Folding and Functional Complementation of Engineered Fragments from Yeast Phosphoglycerate Kinase†

Frédéric Pecorari, Christophe Guilbert, Philippe Minard, Michel Desmadril, and Jeannine M. Yon*

Laboratoire d'Enzymologie physico-chimique et moléculaire, Unité associée du Centre National de la Recherche Scientifique, Université de Paris-sud, 91405, Orsay, France

Received August 18, 1995; Revised Manuscript Received December 22, 1995⊗

ABSTRACT: A set of protein fragments was produced by site-directed mutagenesis followed by chemical cleavage of phosphoglycerate kinase according to a previously described method [Pecorari *et al.* (1993) *Protein Eng. 6*, 313−325]. The cleavage positions were chosen in order to correspond to limits between structural subdomains. These isolated fragments were studied by circular dichroism, folding transitions, and cross-linking analyses. It appears that fragments corresponding to globular subdomains in the protein can recover the expected helix content. However, the cooperativity classically observed in the folding transitions of natural proteins is only observed for fragments larger than a domain. Previous studies have shown that the isolated C-terminal domain is an autonomous folding unit which displays a single cooperative transition [Missiakas *et al.* (1990) *Biochemistry 29*, 8683−8689]. The results presented here show that the presence in a fragment of a sequence overpassing that of the C-terminal domain modifies its folding process. Reassociation experiments suggest that the efficiency of the complementation process is not related to the folding autonomy of the isolated fragments.

Recent technological advances, especially in rapid mixing methods, have provided important insights into the mechanism by which globular proteins fold. Indeed, during the past few years it has been established by stopped-flow circular dichroism that a fraction of the secondary structures appears early in the folding process (Kuwajima *et al.*, 1987). Furthermore, nuclear magnetic resonance (NMR)[1] pulse hydrogen exchange labeling methods (Baldwin & Roder, 1991) have identified native elements of secondary structures which are formed in the first milliseconds (Roder *et al.*, 1988; Bycroft *et al.*, 1990; Baldwin, 1993). In subsequent folding steps, aromatic side chains are buried, and the formation of tertiary interactions, such as the appearance of native epitopes in the tryptophan synthase β_2-subunit (Blond-Elguindi & Goldberg, 1990), is observed. Finally, in a rate-limiting step, late events take place, such as precise docking of side chains (Matouschek *et al.*, 1990), subunit association (Jaenicke, 1987), correct pairing of domains (Lecomte & Matthews, 1993), and rearrangements of some disulfide bridges (Creighton, 1978). These observations support the generally accepted view of a sequential protein folding mechanism (Kim & Baldwin, 1990) where secondary structures are formed at the earliest stages, followed by a collapse to a specific globular state through long-range interactions.

From the existence of distinct structural domains within globular proteins, Wetlaufer (1973, 1981) and Levitt and Chothia (1976) have proposed that domains and subdomains could behave as independent folding units which assemble to form the native molecule. According to this model, some stretches of secondary structures are formed in different parts of the molecule and interact to form subdomains and then domains, which then finally merge to form the native structure.

Based on the assumption that the existence of domains or subdomains reflects the dynamics of the folding process, a classical strategy to study folding intermediates under equilibrium conditions is to study protein fragments. The protein folding being a highly cooperative process, intermediate species are not generally sufficiently populated under equilibrium conditions. In this regard, protein fragments can be useful models of incompletely folded polypeptide chains and can be studied under equilibrium conditions. Further questions can be addressed by the study of the reassociation between complementary fragments: should the cleavage point be located at the limit between two structural units, such as domains or subdomains fragments, in order to give rise to a productive reassociation? Is the ability of the fragments to fold independently a prerequisite to their docking and reassociation? In other words, does protein folding proceed *via* the assembly of preformed structural blocks or does it proceed *via* a structural induction process involving long-range interactions? Experimental answers to these questions are crucial for understanding the folding process.

Fragment studies related to protein folding were reported many years ago [see Wetlaufer (1981), Ghélis and Yon (1982), and Jaenicke (1987, 1991) for reviews]. However, a renewed interest in this subject due to progress in methodology has appeared recently. Indeed, fragments can now be produced by chemical synthesis or by genetic methods and can be chosen from previously reported three-dimensional structures.

† This work was supported by the Centre National de la Recherche Scientifique, l'Université de Paris-Sud, and by a contract with the Centre National de la Transfusion Sanguine. F.P. was supported by a grant from the Société de Secours des Amis des Sciences.

* To whom correspondences should be addressed.

⊗ Abstract published in *Advance ACS Abstracts,* March 1, 1996.

[1] Abbreviations: bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane; BPTI, bovin pancreatic trypsin inhibitor; GdmCl, guanidinium chloride; HPLC, high-pression liquid chromatography; NMR, nuclear magnetic resonance; SDS−PAGE sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; Tris, tris(hydroxymethyl)aminomethane; PGK, phosphoglycerate kinase (EC 2.7.2.3).

0006-2960/96/0435-3465$12.00/0

Although it is clearly established that isolated domains behave as independent and stable folding units and display cooperative unfolding transitions (Minard *et al.*, 1989; Missiakas *et al.*, 1990; Sharma *et al.*, 1990; Harold *et al.*, 1991; Jecht *et al.*, 1994), very few complementation experiments between fragments corresponding exactly to the structural domains from a protein have been reported. This is due to the fact that in many cases the sites cleaved by limited proteolysis correspond to exposed loops rather than to a domain's limit, and also to the difficulty to purify the different fragments required for such experiments. Burbaum and Schimmel (1991) have obtained by genetic methods six pairs of fragments corresponding to the two structural domains of methionyl tRNA synthetase for different junction points. While the domain's limit is between residues 360 and 364, the fragments corresponding to junctions 355, 358, and 363 do not complement, neither *in vitro* nor *in vivo*. In contrast, the fragments corresponding to junctions 367, 371, and 374 complement only when the fragments are refolded together *in vitro*, suggesting that crucial interactions occur between domains during their refolding that cannot take place once domains are refolded.

At a lower level in the protein structure hierarchy, the existence of folding units smaller than domains has been studied, and many studies of protein fragments smaller than a structural domain have been reported. In experiments of isolated fragments from staphylococcal nuclease (Taniuchi & Anfinsen, 1968, 1969, 1971; Taniuchi *et al.*, 1977), complementation occurs during the refolding process. It was suggested that a disordered complex of the overlapping fragments would be an intermediate in the formation of the active enzyme, which further rearranges to produce two alternative species of the functional protein. More recently, studies of fragments from staphylococcal nuclease have shown that the conformation of N-terminal fragments is less organized in shorter fragments than in longer ones (Shortle & Meeker, 1989). Dalzoppo *et al.* (1985) have studied fragments from thermolysin smaller than the C-terminal structural domain. Even the smallest fragments were compactly folded, showed cooperative reversible unfolding transitions, and behaved as autonomous folding units. Fragments smaller than a structural domain have been obtained from the β-subunit of tryptophan synthase (Chaffotte *et al.*, 1991), from SH2 domains of proteins p60 and p85 (Williams *et al.*, 1993), from barnase (Sancho & Fersht, 1992; Kippen *et al.*, 1994), and from the barley chymotrypsin inhibitor 2 (Prat Gay *et al.*, 1994). Although they have a poor structural content when isolated, fragments generate, upon mixing, associated species which have spectroscopic properties close to the native ones. Tasayco and Carey (1992) have obtained two fragments, 8−71 and 72−108, from the tryptophan repressor (108 residues) and peptide 94−106 by chemical synthesis. When isolated, fragment 8−71 had native properties while fragment 72−108 and peptide 94−106 had disordered conformations. However, the two fragments were able to associate and, thus, had an NMR spectrum close to the native one and peptide 94−106 was able to fold when mixed to fragment 8−71. Similar results have been obtained with peptides from cytochrome *c* and from yeast phosphoglycerate kinase that regain their native conformations only upon mixing with a larger fragment (Wu *et al.*, 1993; Ritco *et al.*, 1995).

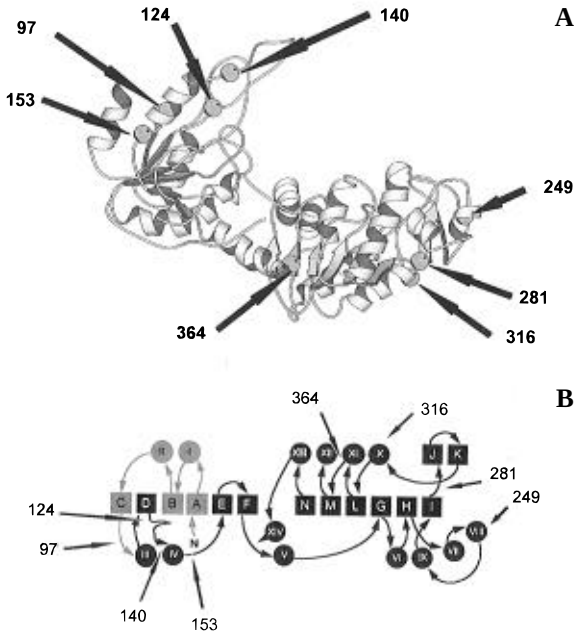

FIGURE 1: Schematic representation of phosphoglycerate kinase made using MOLSCRIPT (Kraulis, 1991) indicating by an arrow the different points of cleavage. (A) Crystallographic structure. (B) Topological scheme of the phosphoglycerate kinase structure. The grayed region of the structure corresponds to fragment 1−96, and shows the topological discontinuity for the pair of fragments 1−96/97−415.

