_0 + C^n)$$

For CD transitions, linear dependencies of the signal for native and denatured proteins were taken into account during the calculation procedure before normalized curves were plotted.

For transition curves observed by fluorescence intensity, the whole transition was fitted by a linear combination of two single transition curves with opposite amplitudes. The fluorescence intensity data, for both PGK and the C-domain, cannot be correctly fitted on the assumption of a single

[1] Abbreviations: PGK, phosphoglycerate kinase (EC 2.7.2.3); EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; Gdn·HCl, guanidine hydrochloride; 1,8-IAEDANS, *N*-[[(iodoacetyl)amino]ethyl]-8-naphthylamine-1-sulfonic acid; CD, circular dichroism; NMR, nuclear magnetic resonance.

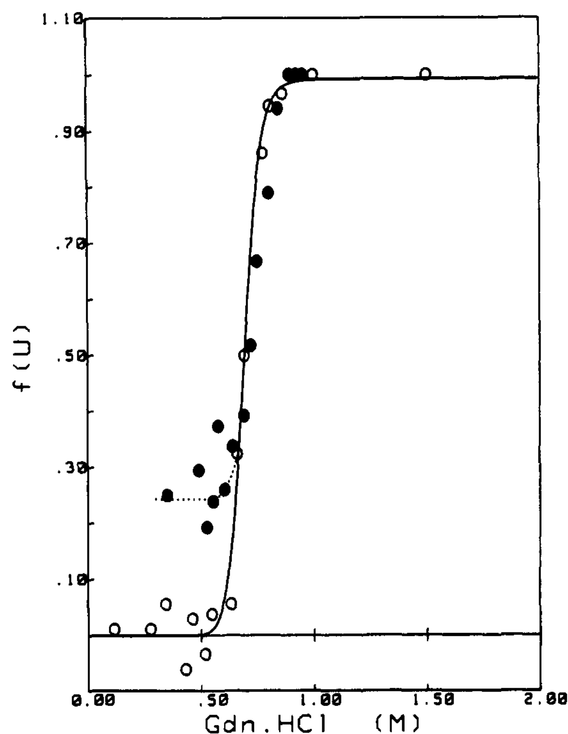


FIGURE 2: Unfolding–refolding transition of yeast PGK as followed by the enzyme activity. The transition curve is normalized: $f(U)$ is the fraction of protein unfolded. (○) Unfolding process; (●) refolding process.

transition. During the calculation procedure, linear dependencies of the fluorescence intensity of the native and denatured forms were taken into account in both cases.

Results

Unfolding–Refolding Transition of PGK Followed by the Enzyme Activity. The normalized transition, as followed by the enzyme activity, is shown in Figure 2. The enzyme, previously denatured in 6 M Gdn·HCl, did not completely recover its activity after renaturation: 25% of activity was lost. The fact that the enzyme activity is not totally recovered is probably due to a very local effect, since this signal is more sensitive than the conformational probes such as CD. The curve displayed a very cooperative process, with a c_m value corresponding to the midpoint of the transition curve of 0.70 ± 0.02 M Gdn·HCl.

Unfolding–Refolding Transitions of PGK and the Isolated Domains Followed by Circular Dichroism. The transitions of PGK and the two isolated domains were followed by measuring the variation in ellipticity at 218 nm, which reflects the decrease in ordered structures for increasing concentrations of Gdn·HCl. Figure 3 shows the normalized transition curves. Each curve was found to be symmetrical, monophasic, and completely reversible. For PGK and the N-domain, the same c_m value of 0.80 ± 0.02 M Gdn·HCl was obtained, whereas for the C-domain, a smaller value of c_m was determined, 0.63 ± 0.02 M Gdn·HCl.

Unfolding–Refolding Transitions of the C-Domain and PGK As Assessed by the Fluorescence of Tryptophans. The two tryptophan residues of PGK are localized in the C-domain; Trp308 is situated in a β-turn and is exposed to the solvent, while Trp333 is buried at the end of the β_L segment. Thus, the effects of Gdn·HCl on the fluorescence properties of PGK reflect the conformational events that occur in the C-domain.

