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Kinetic studies of the refolding of yeast phosphoglycerate kinase: Comparison with the isolated engineered domains

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Abstract

Unfolding and refolding kinetics of yeast phosphoglycerate kinase were studied by following the time-dependent changes of two signals: the ellipticity at 218 nm and 222 nm, and the fluorescence emission at 330 nm (following excitation at 295 nm). The protein is composed of two similar-sized structural domains. Each domain has been produced by recombinant DNA techniques. It has been previously demonstrated that the engineered isolated domains are able to fold into a quasinative structure (Minard, P., et al., 1989b, *Protein Eng. 3*, 55-60; Missiakas, D., Betton, J.M., Minard, P., & Yon, J.M., 1990, *Biochemistry 29*, 8683-8689). The behavior of the isolated domains was studied using the same two conformational probes as for the whole enzyme. We found that the refolding kinetics of each domain are multiphasic. In the whole protein, domain folding and pairing appeared to be simultaneous events. However, it was found that some refolding steps occurring during the refolding of the isolated C-domain are masked during the refolding of yeast phosphoglycerate kinase. The N-domain was also found to refold faster when it was isolated than when integrated.

Keywords: domains; folding; kinetics; phosphoglycerate kinase

The folding mechanism of proteins is a highly cooperative process, at least for small proteins. Only the native and unfolded forms are stable under equilibrium conditions. Protein folding intermediates are only transiently populated and, as a consequence, very little is known about their structures (Kim & Baldwin, 1990).

Many experimental approaches have been developed to characterize the structural nature of intermediates, e.g., stopped-flow circular dichroism (CD) (Labhart, 1986) or amide proton labeling coupled to two-dimensional NMR (Roder et al., 1988; Udgaonkar & Baldwin, 1990). The existence of slow steps during the folding process has been demonstrated in some particular cases. A cis-trans isomerization of certain prolines has been demonstrated to occur in the unfolded state (Brandts et al., 1975). It follows that the unfolded population is heterogeneous, and two different pathways for folding have to be taken into account. The formation of disulfide bonds has also been used to characterize intermediates occurring during the slow step of folding (Ristow & Wetlaufer, 1973; Creighton, 1974; Weissman & Kim, 1991).

Multidomain proteins frequently displayed multiphasic transitions, suggesting that intermediates are stable under equilibrium conditions. This might be related to a difference in stability among the domains of the protein and also by the importance of the interdomain interactions. Domains in proteins have been defined as structural units sometimes bearing a functional site (Rossmann & Liljas, 1974; Rose, 1979; Wodak & Janin, 1980). It was proposed by Wetlaufer (1973) that domains are independent folding units. Based on this idea, many approaches, such as chemical cleavage or limited proteolysis, have been used to isolate putative protein domains as reviewed by Wetlaufer (1981), Ghélis and Yon (1982), and Jaenicke (1987, 1991). In many cases, protein fragments were found to be stable entities once isolated in vitro, which were either able to reassociate or to retain a quasi-native-

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like three-dimensional structure. Some of these protein fragments did not correlate with the structural and functional domains of the uncleaved protein. Moreover, very little is known about the kinetic behavior of the isolated fragments during refolding. A well-documented example is provided by the β_2 subunit of tryptophan synthase and its proteolytic fragments (Blond & Goldberg, 1986; Blond-Elguindi & Goldberg, 1990); however, the F1 and F2 proteolytic fragments do not correspond to the crystallographic domains.

We have focused our interest on this last aspect of protein folding. For this purpose we have chosen a well-suited protein: yeast phosphoglycerate kinase (yPGK). The protein is folded into two domains as determined by X-ray crystallography (Watson et al., 1982). We also have used a new experimental approach to produce protein fragments corresponding to the structural domains of yPGK – site-directed mutagenesis. The engineered N-domain is composed of the first 185 amino acids of the protein, and the engineered C-domain is composed of the last 230 amino acids from amino acid 186 to 415 (Fig. 1; Kinemages 1, 2). Hence, there are no overlapping sequences between the two domains.

We have shown in a previous study that the engineered isolated domains could be overproduced in yeast and purified to homogeneity (Minard et al., 1989a). The isolated domains reached a quasi-native structure after in vivo folding (Fairbrother et al., 1989; Minard et al., 1989a), but they did not reassociate, either to give an active enzyme or a structurally complemented protein. They behave as totally independent folding units. We have also studied the conformational transitions of the domains and compared them to those of the whole enzyme. It has been shown that after total denaturation, each domain is able to refold in vitro into its initial structure independently of further interactions. Each isolated domain was found to have similar stability (Missiakas et al., 1990).

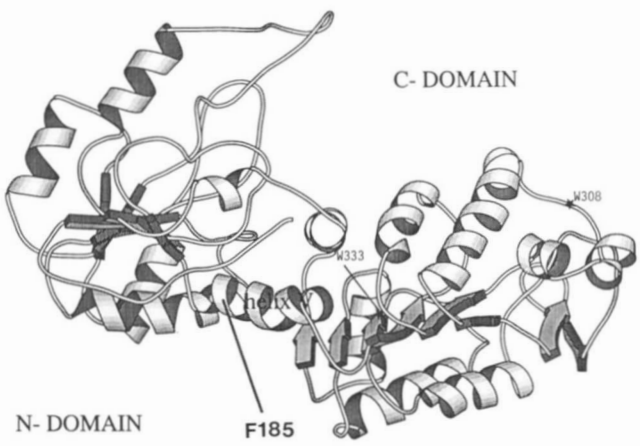

Fig. 1. X-ray structure of yeast phosphoglycerate kinase from Watson et al. (1982). F185, the site chosen as the limit between the two domains, is indicated.