To summarize, there are very few reported examples of complementation experiments between fragments corresponding accurately to structural domains, while there is a large set of experiments suggesting that a productive reassociation can be observed with fragments that do not correspond to structural units or to fragments able to fold independently. From this later observation, the folding process appears more as an induction process rather than as a stepwise and hierarchical association of preformed elements of structure.

Phosphoglycerate kinase is a classical exemple of a two-domain monomeric protein (Figure 1). The three-dimensional structures of horse muscle (Banks *et al.*, 1979), yeast (Watson *et al.*, 1982), pig muscle (Harlos *et al.*, 1992), and *Bacillus stearothermophilus* (Davies *et al.*, 1993) enzymes are very similar. Previous studies have shown that engineered isolated domains of the yeast enzyme recover a quasi-native structure *in vivo* as well as *in vitro* (Minard *et al.*, 1989; Fairbrother *et al.*, 1989). However, they cannot reassociate to generate a functional enzyme. In contrast, two pairs of complementary fragments 1−96/97−415 and 1−248/249−415, have shown a significant functional complementation (Pecorari *et al.*, 1993). These results have suggested that the correct folding of the isolated fragments is not a prerequisite for their complementation. In the present work, several pairs of fragments were produced and studied. They were designed on consideration of the phosphoglycerate kinase structure in order to explore the folding behavior of phosphoglycerate kinase fragments corresponding to subdomains. The other purpose of this study was to analyze the requirements for a pair of fragments to achieve a functional complementation. We produced these fragments by previous reported techniques (Pecorari *et al.*, 1993). A unique cysteine residue was introduced by site-directed mutagenesis at a definite position as a specific site for chemical cleavage

by 5,5′-dithiobis(2-nitrobenzoate)/potassium cyanide. The structure and stability of each fragments were studied, and complementation experiments with the different pairs of fragments were monitored under various conditions.

MATERIALS AND METHODS

Fragment Design, Mutagenesis, and Protein Purification. The positions of cleavage sites into yeast phosphoglycerate kinase (PGK) were chosen at the limits between subdomains in the native structure. The limits were computed with an algorithm adapted from Wodak and Janin (1980). Briefly, in a curve where the interface area between two contiguous fragments is plotted for all the positions in the sequence, the domain's limit appears as a deep minimum. The limits between structural subdomains were chosen as cleavage positions in the present work and appear as secondary minima in this interface area plot. The following mutants of yeast phosphoglycerate kinase (PGK) were obtained from a first mutant in which the unique cysteine residue (Cys97) was replaced by an alanine according to Minard *et al*. (1989): C97A,I124C; C97A,S140C; C97A,S153C; C97A,-V281C; C97A,E316C; C97A,N364C. The wild-type and mutants proteins were overexpressed in yeast. They were purified according to the method previously described by Pecorari *et al*. (1993).

Enzyme Activity. The enzyme activity was measured according to the method published by Büchner (1955), slightly modified by Betton *et al.* (1984). The absorbance was recorded on a Cary 219 spectrophotometer. An extinction coefficient $\epsilon = 0.49$ (mg/mL)/cm was used to determine the protein concentration.

Production and Purification of the Fragments. Except for fragment 1−172 which was obtained by cleavage at the methionine positions by CNBr according to Adams *et al.* (1985), the cleavage at the cysteinyl residue was monitored according to the two-step procedure adapted from Vanaman and Stark (1970) by Pecorari *et al*. (1993). The yield of cleavage being smaller than 100%, uncleaved PGK was present in solution with the fragments.

For mutants C97A,I124C and C97A,N364C, each mutant in solution in a 50 mM Tris-HCl buffer, pH 8, containing 3 M GdmCl, was concentrated on Centriprep-10 and loaded onto a Sephacryl HR100 column equilibrated with a 20 mM phosphate buffer, pH 7.5, containing 3 M GdmCl. The second elution peak corresponded to fragment 1−123 or 364−415. It was not possible to achieve a separation of fragment 1−364 from PGK. Fragment 124−415 was separated from uncleaved PGK by ion exchange chromatography on a S-Sepharose column equilibrated with 20 mM bis-Tris buffer, pH 6.8, containing 5 M urea. Under these conditions, fragment 124−415 was eluted, whereas uncleaved PGK was retained on the column.

Fragments 1−139 and 1−152 were purified on a DEAE-Sepharose column following exchange of the 50 mM Tris-HCl buffer, pH 8, containing 3 M GdmCl by a 20 mM Tris-HCl buffer, pH 8.4, containing 5 M urea. These fragments were eluted and separated from their complementary fragment while PGK was retained on the column. Fragments 140−415 and 153−415 and PGK were further eluted by increasing the ionic strength (0.2 M NaCl). A separation of each of these fragments from PGK was achieved by a chromatography on a S-Sepharose column equilibrated with a 20 mM bis-Tris buffer, containing 5 M urea, adjusted at pH 6.8 for fragments 140−415 and 153−415, and at pH 6.0 for fragment 281−415. Fragment 1−280 was purified on a Q-Sepharose column equilibrated with a 20 mM bis-Tris buffer, pH 7.0, containing 5 M urea. The N-terminal fragment was eluted whereas PGK was retained on the column.

All fragments purified in a buffer containing urea were concentrated and loaded onto a G25 Sephadex column equilibrated with a 10 mM phosphate buffer, pH 7.5, containing 3 M GdmCl. The fractions thus obtained represented stock solutions of the fragments.

To avoid contamination by cyanates (Stark, 1965), urea was deionized on an Amberlite column and then used within 1 day.

The concentration of the purified fragments denatured in 6 M GdmCl was measured by the absorbance, using their molar extinction coefficients calculated according to Stanley *et al.* (1989), at 276 nm for the N-terminal fragments which contain no tryptophan residue, and at 280 nm for the C-terminal fragments which contain two tryptophan residues. They were respectively $\epsilon_{1-123} = \epsilon_{1-139} = \epsilon_{1-152} = 5800\ M^{-1}\ cm^{-1}$, $\epsilon_{1-280} = 8700\ M^{-1}\ cm^{-1}$, $\epsilon_{124-415} = \epsilon_{140-415} = \epsilon_{153-415} = 15\,220\ M^{-1}\ cm^{-1}$, and $\epsilon_{281-415} = 12\,660\ M^{-1}\ cm^{-1}$.

Molecular Weight Determinations. The hydrodynamic properties of the refolded fragments were determined by HPLC gel filtration on a Superose 12 column (flow rate 0.2 mL/min) equilibrated with a 50 mM phosphate buffer, pH 7.5, containing 0.2 M NaCl. The fragments were renatured during 1 h at room temperature in the same buffer before injection. Transferrin, albumin, β-lactalbumin, carbonic anhydrase, and α-lactalbumin were used as standards.

Cross-Linking Experiments. The possible association of the fragments was analyzed by cross-linking with glutaraldehyde according to Herman *et al*. (1979); 1% (v/v) glutaraldehyde was added to each fragment sample and allowed to react for 1 min; then the reaction was stopped by the addition of 2 M $NaBH_4$ in 0.1 M NaOH. When the GdmCl concentration of the cross-linked sample was higher than 0.5 M, the solution was dialyzed overnight at 4 °C against a 10 mM phosphate buffer solution, pH 7.5, to remove the denaturant. Then 500 μL of the protein solution was concentrated by a trichloroacetic acid precipitation (50 μL of TCA, 50% v/v, + 2 μL of deoxycholate, 1% m/v). The pellet was dissolved in an electrophoresis sample buffer and subjected to SDS−PAGE.

Refolding of the Fragments. Reference circular dichroism spectra of fragments, in a 10 mM phosphate buffer, pH 7.5, were recorded at 20 °C with a Jobin & Yvon Mark V dichrograph between 195 and 250 nm. The fragments' final concentrations were 2 μM. The secondary structure content was evaluated from the circular dichroism spectra using the procedure of Chang *et al*. (1978).

The refolding transitions of the fragments were assessed by circular dichroism and fluorescence measurements. The fragments were diluted to different final concentrations of GdmCl ranging from 0.08 M to 5 M, in a 10 mM phosphate buffer, pH 7.5, at a temperature of 20 °C. The denaturant concentrations were determined by refractometry according to Nozaki (1970). The fragments' final concentration was 2 μM. After a 1 h minimum incubation to reach the equilibrium, the spectra were recorded at 20 °C. Due to GdmCl absorbance for the transitions determined by circular

dichroism, the spectra were recorded only between 210 and 250 nm. The structural transitions were determined by the variations in ellipticity at 218 nm.