For PGK and the C-domain, the maximum emission wavelength was 330 nm for the native state and 345 nm for the denatured state. The fluorescence of Trp for PGK and

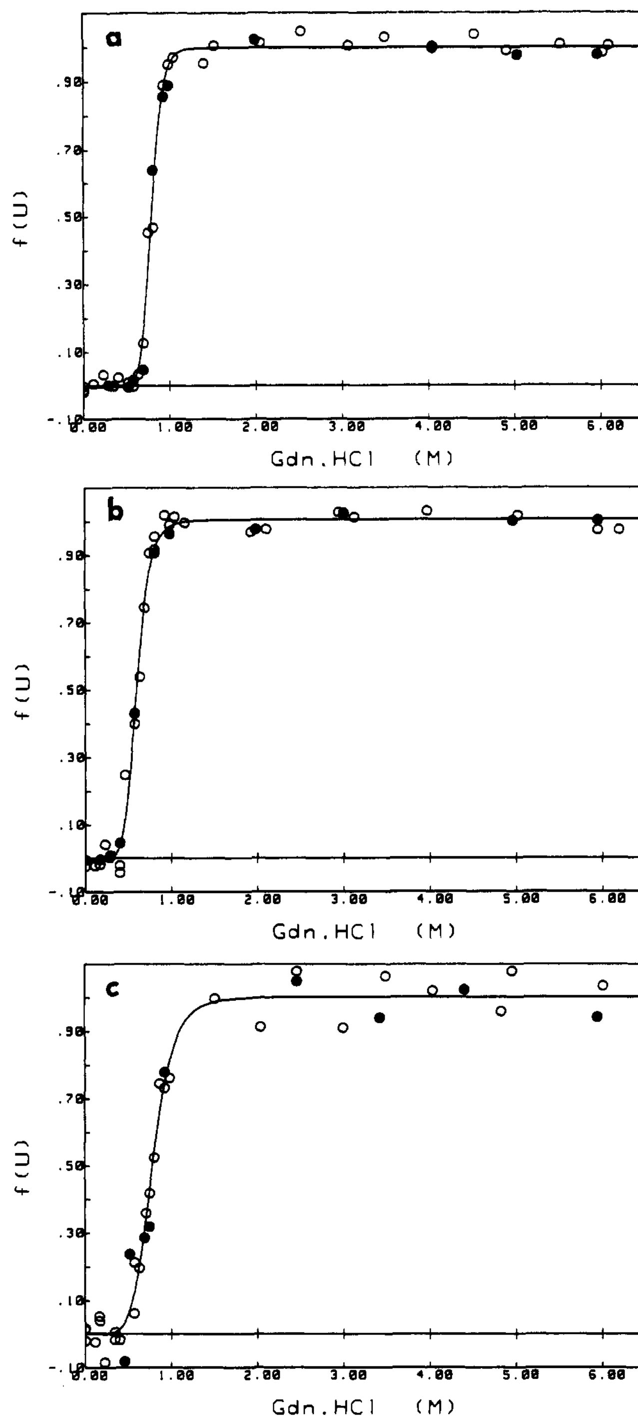


FIGURE 3: Unfolding–refolding transition as assessed by circular dichroism at 218 nm. Normalized curves for (a) PGK, (b) C-domain, and (c) N-domain. $f(U)$ is the fraction of protein unfolded. (○) Unfolding process; (●) refolding process. The three experimental curves were fitted according to a single transition, the total amplitudes being 2.47, 2.87, and 1.53 ($\Delta\epsilon$/residue) for curves a, b, and c, respectively. The slopes corresponding to the linear dependency of the signal versus Gdn·HCl were −0.02, 0.80, and 0.03 for the native protein and 0.12, 0.07, and 0.03 for the denatured protein, for PGK, and for the C- and N-domains, respectively.

the C-domain under denaturing conditions exhibited two different effects: a complex variation of the intensity and a 12-nm red shift. Both effects are apparent in Figures 4 and 5.