In the present work, we studied the kinetics of folding of the isolated engineered domains and compared them to those of the whole enzyme. We tried to determine if the folding of each domain follows a succession of distinct steps. The refolding of the enzyme occurs in several steps. By comparing the results obtained with yPGK to those obtained for the two isolated domains, we determined first whether the domains refold following the same pathway when isolated compared to when they are integrated, and second if the slow refolding step of yPGK involves the structural formation of one domain, or an arrangement between two domains that are already folded.

The unfolding and refolding kinetics were studied using two methods, CD and tryptophan fluorescence. The slow-folding rates were readily determined by manual mixing methods. Mechanical rapid mixing techniques were used to follow the fast conformational changes occurring during refolding of yPGK.

Results

Kinetics of unfolding and refolding as assessed by CD

Unfolding–refolding of yPGK

A monophasic process was observed for the unfolding of yPGK, whereas the refolding kinetics consisted of two phases: a rapid phase that was not detectable by manual mixing, followed by a slow phase. The macroscopic rate constant value of unfolding, λ_u, increased with the concentration of denaturant. However, the variation of the macroscopic rate constant value, λ_r, of the slow refolding phase with respect to guanidinium chloride (Gdn.HCl) concentration was complex (Fig. 2A). The λ_r values increased between 0.05 and 0.20 M Gdn.HCl, decreased between 0.20 and 0.70 M Gdn.HCl, and finally increased above this last concentration. For these latter concentrations, the rate constant values of the slow refolding phase were found to be similar to those corresponding to the unique unfolding phase.

With the goal of investigating the plausibility of proline isomerization as a slow step in the refolding of yPGK, double-jump experiments, similar to those described by Brandts et al. (1975), were performed. Neither the apparent macroscopic rate constant nor the amplitude of refolding was found to depend on the time of incubation.

The fast refolding step was also studied for yPGK by performing fast mixing with a stopped-flow apparatus, using the same conditions as described above. Figure 3 shows the kinetic progress curve measured by CD at 222 nm. The kinetic process was analyzed by the sum of three exponential terms; however, one phase was unobservable, as it was achieved during the 4.5-ms dead-time of the experiment. The kinetic data obtained for the slow

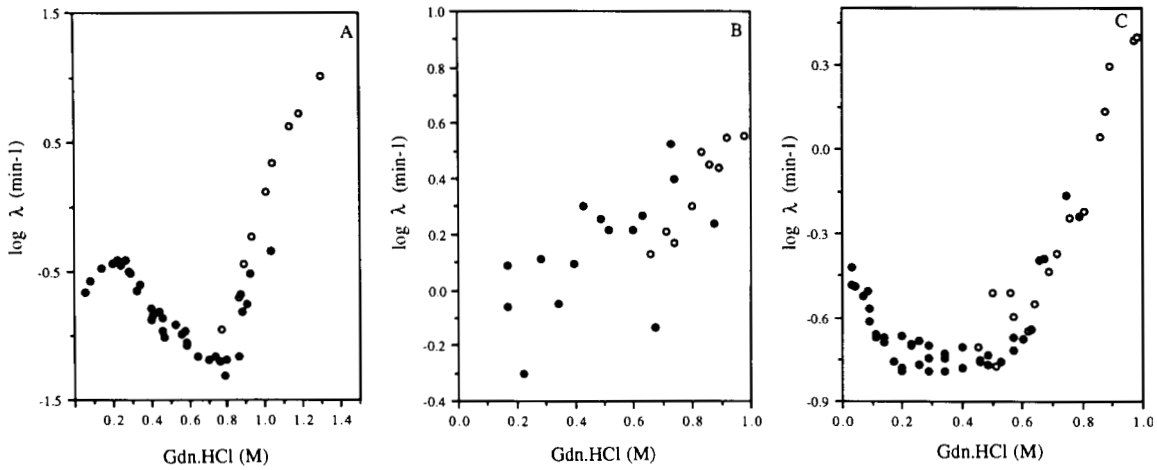

Fig. 2. Logarithmic variations of the macroscopic rate constant values of unfolding, λ_u (○), and of the slow refolding phase, λ_r (●), as a function of Gdn.HCl concentration, **(A)** for yeast phosphoglycerate kinase, **(B)** for the isolated N-domain, and **(C)** for the isolated C-domain. The kinetics were performed after manual mixing and assessed by CD at 218 nm, at 20 °C and pH 7.5.

refolding phase by performing a manual mixing or a mechanical rapid mixing are shown in Table 1. In Table 1, only the macroscopic rate constants are given; the amplitudes obtained from the rapid mixing experiments were not accurate enough, but the λ values thus obtained were significant.