For the transitions as assessed by fluorescence measurements, the emission fluorescence spectra following excitation at 293 nm were recorded from 280 to 400 nm with a SPEX fluorimeter equipped with a thermostated cell.

Analysis of the Transition Curves. The experimental results were fitted according to the equation derived from the denaturant binding model: $f_U = c^n/(c_m^n + c^n)$. In this equation, f_U is the fraction of unfolded protein, c is the denaturant concentration, n is a cooperativity index, and c_m is the midpoint transition. The linear dependency of the signals upon denaturant concentration for unfolded proteins was taken into account. The complex transition curves obtained were fitted to an equation in which both the base lines and the transition regions of the curve are fitted simultaneously. The transition region was described by a linear combination of one or two single-transition curves.

The transition equilibrium between an unfolded (U) and a folded (F) protein under the two-state approximation is defined as follows:

$$K_U = U/F = f_U/(1 - f_U) = \exp(-\Delta G_U/RT)$$

ΔG_0, the variation of free energy of unfolding in the absence of denaturant, was evaluated by a linear extrapolation according to Pace (1986):

$$\Delta G_U = \Delta G_0 - m(\text{denaturant})$$

m being the dependency of ΔG_U on the denaturant concentration.

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

Complementation Experiments. The functional complementation of fragments was assessed by measuring the enzyme activity. For each stock solution of fragment, the absence of any background level of enzyme activity in the purified fragments was checked before complementation experiments. For each pair of fragments, the kinetics of complementation were monitored under different conditions. Either the two denatured fragments, each at 2 μM final concentration, were mixed to refold in their mutual presence, or they were previously refolded separately before mixing for at least 1 h at 20 °C in a 20 mM phosphate buffer, pH 7.5. In another set of experiments, only one of the two fragments was previously refolded before mixing. The final concentration of each fragment was 2 μM in each case. The kinetics of enzyme activity recovery were analyzed with a nonlinear regression method according to Marquard (Press *et al.*, 1986).

RESULTS

Production and Characterization of the Fragments. Structural building blocks were identified by minima of the interface area between contiguous fragments (Figure 2) according to a method adapted from Wodak and Janin (1980). The different cleavage points are indicated in Figure 1. The cleavage positions lead to fragments that could be putative early folding units according to the modular view of the folding process.

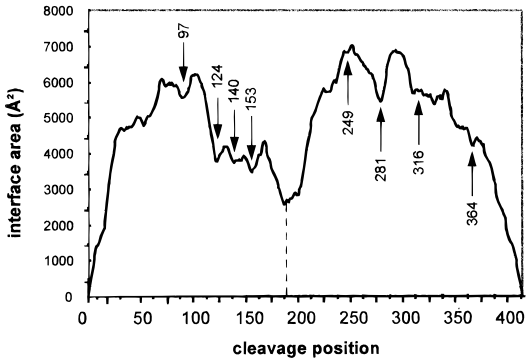

FIGURE 2: Plot of interface area as a function of cleavage position. The vertical dashed line corresponds to domain's limit for phosphoglycerate kinase.

The yield of cleavage was about 70% for most mutants, whereas it was abnormally low, 10 and 0−5% for mutants C97A-V281C and C97A-E316C, respectively. However, the labeling of cysteine residues was nearly 100% for all mutants. A possible explanation of these low cleavage yields could be the presence of a proline adjacent to the cysteine (Pro-Cys) in the sequence of both mutants.

Different fragments were obtained: 1−123, 1−139, 1−152, and 1−280 from the N-terminal part of the sequence, and 124−415, 140−415, 153−415, 281−415, and 364−415 from the C-terminal part. The purifications of the N-terminal fragments were based on the basic character of the PGK N-terminal part, as previously reported (Pecorari *et al.*, 1993). Most of them were obtained by ion exchange chromatography in the presence of urea. It was not possible to achieve a separation of fragment 1−363 from the entire protein, their difference in both size and charge being too small.

For all isolated fragments, a unique band was obtained by SDS−PAGE analysis of the purified fragments, and no measurable enzyme activity was found in the fragment stock solutions. The fragments were also characterized by UV spectroscopy. The spectra of the C-terminal fragments, 124−415, 140−415, 153−415, and 281−415, which contain the two tryptophan residues, showed a maximum at 277 nm and a shoulder at 290 nm (data not shown).

The hydrodynamic properties of the refolded fragments were investigated by HPLC gel filtration. The N-terminal fragments were found to have the expected apparent molecular weight, with the exception of fragment 1−248 as previously reported (Pecorari *et al.*, 1993) and fragment 1−280 whose apparent molecular weight was smaller than expected. According to the circular dichroism spectra, several fragments were found to be poorly structured (see below), but nevertheless were eluted at the elution volume expected for a globular monomeric polypeptide chain. This could be explained by the retention of these fragments on the column. The effect of a possible increase in the Stokes radius might be compensated by an increase in the elution volume due to interactions with the gel. These interactions with the column occur in spite of the salt concentration used in the elution buffer and are probably related to the density of basic residues in the N-domain. Supporting this assumption, fragment 1−139 remained bound to the column and was eluted only by increasing the salt concentration above 0.5 M. In contrast, the apparent molecular weights of the C-terminal fragments were systematically much larger than

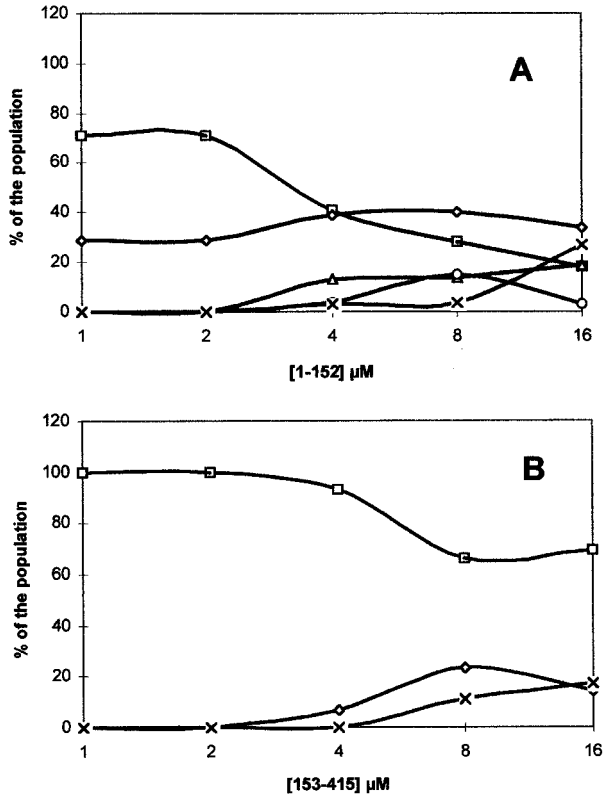

FIGURE 3: Proportion of associated species as a function of fragment concentration determined after analytical densitometry of SDS−PAGE gels following cross-linking (A) for fragment 1−152 and (B) for fragment 153−415. (□) Monomer; (◇) dimer; (△) trimer; (○) tetramer; (×) aggregates.

expected, suggesting that these fragments were either associated or partially extended.

In order to determine a possible oligomerization, cross-linking experiments were carried out. As already reported for fragments 1−96, 1−248, 97−415, and 249−415 (Pecorari *et al*., 1993), all fragments were found to self-associate with their increasing concentration. This oligomerization occurred at concentrations lower for the N-terminal fragments than for the C-terminal ones. At concentrations smaller than 8 μM, there is no significant formation of dimeric species for the C-terminal fragments, whereas concentrations as small as 1 μM result in 30% dimeric species for the N-terminal fragments (see Figure 3A,B for fragments 1−152 and 153−415, respectively). Similar results were obtained with the other fragments (data not shown). The proportion of the associated species decreased in the presence of GdmCl (Figure 4). Under denaturing conditions (6 M GdmCl), there was no further aggregation, suggesting that the associated species observed are destabilized by GdmCl and do not result from artifactual cross-linking events subsequent to intermolecular collisions.

The short fragment 364−415 was subjected to electron spray mass spectrometry. The observed molecular mass (5268.8 Da) was found to be in good agreement with the expected mass (5260.0 Da).