The transitions presented in Figure 4 were obtained from the changes in the fluorescence intensity at 330 nm. The transitions were found to be reversible; the presence of a hyperfluorescent intermediate was detected with a maximum emission at 0.9 M Gdn·HCl for PGK and 0.5 M Gdn·HCl for

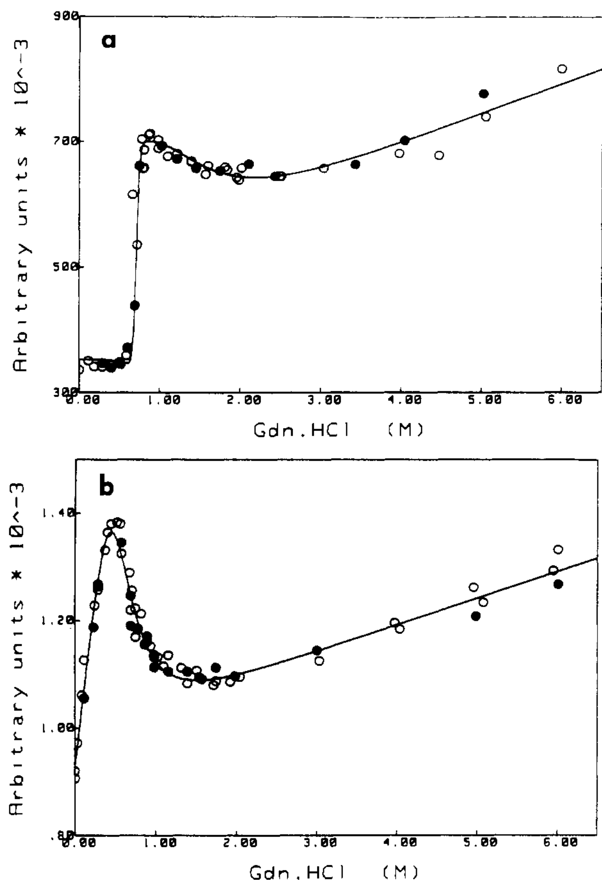


FIGURE 4: Unfolding–refolding transition of the C-domain followed by the fluorescence intensity at 330 nm (excitation wavelength: 295 nm): (a) C-domain in PGK and (b) C-domain prepared by site-directed mutagenesis. The fluorescence intensity is expressed in arbitrary units; the ratio of the fluorescence intensity of the native C-domain over the fluorescence intensity of native PGK is 1.6. (○) Unfolding process; (●) refolding process. The total amplitudes are 150 and 62, and the amplitudes of the first transitions are 195 and 99 for PGK and the C-domain, respectively. The slopes of the linear dependency of the signal versus Gdn·HCl concentration were 0 and 1260 for the native protein and 48 and 707 for the denatured one for PGK and the C-domain, respectively.

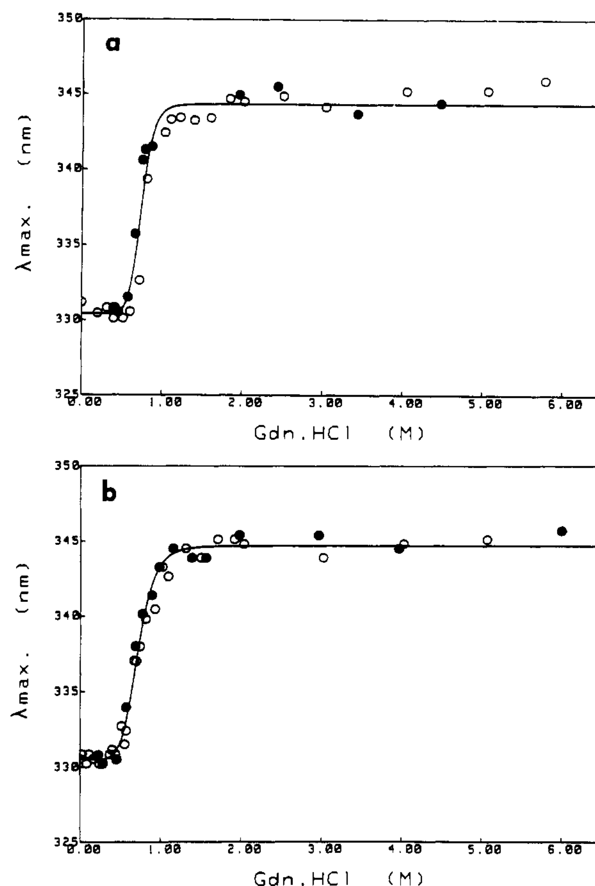


FIGURE 5: Unfolding–refolding transition of the C-domain followed by the variation in the maximum fluorescence emission wavelength (excitation wavelength: 295 nm): (a) C-domain in PGK and (b) C-domain prepared by site-directed mutagenesis. (○) Unfolding process; (●) refolding process.