Unfolding–refolding of the N-domain

The unfolding kinetics of the N-domain were also monophasic and analyzed by a single exponential term. The macroscopic rate constant values of unfolding, λ_u, were decreasingly shifted when comparing the N-domain to yPGK (Fig. 2B). As for yPGK, the kinetics of refolding were found to be biphasic, the fast step being completed during the 15-s dead-time of mixing. The variation of the macroscopic rate constant value of the slow refolding phase, λ_r, with respect to Gdn.HCl concentration is presented in Figure 2B. The λ values increased with increasing Gdn.HCl concentration. This corresponded to the unique unfolding phase. For total renaturation, 0.08 M Gdn.HCl, the slow refolding phase accounted for only 15% of the total signal, i.e., 85% of the refolding process was achieved during the dead-time of mixing. Furthermore, the normalized amplitudes extrapolated to $t \rightarrow \infty$ (data not shown) were superimposable with the CD transition curve determined under equilibrium conditions (Missiakas et al., 1990). Thus, no aggregation of the N-domain occurred during the experiment.

Table 1. *Renaturation followed by CD and fluorescence, at 20 °C and pH 7.5*[a]

Technique	Phase	λ_r min^{-1}
Yeast phosphoglycerate kinase		
Stopped-flow CD dead-time 4.2 ms	1	Undetermined
	2	49 ± 6
	3	0.71 ± 0.06
	4	0.15 ± 0.006
Manual mixing CD dead-time 15 s	Unique one recorded	0.228 ± 0.01 (70%)
Fluorescence	Unique one recorded	0.23 ± 0.01
C-domain		
Manual mixing CD	1	3.33 ± 1.00
	2	0.35 ± 0.03 (34%)
Fluorescence	Unique one recorded	0.35 ± 0.03
N-domain		
Manual mixing CD	Unique one recorded	0.61 ± 0.10 (15%)

[a] All experiments were conducted under the same final conditions in a final concentration of 0.05 M guanidinium chloride. The amplitudes when determined are indicated in parentheses.

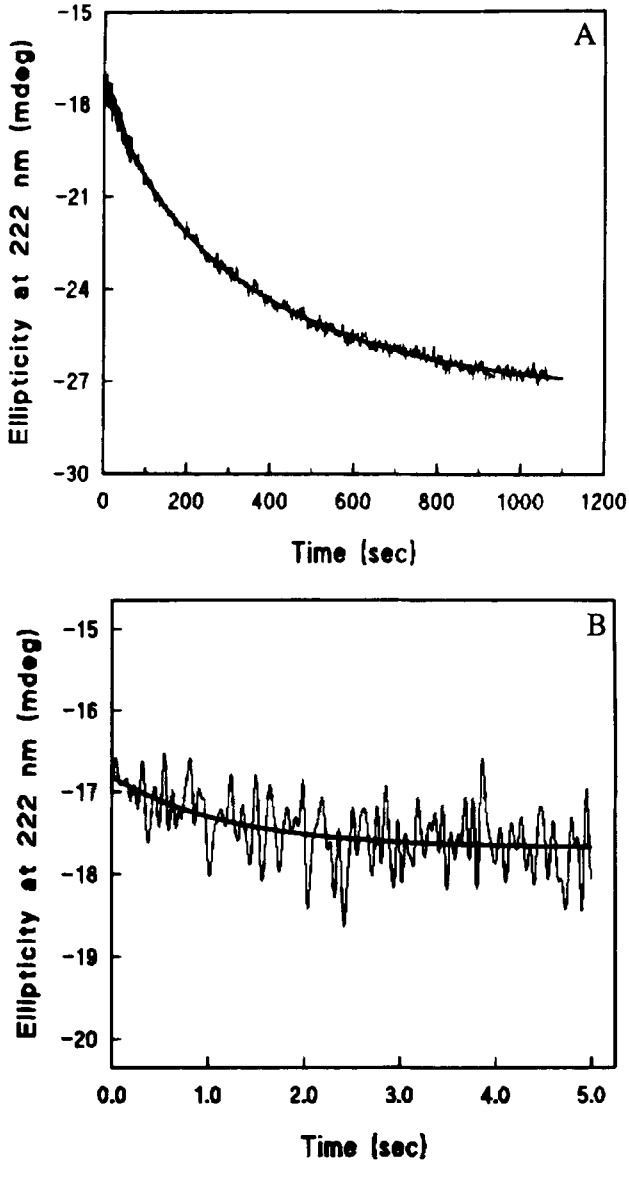

Fig. 3. Kinetics of refolding of yeast phosphoglycerate kinase followed by stopped-flow CD at 222 nm, 20 °C, and pH 7.5. The refolding was initiated by a concentration jump of Gdn.HCl from 6 M to 0.076 M, with a stopped-flow apparatus. The dead-time of the experiment was 4.2 ms. **A**: This kinetic progress curve represents the average of four accumulated kinetics, each recorded in the same conditions during 1,065 s. **B**: This curve represents the average of 40 runs, each recorded in the same conditions during the first 5 s of refolding.

Unfolding-refolding of the C-domain

As for yPGK and the N-domain, the unfolding kinetics were described by a monophasic process. The λ_u values are included between those of yPGK and the N-domain (Fig. 2C). The refolding kinetics were found to be biphasic for denaturant concentration higher than 0.2 M Gdn.HCl; below 0.2 M Gdn.HCl, three phases were observed. Thus, depending on the range of denaturant concentrations used, the slow phase presented different characteristics:

1. Between 1 and 0.20 M residual Gdn.HCl concentration, a unique slow phase was recorded. For 0.20 M final denaturant concentration, the λ_r value was 0.185 ± 0.005 min^{-1} with an associated amplitude of 55%.
2. Below 0.20 M residual Gdn.HCl concentration (0.20–0.03 M), the refolding process was found to be triphasic, with a very rapid phase, an intermediate, and slower phases. For total renaturation, 0.08 M residual Gdn.HCl, the amplitude of the slowest step accounted for 34% of the total signal, and the macroscopic rate constant value, λ_r, was 0.30 ± 0.01 min^{-1}. The rate constant values, λ_r, of the slowest step are reported in Figure 2C. They correspond to those of the unique phase of unfolding. The intermediate refolding phase was too fast to determine accurately the amplitude and rate constant values by the manual mixing technique.