The far-UV CD spectra of the refolded fragments were systematically determined. It is expected that the putative folding unit should be able to recover the helical content expected from the crystallographic structure. Furthermore, a characteristic property of native proteins is the cooperativity of the folding−unfolding transition curves. Although not

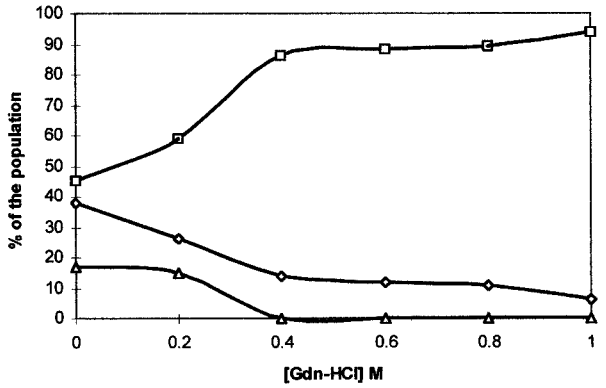

FIGURE 4: Proportion of associated species as a function of GdmCl concentration for fragment 1−123. (□) monomer; (◇) dimer; (△) trimer.

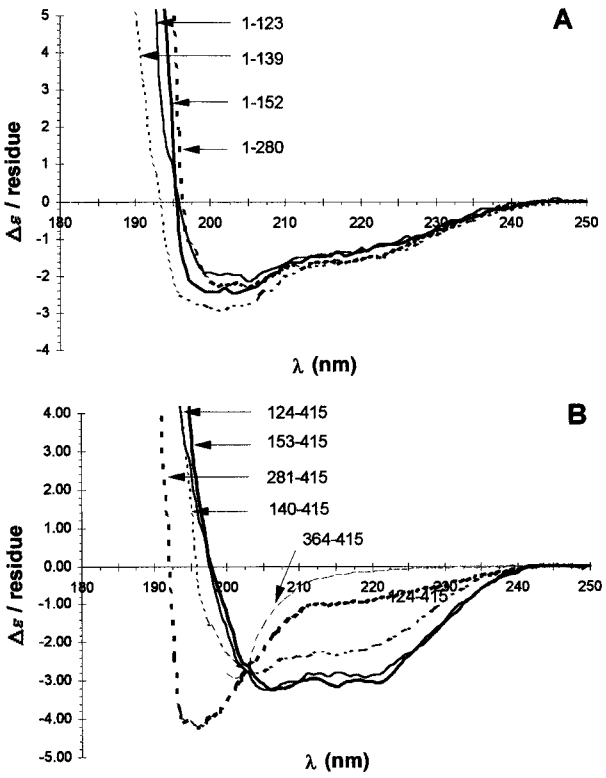

FIGURE 5: Circular dichroism spectra: (A) N-terminal fragments; (B) C-terminal fragments.

sufficient to demonstrate that a fragment is folded as the corresponding region in the native protein, these two criteria (far-UV CD spectra and transition curves) can discriminate poorly structured or highly unstable fragments from correctly folded fragments such as the isolated domains of yeast PGK.

Secondary Structure of the Isolated Fragments. The fragments were renatured by dilution in a regeneration mixture (0.08 M GdmCl final concentration) for at least 1 h to reach the equilibrium. Circular dichroism spectra of the renatured fragments were recorded between 210 and 250 nm, the presence of residual GdmCl preventing measurements at lower wavelengths. These spectra clearly showed the presence of secondary structure in all fragments with the exception of fragment 364−415 (Figure 5). The helix content was evaluated from deconvolution of the spectra and compared with that expected from the X-ray structure of PGK. Table 1 summarizes the results; it also includes for comparison the data previously obtained for the isolated

Table 1: Helix Content of the Fragments

fragment	% helix		
	expected	observed	expected when only domain is structured
N-Terminal Fragments			
1−96[a]	27	8	
1−123	29	31	
1−139	25	20	
1−152	30	28	
N-domain[b]	24	23	24
1−248[a]	33	19	19
1−280	36	32	16
C-Terminal Fragments			
97−415[a]	38	16	31
124−415	38	36	34
140−415	41	19	36
153−415	40	40	38
C-domain[b]	40	32	40
249−415[a]	40	7	
281−415	34	4	
364−415	44	0	

[a] From Pecorari *et al.* (1993). [b] From Minard *et al.* (1989).

domains (Minard *et al*., 1989) and for other fragments (Pecorari *et al*., 1993).

Several fragments smaller than their parent domain (1−96, 249−415, 281−415, 364−415) retain a very low helix content, while others recover the expected content. We observed that a fragment smaller than a domain, and possessing a globular structure in the whole molecule without topological discontinuity in the β-sheets (Figure 1B), is able to regain the expected helical content once isolated.

Several fragments larger than one domain (1−280, 124−415, 153−415) recover all the expected helical content. This suggests that the whole domain as well as the fraction of the second domain included in these fragments recovered the expected helical content. Again, there is a correlation between the ability of these fragments to recover the expected helical content and the absence of a topological discontinuity in the β-sheet of the cleaved domain.

Fragment 1−248 has the helical content expected if only the N-domain region of this fragment is folded. Two fragments larger than the C-domain, 97−415 and 140−415, have a relatively low helical content, suggesting that the C-domain region included in these fragments is not properly folded, while it has been shown that the C-domain is able to fold autonomously. This suggests that the fraction of the N-domain included in this fragment introduces some illicit interactions which could prevent the correct folding of the C-domain although the latter is an autonomous folding unit.

Refolding Transition of the Fragments. Refolding transitions of the different fragments were assessed by the variations in ellipticity at 218 nm and, for the C-terminal fragments which contain tryptophan residues, by the shift in the maximum fluorescence emission wavelength.

For all fragments, a significant transition was observed by circular dichroism as shown in Figure 6. However, the transition of the short fragment 281−415 displays a small amplitude as already reported for fragments 1−96 and 249−415 (Pecorari *et al*., 1993). Similarly, it is very sensitive to the denaturant, even to very low GdmCl concentrations. The same observation applies to fragments 1−123, 1−139, and 1−152, although the transition shows a larger amplitude. In contrast, the simple transition curve corresponding to fragments 1−280 is very similar to those usually observed for stable folding units as previously found for fragment 1−248 (Pecorari *et al*., 1993). The refolding of fragments 124−415, 140−415, and 153−415 presents a biphasic transition. A better fit of the transition curve is obtained with two transitions. A first transition with a very low cooperativity (cooperativity index $\approx$ 1.5) was observed at denaturant concentrations between 0.0 and 0.5 M GdmCl. For higher denaturant concentrations, the transitions were cooperative. In summary, the cooperativity of the transition for fragments smaller than a domain, even when they recover practically all the expected secondary structure, is obviously lower than the cooperativity observed with a whole domain.

Since the C-terminal fragments contain the two tryptophan residues of PGK (Trp 308 and 333), their transitions were assessed by the variations of the maximum emission wavelength following excitation at 293 nm. The refolding transition curves thus obtained are illustrated in Figure 7. The transition observed for fragment 281−415, corresponding only to a fraction of the C-domain, is very different from the transition observed with the three other fragments, 124−415, 140−415, and 153−415, which are longer than the C-domain. It appears that in the short fragment 281−415 the tryptophan residues are almost as accessible to the solvent in the refolded form, as they are in the unfolded form. In contrast, the refolding of the three fragments longer than the C-domain is best described by two transitions.

The parameters of the transition curves were evaluated for the cooperative transitions (Table 2) and compared with those of the isolated domains and the entire PGK. It clearly appears that the same c_m values were obtained by both circular dichroism and fluorescence for most of the large C-terminal fragments, indicating that within the cooperative transition these fragments regain part of their secondary structure and tertiary structure as assessed by the change in the tryptophan environment for the same GdmCl concentration. The variations in λ_{max} lead to c_m values around 0.63 for the C-terminal fragments larger than the C-domain, whereas this value is higher (0.73) for the isolated C-domain. The c_m values observed for fragments larger than a domain are close to the c_m value of the domain included in these fragments.