the C-domain. Above 2 M Gdn·HCl, the fluorescence intensity increased linearly with the concentration of denaturant, indicating a simple solvent effect. The existence of this intermediate has not been described in previously published folding studies of yeast PGK (Nojima et al., 1977; Adams et al., 1985); instead, the decrease in fluorescence intensity between 0.9 and 2 M Gdn·HCl was attributed to a linear dependency of the fluorescence upon the denaturant concentration. However, when higher denaturant concentrations are taken into account, it is clear that the fluorescence increases linearly only at Gdn·HCl concentrations above 2 M where the protein was completely unfolded. Therefore, the decrease in fluorescence between 0.9 and 2 M Gdn·HCl corresponds to the unfolding process.

In Figure 5, the variation in the maximum fluorescence emission wavelength, $\Delta\lambda_{max}$, was used to describe the transition. The transitions were found to be completely reversible and symmetrical with a c_m value of 0.73 ± 0.02 M Gdn·HCl for both PGK and the C-domain.

Unfolding–Refolding Transitions of the N-Domain and PGK Followed by the Fluorescence of AEDANS Linked to Cys97. The unique cysteinyl residue, Cys97, in PGK and in the N-domain was modified by a fluorescent probe, 1,8-IAEDANS. The yield of labeling was about 98% for PGK and 95% for the N-domain. This modification affected slightly the stability of the two molecules, as determined by circular dichroism (Table I); however, the unfolding–refolding process remained reversible, and the secondary structures of the labeled molecules were very similar to those of the native PGK and N-domain. Since the fluorescent properties of AEDANS are very sensitive to the polarity of the environment, AEDANS was used as a conformational probe of the N-domain. Each labeled molecule had a spectrum exhibiting a maximum emission at 470 nm for the native and at 500 nm for the denatured states (in 6 M Gdn·HCl) following excitation at 350 nm; thus a similar 30-nm red shift occurred for the AEDANS-N-domain and for AEDANS-PGK. The unfolding–refolding transitions of the labeled proteins were studied by measuring the variations in fluorescence intensity at 470 nm (Figure 6). For PGK, the unfolding and refolding processes did not coincide at low denaturant concentrations (<0.5 M Gdn·HCl); there is no clear interpretation of this phenomenon. Above 0.5 M Gdn·HCl, the fluorescence intensity decreased with increasing concentrations of Gdn·HCl up to 2 M. This latter transition was found to be reversible with a c_m value of 0.89 ± 0.05 M Gdn·HCl. This same transition was also observed for the isolated N-domain with a c_m value of 0.73 ± 0.05 M Gdn·HCl.

Thermodynamic Analysis. The stabilities of the isolated domains and of yeast PGK were compared after a thermodynamic analysis of the transition curves established by circular dichroism. Despite the detection of intermediates by fluorescence measurements, the transitions as assessed by CD were found to be symmetrical, indicating that, under equilibrium conditions, intermediate states were not detected. Thus, the transition between the folded (F) and unfolded (U)

Table I: Unfolding–Refolding Parameters of Yeast Phosphoglycerate Kinase and the Isolated Domains

protein	CD	Trp fluorescence		AEDANS fluorescence intensity	enzyme activity
		$\Delta\lambda_{max}$	intensity		
PGK					
c_m[a]	0.80 ± 0.02	0.73 ± 0.02	0.70 ± 0.02 (first effect)		0.70 ± 0.02
n[b]	12.8	9	13		19
c_m			1.38 ± 0.1 (second effect)		
n			3.8		
C-domain					
c_m	0.63 ± 0.02	0.73 ± 0.02	0.57 ± 0.08 (first effect)		
n	7.8	6.8	4.5		
c_m			1.36 ± 0.1 (second effect)		
n			1.3		
N-domain					
c_m	0.80 ± 0.02				
n	6.5				
labeled PGK					
c_m	0.55 ± 0.05			0.89 ± 0.05 (half effect)	
n	5			6.7	
labeled N-domain					
c_m	0.68 ± 0.05			0.73 ± 0.05	
n	4			4	

[a] c_m is expressed in molar Gdn·HCl. [b] n is the cooperativity index evaluated from the relationship $K_{app} = K_0C^n$.