We have also tested the possibility of a heterogeneous denatured state for yPGK by performing a double-jump experiment. The domain was incubated for various periods in 5 M Gdn.HCl, and the refolding kinetics were studied as a function of the time of incubation in 5 M Gdn.HCl. The amplitude of the slower refolding phase was found to depend on the time of incubation (Fig. 4). The rate constant of this phenomenon was about 0.08 min^{-1}.

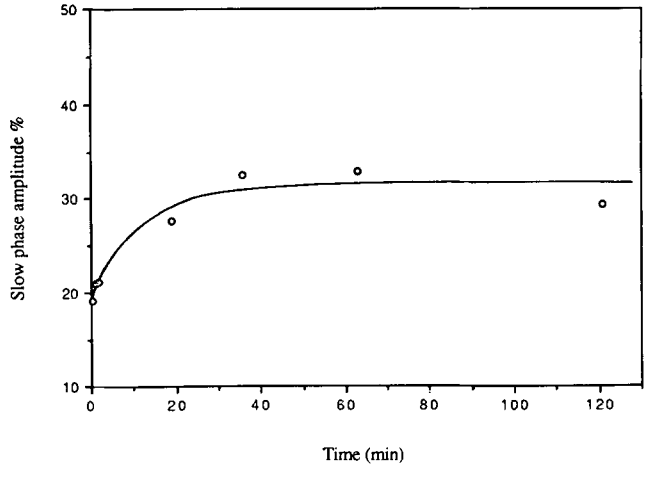

Fig. 4. Double-jump experiment performed with the isolated C-domain. Variation of the amplitude of the slower refolding phase as a function of the time of incubation in 5 M Gdn.HCl. The kinetics were followed after a 1:100 dilution of the denatured protein and assessed by CD at 218 nm, 20 °C, and pH 7.5. The manual method of mixing was used. The kinetics were found to be triphasic. The amplitude associated with the slower phase is reported on the figure.

Kinetics followed by the emission of tryptophan residues

The two tryptophan residues contained in yPGK are localized in the C-domain; Trp 308 is situated in a β-turn between the segment βK and helix αX, and Trp 333 is buried at the end of the βL segment (Fig. 1; Kinemage 3).

We have previously studied the unfolding-refolding transition of yPGK and the C-domain by following the fluorescence of the tryptophan residues. Their fluorescence changed markedly during unfolding. In both cases, an increase in the fluorescence intensity was observed followed by a decrease in this fluorescence, which was accompanied by a shift of the maximal emitted light from 330 nm to 345 nm. The first phenomenon has been attributed to the removal of a quenching amino acid from near Trp 308 (Nojima et al., 1976; Missiakas et al., 1990). These results have shown the existence of a hyperfluorescent intermediate for both isolated and integrated domains.

The kinetics of refolding recorded at 333 nm following excitation at 295 nm, for both yPGK and the C-domain, showed the process to be at least biphasic. The first phase was too fast to be analyzed by manual mixing. However, this phase was detected because its amplitude reached an intensity value higher than those recorded for the native and the denatured forms of the C-domain, or of the whole protein. During the slow refolding phase, the fluorescence emission intensity decreased. This phase was analyzed by a single exponential term. Figure 5 indicates the variation in the macroscopic rate constant value with respect to the concentration of denaturant. The values of this constant are indistinguishable from the values of the macroscopic rate constant measured by CD. Both for yPGK and the C-domain, the fluorescence and the CD signals led to the detection of the same slow phase.

We tried to determine the fluorescence emission spectra of the intermediates of yPGK and the isolated C-domain. Refolding runs were carried out in a final concentration of denaturant of 0.05 M Gdn.HCl, and followed at various emission wavelengths after excitation at 295 nm. The amplitude of the slow phase was extrapolated at $t = 0$ for each wavelength by using the following relationship:

$$y = A_1 + A_2 e^{-\lambda t}.$$

The results are shown in Figure 6. In both cases, the maximum emission wavelength spectra of the intermediates were the same as for the spectra of the unfolded molecule, i.e., 345 nm. The fluorescence intensity was higher for both intermediate spectra than for the unfolded or native molecule spectra.