The CD transition curves observed for C-terminal fragments longer than the C-domain are clearly biphasic. To explain these biphasic transitions, one possibility is that the C-domain folds correctly in the main transition (between 0.6 and 1.0 M GdmCl) while the fraction of the N-domain gives rise to a less stable structure corresponding to the other transition (0.1−0.5 M GdmCl). However, the fluorescence transition curve suggests that this is not a satisfying interpretation. Indeed, the two tryptophan residues are located in the C-domain, and the transition curves monitored by fluorescence are expected in this case to give a single transition corresponding to the putative unfolding of the C-domain (the second phase of the CD transition). A single transition is obtained with the isolated C-domain and the whole protein (Missiakas *et al*., 1990) when the maximum wavelength of fluorescence emission is used to monitor the refolding process. The result actually obtained is that two transitions are also observed for these fragments, suggesting that the events at the origin of the first CD transition also involve the unfolding of the C-domain in at least a fraction of the molecules. Again, this result strongly suggests that

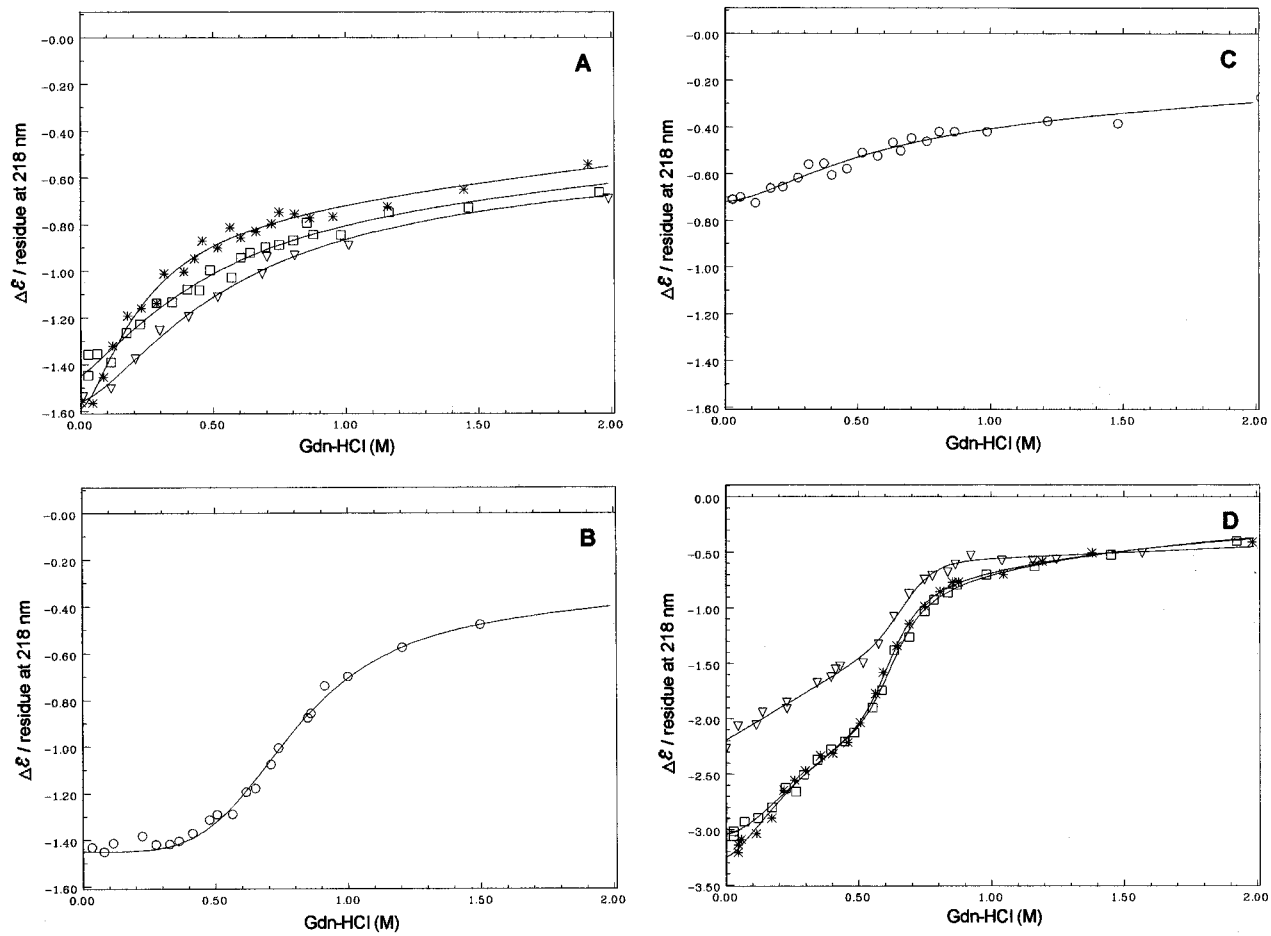

FIGURE 6: Refolding transition of the fragments as assessed by circular dichroism. (A) Short N-terminal fragments: (□) 1−123; (▽) 1−139; (*) 1−152. (B) Longer N-terminal fragment: (○) 1−280. (C) Short C-terminal fragment: (○) 281−415. (D) Longer C-terminal fragments: (*) 153−415; (▽) 140−415; (□) 124−415. The final concentration of fragments was 2 μM.

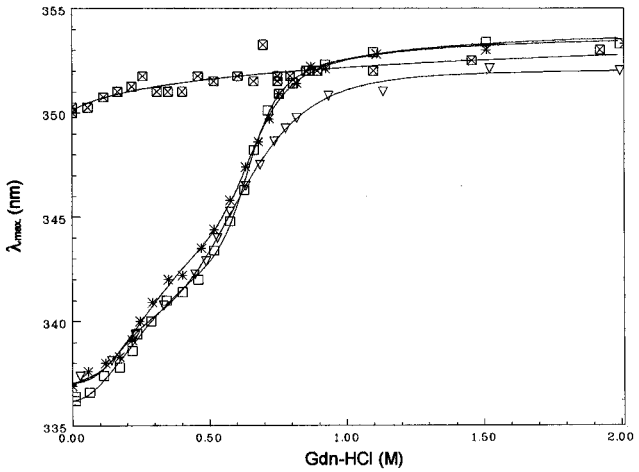

FIGURE 7: Refolding transition of the C-terminal fragments as assessed by the variations in the maximum emission wavelength after excitation at 293 nm: (x inside open box) 281−415; (*) 153−415; (▽) 140−415; (□) 124−415. The final concentration of fragments was 2 μM.

while the isolated C-domain is an autonomous folding unit, the presence of an additional polypeptide sequence in a fragment longer than the C-domain can interfere with its folding process. In other words, this suggests that certain sequences within the C-terminal domain can explore interactions outside its folding unit.

Values for ΔG_0 corresponding to this second transition were evaluated assuming that they correspond to a two-state process for a fraction of the population. These values, reported in Table 2, suggest that the stabilities of these fragments are very close to the stability of the C-terminal domain.

The C-terminal fragments were found to have a tendency to aggregate at increasing concentrations. Aggregation is classically related to hydrophobic interactions which are weakened when the temperature decreases. For this reason, the effect of low temperatures on transition was studied. Fluorescence transition curves obtained at three temperatures, 6, 20, and 30 °C, are presented in Figure 8. They are biphasic, and surprisingly, at 6 °C, the two conformational transitions exhibit a very low cooperativity, contrary to that observed at 30 and at 20 °C. This low cooperativity indicates a greater instability of the fragment at low temperature, and suggests a strong contribution of the hydrophobic interactions in the stability of this C-terminal fragment. Moreover, the existence of two transitions, even at low temperature, strongly suggests that the first transition does not result from aggregation.

Complementation of Complementary Fragments. It has been shown previously (Pecorari *et al.*, 1993) that two pairs of fragments, 1−96/97−415 and 1−248/249−415, yielded significant enzyme activity upon complementation. The same kinds of experiments were monitored for the different pairs of fragments. Kinetics of complementation were carried out under different conditions: either by mixing the two unfolded fragments or by mixing one or two fragments

Table 2: Thermodynamic Parameters Deduced from the Refolding Transition Curves

	N-Terminal Fragments			
fragment	1−248[e]	1−280	N-domain[c]	PGK[d]
c_m[a]	0.85 ± 0.01	0.77 ± 0.06	0.80 ± 0.02	0.80 ± 0.02
n[a]	5.9 ± 0.6	4.3 ± 1	6.5 ± 0.7	12.8 ± 1.3
ΔG_0[a]	−2.5 ± 0.4	−2.9 ± 0.1	−4.5 ± 0.5	−7.8 ± 0.5

	C-Terminal Fragments				
fragment	97−415[e]	124−415	140−415	153−415	C-domain[c]
c_m[a]	0.073 ± 0.05	0.63 ± 0.06	0.70 ± 0.06	0.62 ± 0.06	0.63 ± 0.02
c_m[b]		0.65 ± 0.01	0.63 ± 0.06	0.65 ± 0.02	0.73 ± 0.02
n[a]		10.9 ± 4.1	11.2 ± 4.5	10.8 ± 3.5	7.8
n[b]	nd	12.2 ± 2.2	6.2 ± 1.5	10.2 ± 2.8	6.8
ΔG_0[a]		−3.7 ± 0.2	−3.5 ± 0.9	−3.9 ± 0.3	−4.5 ± 0.5
ΔG_0[b]		−4.5 ± 0.8	−3.5 ± 0.4	−3.9 ± 0.8	nd

[a] From the second transition as assessed by circular dichroism. [b] From the second transition as assessed by the variation of the maximum fluorescence emission wavelength. [c] From Missiakas *et al.* (1990). [d] From Betton *et al.* (1984). [e] From Pecorari *et al.* (1993).