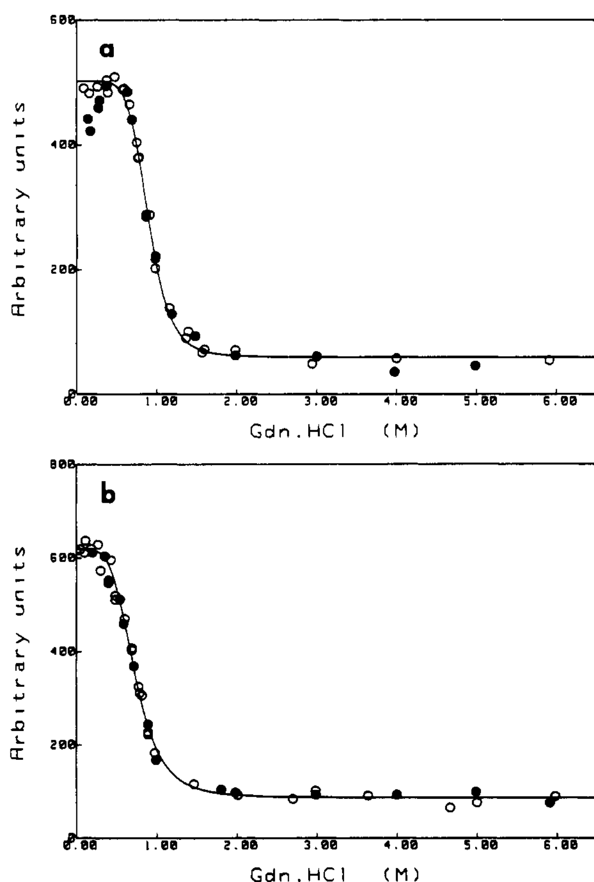
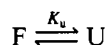


FIGURE 6: Unfolding–refolding of the N-domain as assessed by the fluorescence intensity of AEDANS at 470 nm (excitation wavelength: 350 nnm). The fluorescence is expressed in arbitrary units. (a) N-domain in PGK and (b) N-domain prepared by site-directed mutagenesis. (O) Unfolding process; (●) refolding process.

protein can be described, to a first approximation, by a two-state mechanism:



$$F \overset{K_u}{\rightleftharpoons} U$$

The equilibrium constant, K_u, and the free energy, ΔG_u, were calculated for the Gdn·HCl concentrations within the transition region according to the relationship:

$$K_u = U/F = f_u/(1 - f_u) = \exp(-\Delta G_u/RT)$$

Table II: Thermodynamic Parameters of the Unfolding–Refolding Transition As Assessed by Circular Dichroism for Phosphoglycerate Kinase and the Isolated Domains

protein	ΔG_0 (kcal/mol)	m (kcal/mol^2)
PGK	7.8 ± 0.5	9.7 ± 1.3
C-domain	4.5 ± 0.5	7.6 ± 1.5
N-domain	4.0 ± 0.5	5.1 ± 1.1

f_u being the fraction of unfolded protein. The free energy of unfolding in the absence of denaturant, ΔG_0, was evaluated from a linear extrapolation of the values of ΔG_u versus the denaturant concentrations according to Pace (1986):

$$\Delta G_u = \Delta G_0 + mc$$

The corresponding values of ΔG_0 are given in Table II. The slope, m, was weaker for each of the domains than for PGK (Table II).

Discussion

In the present study, the role of domains as folding units in yeast PGK was investigated by comparing the unfolding–refolding transitions of the native protein with those of the domains prepared by site-directed mutagenesis. The transitions induced by Gdn·HCl were followed under equilibrium conditions by using several signals, circular dichroism, fluorescence of tryptophan residues located in the C-domain, fluorescence of an extrinsic probe bound to the unique cysteinyl residue (Cys97) in the N-domain, and, for the whole enzyme, the catalytic activity.