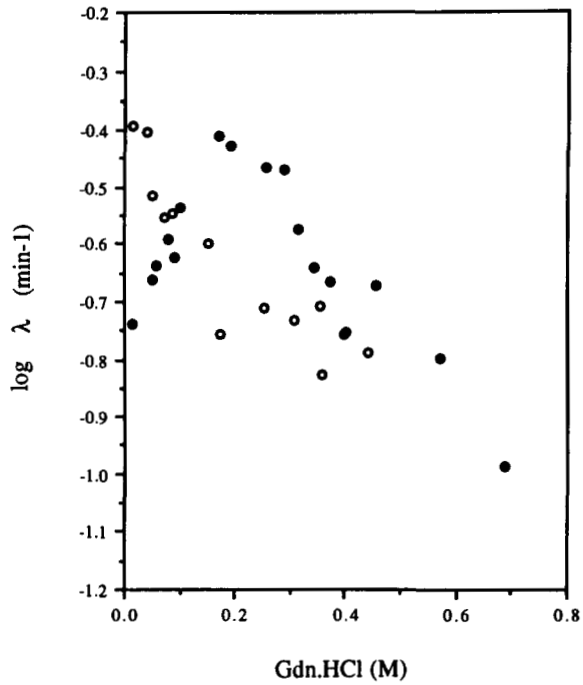

Fig. 5. Plots of the macroscopic rate constant values of the slow refolding phase, λ_r, as a function of Gdn.HCl concentration as assessed by fluorescence measurements of the emitted intensity at 330 nm after excitation at 295 nm. The kinetics were performed by manual mixing at 20 °C and pH 7.5. Values obtained for yeast phosphoglycerate kinase (●); values obtained for the C-domain (○).

Discussion

The results obtained studying the refolding kinetics of the engineered isolated domains of yPGK showed that their folding is a complex process. The kinetics monitored by CD showed the existence of at least one folding intermediate appearing after a fast phase. The tryptophan residues did not recover their native environment until the end of the slow step of folding, neither for the C-domain, nor for yPGK. The use of a stopped-flow technique for performing a rapid mixing allowed the detection of a minimum number of folding steps for yPGK.

The refolding kinetics of the isolated N-domain followed by CD showed two phases. Refolding of the isolated domain was a very rapid event. In the absence of interactions with the C-domain, this domain refolds faster than when it is integrated in the whole enzyme, and this was true regardless of the residual concentration of denaturant. The simplest scheme accounting for the results is the following:

$$U_N \leftrightarrow X_N \leftrightarrow F_N,$$

U_N, X_N, and F_N being, respectively, the unfolded, intermediate form, and folded N-domain.

The refolding process of the isolated C-domain was more complex. A rapid phase of refolding was completely achieved during the dead-time of mixing. Then, the refolding process was described by a phenomenological equation consisting of the sum of two exponential terms.

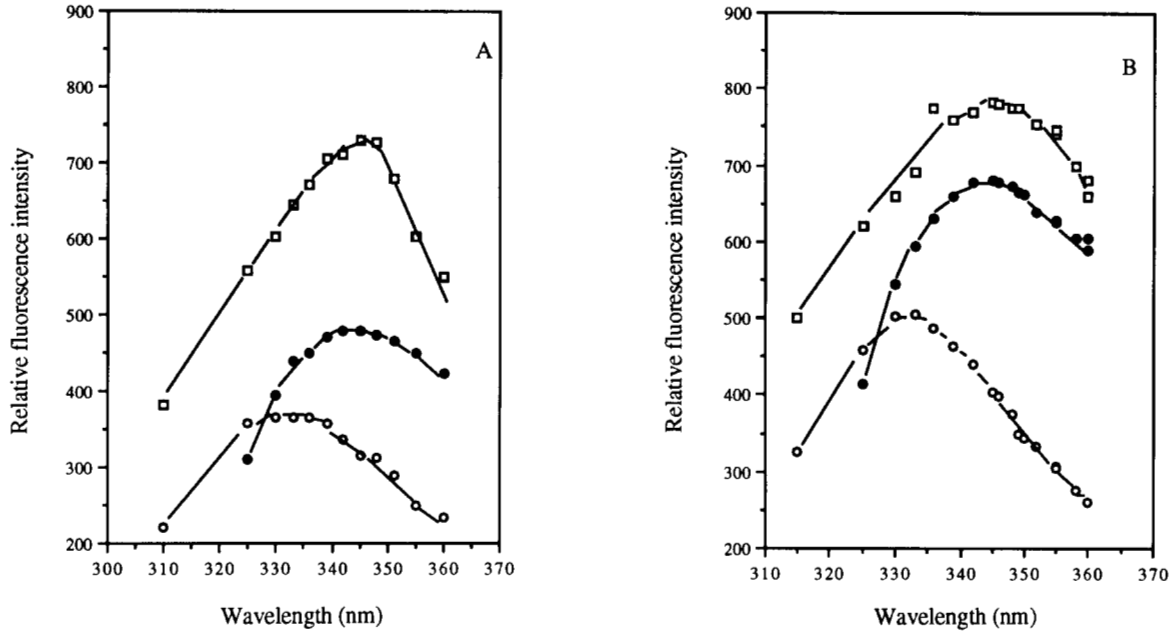

Fig. 6. Fluorescence emission spectra of the intermediate accumulated during the fast phase of refolding (**A**) for yeast phosphoglycerate kinase (yPGK) and (**B**) for the isolated C-domain. Yeast PGK and the isolated domain were first denatured in 6 M Gdn.HCl and then diluted to a final denaturant concentration of 0.05 M. All experiments were performed at 20 °C and pH 7.5 after excitation at 295 nm. ●, Fluorescence intensity of the denatured protein; ○, fluorescence intensity of the renatured protein, extrapolation at $t \rightarrow \infty$; □, fluorescence intensity of the intermediate form, extrapolation to zero time of the slow phase.