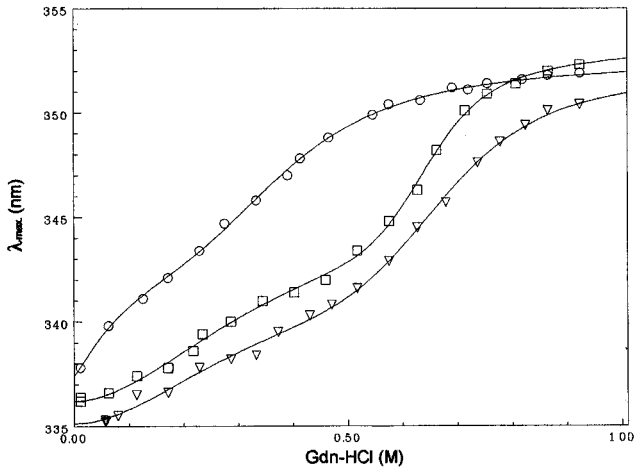

FIGURE 8: Temperature effect on the refolding of fragment 124−415 as assessed by the variation in the maximum wavelength of fluorescence emission following excitation at 293 nm. The final concentration of fragment was 2 μM: (○) 6 °C; (□) 20 °C; (▽) 30 °C.

Table 3: Functional Complementation of the Pair of Fragments 1−139/140−415 under Different Conditions[a]

	rate constant, s^{-1}	amplitude, IU/mg
1−139 unfolded 140−415 unfolded	$6.2 \times 10^{-3} \pm 1.10^{-3}$	14 ± 1
1−139 unfolded 140−415 refolded	very fast	13 ± 1
1−139 refolded 140−415 unfolded	$6.6 \times 10^{-3} \pm 1.10^{-3}$	14 ± 1
1−139 refolded 140−415 refolded	very fast	14 ± 1

[a] Fragments were at 2 μM final concentration, and the residual concentration of GdmCl was 0.04 M in all cases.

previously renatured. The pairs of fragments 1−139/140−415 yielded 0.7% of the wild-type enzyme activity. The same final activity was obtained whether the isolated fragments were previously refolded before association or not. However, the independent refolding of fragment 140−415 enhanced the rate of complementation (Table 3). The pair of fragments 1−123/124−415, 1−152/153−415, and 1−280/281−415 did not generate any enzyme activity regardless of the mixing procedure.

The reassociation of fragments was also investigated by cross-linking experiments, each fragment being at 2 μM final concentration. The pair of fragments 1−139/140−415 produced a species which corresponds to the molecular weight of PGK. However, the isolated fragment 1−139 forms trimeric species with a molecular weight very close to that of PGK. Therefore, it was not possible to determine whether the band on SDS−PAGE corresponded to the association of fragments 1−139 and 140−415 or to the trimeric species of the fragment 1−139. No species corresponding to the molecular weight of PGK was detected on the gel for the pair 1−152/153−415.

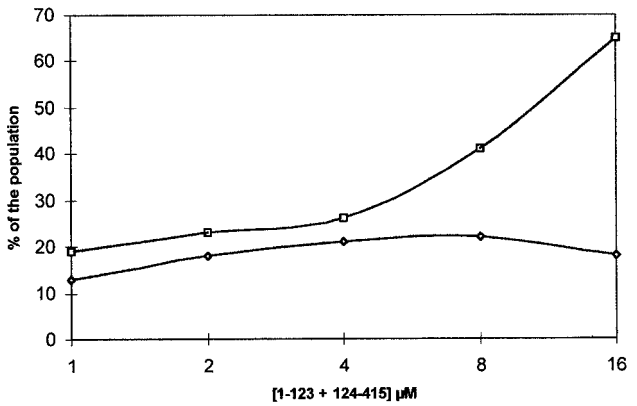

FIGURE 9: Concentration effect on the yield of association of fragments 1−123/124−415: (◇) PGK; (□) multimeric species.

The pair of fragments 1−123/124−415, which did not yield any functional complementation, generated an associated species corresponding to the molecular weight of PGK as assessed by SDS−PAGE. This was clearly distinguishable from multimeric species, with a yield of 12−22% depending upon the fragment concentration. This yield increased with fragment concentration until 8 μM and then decreased, the formation of multimeric species increasing significantly, as shown in Figure 9. The same situation was observed for the pair of fragments 1−139/140−415 (data not shown).

Complementation of Overlapping Fragments. A significant functional complementation (5−6% of the activity of wild-type PGK) was obtained upon mixing overlapping fragments 1−139/124−415 and 1−152/124−415. For these pairs of overlapping fragments, the kinetics of complementation were recorded under different conditions, and the results are reported in Table 4. As already observed (Pecorari *et al.*, 1993), the pre-refolding of the fragment which contains the C-terminal domain sequence increases the rate of complementation. Different combinations of

Table 4: Functional Complementation of Overlapping Fragments 1−139/124−415 and 1−152/124−415[a]

	rate constant, s^{-1}	amplitude, IU/mg
1−139 unfolded 124−415 unfolded	$4.8 \times 10^{-3} \pm 0.4 \times 10^{-3}$	93 ± 5
1−139 unfolded 124−415 refolded	$2.0 \times 10^{-2} \pm 0.2 \times 10^{-2}$	98 ± 2
1−139 refolded 124−415 unfolded	$4.7 \times 10^{-3} \pm 0.3 \times 10^{-3}$	97 ± 2
1−139 refolded 124−415 refolded	$1.9 \times 10^{-2} \pm 0.2 \times 10^{-2}$	92 ± 2
1−152 unfolded 124−415 unfolded	$5.9 \times 10^{-3} \pm 0.4 \times 10^{-3}$	110 ± 3
1−152 unfolded 124−415 refolded	$2.3 \times 10^{-2} \pm 0.2 \times 10^{-2}$	111 ± 2
1−152 refolded 124−415 unfolded	$5.1 \times 10^{-3} \pm 0.3 \times 10^{-3}$	113 ± 3
1−152 refolded 124−415 refolded	$2.3 \times 10^{-2} \pm 0.2 \times 10^{-2}$	109 ± 3

[a] Final concentration of fragments was 2 μM, and residual GdmCl concentration was 0.11 M in all cases.

Table 5: Functional Complementation of Fragments from Different Pairs[a]

fragment	97−415	124−415	140−415	153−415	249−415	281−415
1−96	0.25%					
1−123	nd	0%				
1−139	nd	5%	0.7%			
1−152	nd	6%	0%	0%		
1−172	nd	0%	0%	0%		
1−248	0%	nd	nd	nd	8%	
1−280	nd	0%	0%	0%	nd	0%

[a] nd: not determined. All fragments (2 μM final concentration) are refolded together and the activity was measured after 24 h incubation at 20 °C.

fragments were tested and these results are given in Table 5.

DISCUSSION

The fragments described in this study have a tendency to form multimeric species. The N-terminal fragments form oligomeric species, even at very low concentration (1 μM and lower), and form insoluble aggregates with increasing concentrations. The C-terminal fragments aggregate only at higher concentrations (8 μM). This property, frequently observed with fragments, prevents any study by NMR, and thus, circular dichroism and tryptophan fluorescence were the only available spectroscopic signals that reflect structural states of fragments. It might seem possible that the association state could interfere with determinations of helical contents, but the experimental results clearly show that secondary structure depends strongly on the nature of each fragment rather than on the presence of the associated species which are present.

Are There Folding Subdomains in PGK? This work and previously reported studies (Pecorari *et al.*, 1993) indicate that the shortest fragments either from the N-domain (1−96) or from the C-domain (364−415) of PGK are capable of recovering a low degree of structure (Table 1). Furthermore, these fragments are very unstable and very sensitive to even small concentrations of denaturant. In contrast, longer fragments, but slightly shorter than the N-terminal domain (1−123, 1−139, and 1−152), recover most of the expected helical content. However, a striking conclusion from this work is that all the transition curves observed with fragments smaller than a domain have a low cooperativity. Natural proteins, or domains such as the two isolated domains of PGK (Missiakas *et al.*, 1990), show folding transitions that are highly cooperative. In contrast to fragments smaller than a domain, it appears that all fragments larger than a domain (N or C) show transitions with cooperative phases. This suggests that some crucial interactions which could stabilize the structure cooperatively are present at least in the isolated domains, since these behave like cooperative units.

An interesting observation is that the fragments with a topological discontinuity in the β-sheets cannot recover helical content similar to those expected from the native structure (Table 1). The importance of a topological discontinuity in the β-sheet has been shown in the peptide model PαPβ($\Delta\beta$) from BPTI (Staley & Kim, 1990) which cannot fold due to the absence of the central β-segment. It is likely that the absence of a β-segment in a region usually in the hydrophobic core drastically destabilizes such isolated fragments.

Interestingly, fragments that have a globular structure in whole PGK, without topological discontinuity in β-sheets, recover practically all their expected helical content (fragments 1−123, 1−139, 1−152). These results support the validity of the methods used to identify domains or structural blocks based on globularity or low interface area (Wodak & Janin, 1980; Zehfus & Rose, 1986).