It has been shown previously that the domains of PGK are able to fold in vivo (Minard et al., 1989a; Fairbrother et al., 1989). The present results show that they are also able to refold in vitro. The transitions assessed by CD for the two isolated domains were found to be reversible; thus each domain possesses intrinsically all the information necessary for proper folding. The transition curve of yeast PGK measured by CD was symmetrical, contrary to the transition curve of horse muscle PGK, which has been found to be clearly asymmetrical. This asymmetry has been ascribed to an increased resistance to Gdn·HCl denaturation of the C-domain (Betton et al., 1984). In the present study, the c_m value of the N-domain was found to be identical with the c_m value of the whole protein whereas the c_m of the C-domain was slightly lower. In spite

of the marked similarity of the three-dimensional structures of horse muscle and yeast PGKs, it appeared clearly that the yeast enzyme was less resistant to Gdn·HCl denaturation than the horse muscle enzyme. Furthermore, in yeast PGK, the C-domain is not a more stable entity at equilibrium contrary to the results obtained for the horse muscle enzyme.

The transitions of yeast PGK as assessed by CD and enzyme activity were very similar: only a variation of 0.1 M Gdn·HCl was recorded between the two c_m's. Moreover, both transitions exhibited a high degree of cooperativity (Table I). The cooperativity constant is related to the extent of the molecule that becomes accessible to the denaturant during the unfolding process (Tanford, 1970). Therefore, the lower cooperativities of the transitions of the isolated domains as assessed by CD are not an unexpected result. The thermodynamic stabilities, as reflected by the ΔG_0 values (Table II), were found to be very similar for the two domains and equivalent to half the value of the $\Delta G_{0,PGK}$ found for the native protein. A comparison of the direct thermodynamic parameters obtained by microcalorimetry for the isolated and integrated domains should give an estimation of the relative importance of the intra- and interdomain stabilities within the intact protein (Brandts, 1989).

The study of the fluorescence emission of the tryptophans provides information on the local changes occurring in the environment of the two tryptophan residues. Since the two tryptophans are located in the C-domain, they were used as specific conformational probes to compare the folding transition of the isolated and integrated C-domain. First of all, the fact that the native PGK and the native C-domain displayed the same emission maximum at 330 nm indicated that the fluorophores were in a similar apolar environment in both proteins. This result was confirmed by the fact that a similar red shift was observed under denaturing conditions. The two transitions assessed by measuring the variations in the maximum fluorescence wavelength indicated completely reversible processes having identical c_m's. Therefore, the unfolding–refolding processes were very similar for both molecules. When the fluorescence intensity was observed, it appeared that the native C-domain was more fluorescent than native PGK. Nojima et al. (1976) have shown that the fluorescence of Trp308 might be quenched by the negative charge of a glutamate residue in native PGK. Therefore, the difference in the observed intensities could be interpreted as resulting from decreased quenching in the C-domain. Nevertheless, the transition of the C-domain, as assessed by fluorescence intensity, displayed the same characteristics as the transition of PGK, the presence of a hyperfluorescent intermediate and an unfolding transition occurring up to 2 M Gdn·HCl. The presence of this qualitatively similar intermediate in the C-domain and in PGK leads to the conclusion that the same folding pathway with identical local rearrangement steps occurs in the isolated and integrated C-domains.

It is notable that the transitions assessed by measuring the maximum wavelength of fluorescence emission, which present the same c_m's for both molecules, occur with lower c_m's than the yeast PGK transition measured by CD but with the same c_m as the transition measured by enzyme activity (Table I). It seems that early unfolding events in the C-domain are related to the loss of enzyme activity. However, the observation of later unfolding events occurring in parallel (transitions followed by measuring the fluorescence intensity) suggests that, locally, some stretches of structure surrounding one of the two tryptophan residues are more resistant to Gdn·HCl in the isolated as well as in the integrated C-domain.

The labeling of Cys97 with 1,8-IAEDANS was an indirect way to obtain local information about the N-domain. Similar changes in the emission spectra were measured during the denaturation of the labeled N-domain and the labeled PGK. The probe experienced the same change in environment: it moved from a rather hydrophobic environment to a more polar one. Surprisingly, the transitions assessed by measuring the fluorescence of the probe occurred at higher Gdn·HCl concentrations than the transitions of the labeled molecules measured by CD (Table I), indicating that a hydrophobic structure surrounding the naphthalene ring remained stable even when most of the secondary structures were disrupted. Cys97 has been found to be more reactive toward NbS_2 in the isolated domain than in the intact protein (Minard et al., 1989a). The same is true with respect to the reactivity toward 1,8-IAEDANS, suggesting that the N-domain becomes more "floppy" when isolated. This observation is also supported by the high hydrogen–deuterium exchange rate observed by NMR (Fairbrother et al., 1989). However, the isolated N-domain was found to be a stable entity by CD measurements. It seems that the integrated domain becomes more rigid due to the interdomain interactions without an enhancement of its thermodynamic stability. Further kinetic analysis of the unfolding process, currently under investigation in our laboratory, should clarify this point.