The intermediate phase was observed only for runs performed with residual concentrations of Gdn.HCl lower than 0.2 M. This last result might reflect a decrease in the intermediate macroscopic rate constant value with lower denaturant concentrations while the overall process of refolding becomes faster. Such a phenomenon could be the consequence of a slow equilibrium between two unfolded states, implying a cis-trans proline isomerization. This hypothesis was tested by performing a double-jump experiment according to Brandts et al. (1975). The results indicated that the amplitude of the slow refolding phase depended on the time of incubation with the denaturant. In fact, this suggests the potential existence of two unfolded forms in slow exchange. Because the amplitude of this slow refolding phase corresponded to 20% of the total amplitude even for short unfolding times (Fig. 4), this phase did not totally result from an equilibrium between two unfolded forms. No further experimental evidence was obtained concerning the state of folding of these forms or concerning the direct implication of a cis-trans isomerization of proline residues. Two possible schemes might be proposed to describe the folding of the isolated C domain, either: $U_C \leftrightarrow X'_C \leftrightarrow X''_C \leftrightarrow F_C$ or

$$\begin{array}{c} U_C \leftrightarrow X'_C \leftrightarrow F_C \\ \updownarrow \\ X''_C, \end{array}$$

U_C, F_C, X′, and X″ being the unfolded and folded C-domain and two intermediate species with the tryptophans in a nonnative environment, but different from their environment in the unfolded form, respectively.

The same double-jump experiment carried out with the whole enzyme led to a totally different result. Neither the amplitude nor the macroscopic rate constant values of the kinetics were found to depend on the time of incubation with the denaturant. Thus, in the folding pathway of the isolated C-domain, a step that was not detected during the refolding of the whole molecule was observed.

In this study, the fluorescence experiments have shown that the tryptophan residues acquired their native form only after the slow refolding phase for both yPGK and the C-domain. The intermediate of the isolated domain formed during the fast folding phase has the same spectroscopic characteristics as the equivalent intermediate of yPGK. This indicates that the refolding pathways for both molecules are rather similar. The integrated domain might behave as the isolated one during refolding; however, one of the folding steps is masked within the whole molecule. It has been shown for octopine dehydrogenase that during refolding, one step that may correspond to proline isomerization is masked by the slow pairing step occurring between the domains of the protein (Teschner et al., 1987). Finally, the macroscopic rate constant values obtained in the present study from measurements of the fluorescence intensity were identical to those obtained by CD

using the same final conditions. The same slow folding phase was recorded independently of the conformational probes used: the variations in ellipticity at 218 nm, which reflect the recovery of the secondary structure, and fluorescence measurements, which reflect local changes in the tertiary structure.

The refolding kinetics of the whole protein as assessed by manual mixing displayed biphasicity. As for the isolated C-domain, the refolding kinetics followed either by fluorescence measurements or by CD displayed the same biphasic rates. The macroscopic rate constants found by both methods were indistinguishable with respect to Gdn.HCl concentration. However, contrary to what was observed for the engineered C-domain, no slow equilibrium depending on the incubation time in the denaturant was found during the refolding of the whole protein. Because the slow refolding phase accounted for about 70% of the CD signal at 218 nm, the situation might be somewhere between two extreme possibilities:

1. Either, during the fast phase, 100% of a 30% structured intermediate X accumulated in solution.
2. Or, during the fast phase, the protein reached an equilibrium between two states in which 70% of the population was totally unfolded (U) and 30% was an intermediate state (X) containing almost all of the folded secondary structure.

It is more likely that this last interpretation reflects the real situation, as it seems doubtful that during the slow relaxation refolding time a partially structured intermediate remains stable in solution. Similar data and several arguments obtained in previous works are in agreement with this assumption. Indeed, on the one hand, Betton et al. (1992), using a limited proteolysis approach, have demonstrated that, during the slow refolding phase, only a few proteolyzable sites remained accessible in yPGK. Most of these sites are situated near the interdomain region; others were mapped within both domains. On the other hand the same conclusion has been obtained from the reactivity of a unique cysteine residue introduced by site-directed mutagenesis at different positions in several mutants of yPGK (Ballery, 1991). These results are in agreement with the present study because they showed that neither domain folding constituted a rate-limiting step.

It should be noted that aromatic residues could account for a part of the protein CD signal recorded at 218 nm. So the slow refolding phase recorded might simply reflect the reorganization of the local environment of aromatic residues rather than a net acquisition of secondary structure. This hypothesis was rejected because the aromatic residues of the whole protein in the near UV region displayed a very weak signal (data not shown).

As viewed in Figure 2A, the macroscopic rate constant value decreased for residual denaturant concentrations between 0.20 and 0.03 M Gdn.HCl. Such a phenomenon was not observed with the isolated engineered domains. Two interpretations might be proposed: (1) a change in the rate-limiting step of refolding occurred under these conditions, suggesting the existence at least of a folding intermediate different from the one already found, the rate constant of folding from this intermediate decreasing with decreasing concentrations of Gdn.HCl. (2) Some off-pathway intermediates of the whole protein might be formed under certain refolding conditions, which slow down the total renaturation process of yPGK. In this last case, a polymorphism of a renatured or partially renatured state might be assumed (Jaenicke, 1987). Proline isomerization appears not to be involved in such a process, as no effect of denaturation time was recorded when double-jump experiments were performed with the whole enzyme.