N-Terminal fragment 1−280 longer than the N-domain was found to regain practically all the expected helical content. Its transition curve is cooperative and very similar to those classically observed for the folding−unfolding of whole proteins. Considering the set of fragments, 124−415, 140−415, and 153−415, it clearly appears that the recovered secondary structure is not simply related to the size of the fragment: fragments 124−415 and 153−415 have the expected helical content whereas fragment 140−415 contains only half. A possible explanation is that helix IV (sequence 142−151) is maintained and stabilized upon its interactions with the loop formed by sequence 116−142. When these interactions are missing, the corresponding sequence of helix IV might introduce some perturbation in the folding of the fragments.

Surprisingly, a biphasic transition was observed by circular dichroism as well as by the fluorescence emission wavelength for fragments 124−415, 153−415, and 140−415. These fragments contain the two tryptophan residues located in their C-domain region (308 and 333). Therefore, the biphasicity of the transitions might correspond to distinct changes in the environment of each of the tryptophans. However, the isolated C-domain (185−415) displays a monophasic transition monitored by the λ_{max} fluorescence (Missiakas *et al.*, 1990). Therefore, the additional transition observed with the fragments larger than the C-domain (153−415 as an example) must result from a perturbation of the overall folding process of the C-domain (185−415) by the extra C-domain sequence (i.e., 153−184). These data are consistent either with a new intermediate state induced by the C-domain extra sequence or, alternatively, with the presence of two populations of refolded molecules with different stabilities. This latter hypothesis implies that the same fragment could give rise to conformational heterogeneity upon refolding. For some fragments longer than a domain, the partial formation

of the structure probably arises from a mixture of molecules with a fully folded domain, and molecules with a less stable folded domain. Thus, the presence of a sequence overpassing that of the C-domain (140−185 and 97−185) might prevent the correct folding of this domain. One possible explanation is that the absence of the totality of the polypeptide chain containing the complete information to drive the protein toward its native structure allows the fragment to explore pathways in the conformational space which are not accessible to the whole protein. Alternatively, parallel pathways also exist in the whole protein producing a heterogeneity of intermediates which drive the totality of the molecules in the correct conformation, but only in the presence of the complete sequence ensuring stabilizing interactions.

There are few reports in the literature concerning fragments longer than a structural domain. Vita *et al.* (1989) have obtained a fragment from thermolysin (sequence 1−205) that overpasses the N-terminal domain; this fragment showed a strong tendency to aggregate. Two fragments from lactate dehydrogenase, longer than the N-domain, exhibited an unfolding−refolding transition with a weak cooperativity compared to that of the whole protein, reflecting their instability; furthermore, the tertiary structure seemed to be strongly affected (Opitz *et al.*, 1987). Fragment F1 from the β_2-subunit of tryptophan synthase overpasses the N-domain. It has a native-like structure, but its fluorescence properties are slightly different from those of the uncleaved β_2-subunit (Högberg-Raibaud & Goldberg, 1977; Friguet *et al.*, 1986).

What Are the Conditions Allowing the Specific Recognition between Two Complementary Fragments? Complementation experiment results summarized in Table 5 indicate, in good agreement with previous studies (Pecorari *et al.*, 1993; Minard et al 1989), that there is no correlation between the efficiency of the reactivation process of two complementary fragments and the ability of those fragments to fold when they are isolated. Particularly, the pairs 1−123/124−415 and 1−152/153−415, in which each isolated fragment recovers practically the totality of the expected helical content, did not generate any functional complementation upon mixing, regardless of the mixing procedure.

It is interesting to note that a pair of fragments obtained from pig muscle PGK equivalent to the pair of yeast PGK fragment 1−152/153−415 was shown to reassociate with a high efficiency (Vas *et al.*, 1990; Semisotnov *et al.*, 1991). Therefore, although the structures of the two parent proteins are obviously very similar, the ability of the fragments to reassociate is not conserved. This is an indication that complementation is not a robust process, related to basic folding properties such as the docking of folding units, but instead is a process very sensitive to local structural details.

For most fragment pairs described in this work, the efficiency of the complementation is limited more by the level of the association than by the activity of the reassociated species. However, the pair of fragments 1−123/124−415 is inactive but is able to associate to a certain extent as assessed by cross-linking experiments. These interactions are thus not correct or strong enough to generate the right conformation of the active site. Alternativly, the folded fragments are in a conformation which differs from the native one.

Several complementation experiments were carried out with overlapping fragments. Among them, two pairs were found to generate an enzyme activity (the pairs of fragments 1−139/124−415 and 1−152/124−415), whereas no activity was produced by mixing fragments 1−172 and 124−415. Figure 10 illustrates the structural elements present in the overlapping sequence. It appears that the interactions required for the appearance of a catalytic activity are only present when the two fragments have a common sequence comprising residues 124−139, which corresponds to the loop connecting segment βD and helix IV. Furthermore, the presence of the region including segment βE in the two fragments prevents a functional complementation. Here again, the stabilization of helix IV seems to have a crucial role in the correct folding of yeast phosphoglycerate kinase, and the complementation appears to be very sensitive to local structural details.

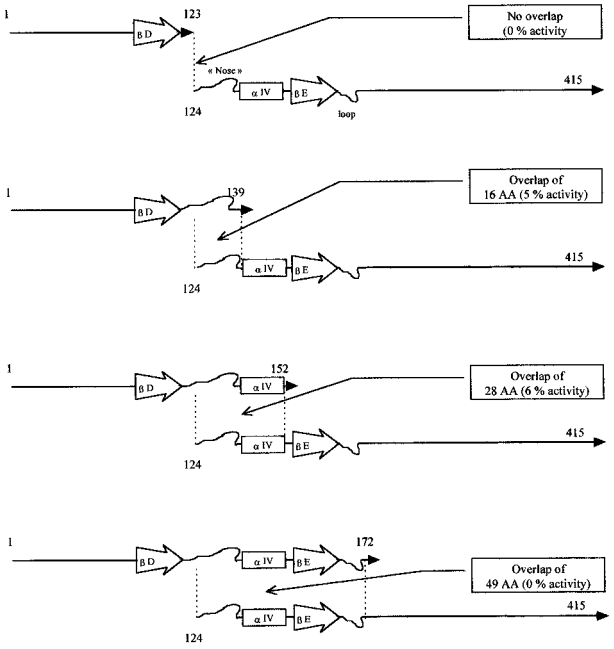

FIGURE 10: Schematic view of the overlapping fragments.

Do the Complementation Kinetics Reflect the Folding Events in PGK? It clearly appears from Tables 3 and 4 that the pre-refolding of the C-terminal fragment accelerates the complementation, which ever N- or C-terminal fragment is present, suggesting that the folding of the C-terminal domain limits the rate of complementation. The limiting steps observed for the pairs 1−139/124−415 and 1−152/124−415 occur with the same rates (mean rate $k \approx 5.1 \times 10^{-3}$ s^{-1}), suggesting that the same phenomenon is observed in these two cases. This mean rate is close to the rate obtained with the pair 1−139/140−415 (mean rate $k \approx 6.4 \times 10^{-3}$ s^{-1}), indicating that 17 residues in addition to fragment 140−415 do not affect the folding rate of the C-terminal part. These rates are close to the folding rate observed for the isolated C-domain, 5.8×10^{-3} s^{-1} (Missiakas *et al.*, 1992), supporting the assumption that this rate-limiting step implies the folding of the C-domain.

Kinetics of complementation suggest another limiting step. Prefolded C-terminal fragments for the pairs 1−139/124−415 and 1−152/124−415 have rate constants close to 2.1×10^{-2} s^{-1}, which is the same order as that observed for the isolated N-domain: 1.0×10^{-2} s^{-1} (Missiakas *et al.*, 1992). Under the same conditions, the kinetics of reactivity were too fast to be determined for the pair 1−139/140−415. It

seems likely that the complementation process depends more on the association rate of the two fragments than on the folding of the N-terminal fragment when the C-terminal fragment is prefolded. Thus, it appears that overlapping sequences introduce an additional constraint reflected by a limiting step, which cannot be observed for the pair 1−139/140−415 and probably consists of rearrangements of common sequences.

Implications for Protein Folding. The results presented in this paper indicate that the fragments corresponding to structural subdomains can recover the expected helical content but not the cooperativity previously observed with the isolated domains. In this respect, the structural domains appear as minimum units of stability in PGK.

The folding of fragments larger than a domain can be perturbed by a polypeptide segment that overpasses the domain and, thus, drives the fragments toward structural heterogeneity. It is likely that, although the domain is an autonomous folding unit, during its folding in the whole protein the domain might explore several refolding pathways. Among them, some pathways could imply illicit extra-domain interactions. Nonnative structures, such as hydrophobic clusters, have been assessed by NMR in isolated fragments of the barley chymotrypsin inhibitor 2 (Prat Gay *et al.*, 1994, 1995; Ruiz-Sanz *et al.*, 1995).