To summarize, the N- and C-domains of yeast PGK have been found to be able to refold, in vitro, independently of each other in a fully reversible process. A hyperfluorescent intermediate, not previously described, occurs during the folding of both the C-domain and the intact PGK. The free energy of stability of each domain is approximately half that of the intact protein, suggesting that the C-domain is not a more stable entity than the N-domain contrary to that reported for the muscle horse enzyme. The isolated domains will be useful tools to investigate the kinetics of the subdomain folding pathway in yeast PGK.

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Registry No. Phosphoglycerate kinase, 9001-83-6.

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Unfolding Behavior of Human α_1-Acid Glycoprotein Is Compatible with a Loosely Folded Region in Its Polypeptide Chain†

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ABSTRACT: The unfolding of human plasma α_1-acid glycoprotein (AGP) induced by heat or guanidine hydrochloride was studied under equilibrium conditions. In thermal unfolding, an intermediate state was detected by the appearance of unusual positive difference absorption bands in the 287-295-nm region, which occurred at lower temperatures than the common denaturation bands at 284 and 291 nm. The formation of this intermediate species apparently involves a local conformational change that perturbs the environment of tryptophyl residues, without affecting the secondary structure of the protein as judged from circular dichroism spectra. On the other hand, denaturation of the glycoprotein induced by guanidine hydrochloride seemed to follow a two-state model with no evidence of any intermediate species; however, the analysis of the transition curve indicated that the change in the accessibility to solvent of amino acid residues of AGP upon unfolding is significantly lower than those observed for other proteins. According to these results, it is proposed that part of the polypeptide chain in native AGP, namely, that from residue 122 to the C-terminus, may be "loosely" folded.

Many small proteins undergo unfolding transitions which closely follow a two-state mechanism under equilibrium conditions (Kim & Baldwin, 1982; Privalov, 1979). The thermodynamic study of these transitions has firmly established that the native state of proteins is only marginally stable; the Gibbs free energy change for the unfolding process, ΔG_U, is typically in the range of 20-60 kJ mol^{-1} under physiological conditions (Privalov, 1979; Pace 1975). This low value of ΔG_U results from a delicate balance between the changes in enthalpy, ΔH_U, and entropy, ΔS_U, that occur during protein denaturation. Both ΔH_U and ΔS_U are strongly temperature dependent due to the difference in heat capacity of denatured and native states, ΔC_{p_U}. It has been shown (Privalov, 1979; Privalov & Gill, 1988) that ΔH_U extrapolated to 110 °C is around 54 J g^{-1} for most globular proteins; however, some proteins suspected to be "loosely" folded display smaller ΔH values. Then, it seems that the unfolding thermodynamics of a protein reflects some features of the structural organization of the macromolecule.

In this work, we studied the unfolding of human α_1-acid glycoprotein (AGP)[1] induced by heat or guanidine hydrochloride (GuHCl). This plasma glycoprotein has a protein moiety with a molecular mass of 21 536 Da (Dente et al., 1985), and about 40% of its total mass is carbohydrate (Schmid, 1975). Although the physiological role of AGP is not clear at present, it has been found that this protein binds steroid hormones (Westphal, 1971) and a number of basic drugs (DeLeve & Piafsky, 1981). On the other hand, apart from an estimated content of secondary structures in the protein moiety (Aubert & Loucheux-Lefebvre, 1976), very little is known on the structure of this conjugated protein. The results presented in this paper indicate that human AGP is less stable than other common globular proteins; this lower stability seems to be attributable to a low degree of

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[1] Abbreviations: AGP, α_1-acid glycoprotein; GuHCl, guanidine hydrochloride; CD, circular dichroism.

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