It appeared that the folding process of each isolated domain was complex. The isolated domains refolded more rapidly than the integrated domains. This suggests that some rearrangements might occur between both integrated domains upon interdomain interactions. Indeed, the tryptophan environment was found to be slightly different in the isolated C-domain, because the fluorescence emission intensity at 333 nm has been found to be 1.3-fold higher than in yPGK (Missiakas et al., 1990). Similarly, Cys 97 has been found to be more accessible to reagents in the isolated N-domain than in the whole enzyme. However, these events were found to occur within the same slow phase when the folding pathway of yPGK was examined. Therefore, according to our results, folding and domain pairing in the whole protein are simultaneous events. Because the isolated domains did not associate, it was not possible to explore the rearrangements occurring upon domain association or to describe the corresponding isomerization steps.

The refolding intermediate characterized for the whole protein is probably highly structured based on CD determinations. But, is it reasonable to assume that this intermediate is in rapid exchange with the unfolded species as proposed above? It seems more probable that the denatured state rapidly reached a more stable and intermediate conformation in a solution containing only a residual concentration of denaturant. In fact, the kinetic results obtained by using the rapid mixing technique argue in favor of this hypothesis. Also, the analysis of the fast refolding phase of yPGK by a stopped-flow technique coupled with CD detection indicated that the protein refolding is more complex than a biphasic process. Indeed, the kinetics for a total renaturation were found to be tetraphasic. The initial step was too fast to be recorded even by this technique of mixing (dead-time of 4.2 ms). The simplest scheme accounting for these results is a pathway consisting of at least three different intermediates. However, no simulation was done to determine the sequence of formation of these species. The intermediate X, which generates the native protein, probably contains a high degree of secondary structure in both domains. It was well

demonstrated that within X, the two tryptophan residues did not possess their native local environment either in the integrated domain or in the isolated domain. The intermediate X, which generates the native protein, is probably not equilibrating with the denatured form, but more probably with another folding intermediate. During the slow refolding phase, this equilibrium is slowly shifted to the native protein.

To summarize, the refolding of the domains within yPGK appeared to be a rather simultaneous process. Part of the secondary structure is rapidly achieved independently in both integrated domains. The isolated N-domain refolded faster than when integrated. The refolding pathway of the isolated C-domain includes a step that is completely masked by a slower rate-limiting step within the whole protein. In the whole protein, relatively slow events implying both domain folding and the interdomain interactions occur simultaneously. The molecular rearrangements arising upon domain pairing were not directly observed, neither in yPGK, nor in the isolated domains since they did not reassociate. Now, to observe the interdomain rearrangements occurring upon possible complementation, it would be of great interest to study the refolding of fragments larger than the structural domains. Such an approach is now under investigation in our laboratory.

Materials and methods

Recombinant yPGK (EC 2.7.2.3) was prepared as described by Minard et al. (1989b). The purifications of the isolated domains have also been reported previously (Minard et al., 1989a). Ultrapure Gdn.HCl was obtained from Pierce.

Unfolding-refolding kinetics

The kinetic processes were followed at 20 °C, using two different signals: (1) the ellipticity at 218 nm, either for yPGK or the isolated domains; (2) the fluorescence intensity at 345 nm following excitation at 295 nm, compared between the C-domain and yPGK.

Experiments using the CD signal were performed in 10 mM phosphate buffer, pH 7.50, containing 0.5 mM EDTA and 1 mM dithiothreitol (DTT) (except for the C-domain, which has no cysteinyl residue). Fluorescence measurements were determined using 50 mM Tris-HCl buffer, pH 7.50, containing 0.5 mM EDTA and 1 mM DTT (except for the C-domain).

The unfolding kinetics were initiated by adding at zero time, 100 μL of native protein to a 1.90-mL assay mixture. Then, 100 μL of 5 M Gdn.HCl-denatured protein were added to initiate the refolding runs. The final concentration of protein was 3.5 μM. Final Gdn.HCl concentrations ranging from 0.03 M to 5 M were tested during kinetic experiments. The mixing of the solutions was achieved by using a magnetic stirrer positioned under the cell holder. The mixing dead-time was about 15 s. Kinetics were followed until equilibrium was reached. The CD kinetics were run on a Mark V dichrograph (Jobin & Yvon) using a 10-mm light path cell. The fluorescence kinetics were performed with a Perkin-Elmer fluorimeter (MPF 44B).

The kinetics were analyzed by a multilinear regression program according to Press et al. (1986), adapted to a Hewlett-Packard 9816 calculator, taking into account either one or two exponential terms.

Double-jump experiments

To determine whether or not the slow step of folding was due to proline isomerization, a double-jump experiment, as described by Brandts et al. (1975), was performed for yPGK and the C-domain. The time of denaturation of the proteins in 5 M Gdn.HCl varied from 0.5 to 120 min. After that, the ellipticity at 218 nm was recorded to follow the kinetics of renaturation as described below (final concentration for proteins 3.5 μM, for Gdn.HCl 0.05 M).

Stopped-flow experiments

The renaturation of yPGK was performed at 20 °C using the BIO-logic stopped-flow SFM-3. The mechanical subsystem consists of three syringes, two mixers, and an observation chamber. The whole system is enclosed in a water jacket to allow temperature regulation of the reactant containers. The SFM-3 syringes are controlled by a BIO-logic MPS-5 microprocessor power supply. Kinetic data were accumulated with a microcomputer.

The kinetics were followed with a CD 6 dichrograph (Jobin & Yvon) at 222 nm, after a 79-fold dilution. The dead-time of mixing was 4.2 ms. Final concentration of yPGK after mixing was 2.8 μM; residual concentration of Gdn.HCl was 0.076 M.

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References

Ballery, N. (1991). Etude de la flexibilité et du repliement de la phosphoglycérate kinase de levure à l'aide de mutants possédant une cystéine. Ph.D. Thesis, Université de Paris-Sud, Orsay.

Betton, J.M., Missiakas, D., & Yon, J.M. (1992). The slow refolding step of phosphoglycerate kinase as monitored by pulse-proteolysis. *Arch. Biochem. Biophys. 296*, 95–101.

Blond, S. & Goldberg, M.E. (1987). Partly native epitopes are already

present on early intermediates in the folding of tryptophan synthase. *Proc. Natl. Acad. Sci. USA 84*, 1147–1151.

Blond-Elguindi, S. & Goldberg, M.E. (1990). Kinetic characterization of early immunoreactive intermediates during the refolding of guanidine-unfolded *Escherichia coli* tryptophan synthase β_2 subunits. *Biochemistry 29*, 2409–2417.

Brandts, J.F., Halvorson, H.R., & Brennan, M. (1975). Consideration of the possibility that the slow refolding step in protein denaturation–renaturation reactions is due to cis–trans isomerism of proline residues. *Biochemistry 14*, 4953–4963.

Creighton, T.E. (1974). The single-disulfide intermediates in the refolding of reduced pancreatic trypsin inhibitor. *J. Mol. Biol. 87*, 603–624.

Fairbrother, W.J., Minard, P., Hall, L., Betton, J.M., Missiakas, D., Yon, J.M., & Williams, R.J.P. (1989). Nuclear magnetic resonance studies of isolated structural domains of yeast phosphoglycerate kinase. *Protein Eng. 3*, 5–11.

Ghélis, C. & Yon, J.M. (1982). Folding and assembly of building blocks. In *Protein Folding*, pp. 448–469. Academic Press, New York.

Jaenicke, R. (1987). Folding and association of proteins. *Prog. Biophys. Mol. Biol. 49*, 117–237.

Jaenicke, R. (1991). Protein folding: Local structures, domains, subunits, and assembly. *Biochemistry 30*, 3147–3161.

Kim, P.S. & Baldwin, R.L. (1990). Intermediates in the folding reactions of small proteins. *Annu. Rev. Biochem. 59*, 631–660.

Labhart, A.M. (1986). Folding intermediates studied by circular dichroism. *Methods Enzymol. 131*, 126–135.

Minard, P., Desmadril, M., Ballery, N., Perahia, D., Mouawad, L., Hall, L., & Yon, J.M. (1989a). Study of the fast-reacting cysteines in phosphoglycerate kinase using chemical modifications and site-directed mutagenesis. *Eur. J. Biochem. 185*, 419–423.

Minard, P., Hall, L., Betton, J.M., Missiakas, D., & Yon, J.M. (1989b). Efficient expression and characterization of isolated structural domains of yeast phosphoglycerate kinase generated by site-directed mutagenesis. *Protein Eng. 3*, 55–60.

Missiakas, D., Betton, J.M., Minard, P., & Yon, J.M. (1990). Unfolding–refolding of the domains in yeast phosphoglycerate kinase: Comparison with the isolated engineered domains. *Biochemistry 29*, 8683–8689.

Nojima, H., Ikai, A., Oshima, T., & Noda, H. (1976). Anomalous fluorescence of yeast 3-phosphoglycerate kinase. *Biochim. Biophys. Acta 427*, 20–27.

Press, W.H., Flannery, B.P., Teukolsky, S.A., & Vetterling, T. (1986). *Numerical Recipes*. Cambridge University Press, New York.

Ristow, S.S. & Wetlaufer, D.B. (1973). Evidence for nucleation in the folding of reduced hen egg lysozyme. *Biochem. Biophys. Res. Commun. 50*, 544–550.

Roder, H., Elöve, G.A., & Englander, S.W. (1988). Structural characterization of folding intermediates in cytochrome c by H-exchange labelling and proton NMR. *Nature 335*, 700–704.

Rose, G.D. (1979). Hierarchic organization of domains in globular proteins. *J. Mol. Biol. 134*, 447–470.

Rossmann, M.G. & Liljas, A. (1974). Recognition of structural domains in globular proteins. *J. Mol. Biol. 85*, 177–181.

Teschner, W., Rudolph, R., & Garel, J.R. (1987). Intermediates on the folding pathway of octopine dehydrogenase from *Pecten jacobaeus*. *Biochemistry 26*, 2791–2796.

Udgaonkar, J.B. & Baldwin, R.L. (1990). Early folding intermediate of ribonuclease A. *Proc. Natl. Acad. Sci. USA 87*, 8197–8201.

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). Sequence and structure of yeast phosphoglycerate kinase. *EMBO J. 1*, 1635–1640.

Weissman, J.S. & Kim, P.S. (1991). Reexamination of the folding pathway of BPTI. Predominance of native intermediates. *Science 253*, 1386–1393.

Wetlaufer, D.B. (1973). Nucleation, rapid folding and globular intrachain regions in proteins. *Proc. Natl. Acad. Sci. USA 70*, 697–701.

Wetlaufer, D.B. (1981). Folding of protein fragments. *Adv. Protein Chem. 34*, 61–92.

Wodak, S. & Janin, J. (1980). Analytical approximation to the accessible surface area of proteins. *Proc. Natl. Acad. Sci. USA 77*, 1736–1740.