According to this work and to recently reported studies (Williams *et al.*, 1993; Sancho & Fersht, 1992; Kippen *et al.*, 1994; Prat Gay *et al.*, 1994; Tasayco & Carey, 1992; Wu *et al.*, 1993; Ritco *et al.*, 1995), it seems that an isolated fragment with low or nondetectable native conformation can recover a native conformation upon mixing with a complementary fragment. This observation might reflect that a protein reaches its functional structure by a mutual induction through the other parts of the molecule involving long-range interactions, rather than by a stepwise process.

The refolding kinetics of the whole PGK molecule involve at least one fast phase, in which a fraction of the far-UV signal is recovered, and a major slow phase, detected by different methods, in which the native and active structure is formed. Double-jump experiments indicate that these two phases are not related to proline isomerization. Fast and slow phases have also been observed with the isolated domains (Missiakas *et al.*, 1992), suggesting that the slow phase of the whole protein cannot be simply assigned to the docking of prefolded domain, but also to some intradomain molecular events. Results obtained with engineered internal cysteines, used as conformational probes, also suggest that some completion of folding, and then association of the domains, occurs simultaneously (Ballery *et al.*, 1993). Taken together, the results described in this paper indicate that the polypeptide chain can explore interactions outside its own folding unit and that a strictly modular, sequential, and hierarchical view of protein folding does not describe the folding process of PGK adequately.

ACKNOWLEDGMENT

We thank Dr. C. Ghélis for stimulating discussions and Dr. Le Caer for mass spectrometry experiments, and we thank Dr. Liepkalns for carefully reading the manuscript and revising the English.

REFERENCES

Adams, B., Burgess, R. J., & Pain, R. (1985) *Eur. J. Biochem. 152*, 715−720.
Baldwin, R. L. (1993) *Curr. Opin. Struct. Biol. 3*, 84−91.
Baldwin, R. L., & Roder, H. (1991) *Curr. Opin. Struct. Biol. 1*, 218−220.
Ballery, N., Desmadril, M., Minard, P., & Yon, J. M. (1993) *Biochemistry 32*, 708−714.
Banks, R. D., Blake, C. C. F., Evans, P. R., Haser, R., Rice, D. W., Hardy, G. W., Merrett, H. M., & Phillips, A. W. (1979) *Nature (London) 279*, 773−777.
Betton, J. M., Desmadril, M., Mitraki, A., & Yon, J. M. (1984) *Biochemistry 23*, 6654−6665.
Blond-Elguindi, S., & Goldberg, M. E. (1990) *Biochemistry 29*, 2409−2417.
Bücher, T. (1955) *Methods Enzymol. 1*, 415−422.
Burbaum J. J., & Schimmel P. (1991) *Biochemistry 30*, 319−424.
Bycroft, M., Matouschek, A., Kellis, J. T., Serrano, L., & Fersht, A. R. (1990) *Nature 346*, 488−490.
Chaffotte, A., Guillou, Y., Delepierre, M., Hinz, H. J., & Goldberg, M. E. (1991) *Biochemistry 30*, 8067−8074.
Chang, C. T., Wu, C. S. C., & Yang, J. T. (1978) *Anal. Biochem. 91*, 13−31.
Creighton, T. E. (1978) *Prog. Biophys. Mol. Biol. 33*, 231−297.
Dalzoppo, D., Vita, C., & Fontana, A. (1985) *J. Mol. Biol. 182*, 331−340.
Davies, G. J., Gamblin, S. J., Littlechild, J. A., & Watson, H. C. (1993) *Proteins*: *Struct., Funct., Genet. 15*, 183−289.
Fairbrother, W. J., Minard, P., Hall, L., Betton, J. M., Missiakas, D., Yon, J. M., & Williams, R. J. P. (1989) *Protein Eng. 3*, 5−11.
Friguet, B., Djavadi-Ohaniance, L., & Goldberg, M. E. (1986) *Eur. J. Biochem. 160*, 593−597.
Ghélis, C., & Yon, J. M. (1982) *Protein Folding*, Academic Press: New York.
Harlos, K., Vas, M., & Blake, C. C. F. (1992) *Proteins: Struct., Funct., Genet. 12*, 133−144.
Harold, M., Leister, B., Hage, A., Luger, K., & Kirschner, K. (1991) *Biochemistry 30*, 3612−3620.
Hermann, R., Rudolph, R., & Jaenicke, R. (1979) *Nature 277*, 243−245.
Högberg-Raibaud, A., & Goldberg, M. E. (1977) *Biochemistry 16*, 4014−4020.
Jaenicke, R. (1987) *Prog. Biophys. Mol. Biol. 49*, 117−237.
Jaenicke, R. (1991) *Biochemistry 30*, 3147−3161.
Jecht, M., Tomschy, A., Kirschner, K., & Jaenicke, R. (1994) *Protein Sci. 3*, 411−418.
Kim, P. S., & Baldwin, R. L. (1990) *Annu. Rev. Biochem. 59*, 631−660.
Kippen, A. D., Sancho, J., & Fersht, A. R. (1994) *Biochemistry 33*, 3778−3786.
Kraulis, P. J. (1991) *J. Appl. Crystallogr. 24*, 946−950.
Kuwajima, K., Yamaha, H., Miwa, S., & Nagamura, T. (1987) *FEBS Lett. 221*, 115−118.
Lecomte, J. T. J., & Matthews, C. R. (1993) *Protein Eng. 6*, 1−10.
Levitt, M., & Chothia, C. (1976) *Nature 261*, 552−558.
Matouschek, A., Kellis, J. T., Serrano, L., Bycroft, M., & Fersht, A. R. (1990) *Nature 346*, 440−445.
Minard, P., Hall, L., Betton, J. M., Missiakas, D., & Yon, J. M. (1989) *Protein Eng. 3*, 55−60.
Missiakas, D., Betton, J. M., Minard, P., & Yon, J. M. (1990) *Biochemistry 29*, 8683−8689.
Missiakas, D., Betton, J. M., Chaffotte A., Minard, P., & Yon, J. M. (1992) *Protein Sci. 1*, 1485−1493.
Nozaki, Y. (1970) *Methods Enzymol. 26*, 43−50.
Opitz, U., Rudolph, R., Jaenicke, R., Erickson, L., & Neurath, H. (1987) *Biochemistry 26*, 1399−1406.
Pace, C. N. (1986) *Methods Enzymol. 131*, 266−280.
Pecorari, F., Minard, P., Desmadril, M., & Yon, J. M. (1993) *Protein Eng. 6*, 313−325.
Prat Gay, G., Ruiz-Sanz, J., Davis, B., & Fersht, A. R. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 10943−10946.
Prat Gay, G., Ruiz-Sanz, J., Neira, J. L., Itzhaki, L. S., & Fersht, A. R. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 3683−3686.
Press, W. H., Flannery, B. P., Teukolsky, S. K., & Vetterling, W. T. (1986) in *Numerical recipes*, Cambridge University Press.

Ritco-Vonsovici, M., Mouratou, B., Minard, P., Desmadril, M., Yon, J. M., Andrieux, M., Leroy, E., & Guittet, E. (1995) *Biochemistry 34*, 833−841.
Roder, H., Elove, G. A., & Englander, S. W. (1988) *Nature 335*, 700−704.
Ruiz-Sanz, J., Prat Gay, G., Otzen, D. E., & Fersht, A. R. (1995) *Biochemistry 34*, 1695−1701.
Sancho, J., & Fersht, A. R. (1992) *J. Mol. Biol. 224*, 741−747.
Semisotnov, G. V., Mas, M., Chemeris, V. V., Kashparova, N. J., Kotova, N. V., Razgulyaev, O. I., & Sinev, M. A. (1991) *Eur. J. Biochem. 202*, 1083−1089.
Sharma, A. K., Minke-Gogl, V., Gohl, P., Siebendritt, R., & Rudolph, R. (1990) *Eur. J. Biochem. 194*, 603−609.
Shortle D., & Meeker, A. K. (1989) *Biochemistry 28*, 936−944.
Staley, J. P., & Kim, P. S. (1990) *Nature 344*, 685−688.
Stanley, C., Gill, S. C., & von Hippel, P. H. (1989) *Anal. Biochem. 182*, 319−326.
Stark, G. R. (1965) *Biochemistry 4*, 1030−1036.
Taniuchi, H., & Anfinsen, C. B. (1968) *J. Biol. Chem. 243*, 4778−4786.
Taniuchi, H., & Anfinsen, C. B. (1969) *J. Biol. Chem. 244*, 3864−3875.
Taniuchi, H., & Anfinsen, C. B. (1971) *J. Biol. Chem. 246*, 2291−2301.
Taniuchi, H., Parker, D. S., & Bohnert, J. L. (1977) *J. Biol. Chem. 252*, 125−140.
Tasayco, M. L., & Carey, J. (1992) *Science 255*, 594−597.
Vanaman, T. C., & Stark, G. R. (1970) *J. Biol. Chem. 245*, 3565−3568.
Vas, M., Sinev, M. A., Kotova, N. V., & Semisotnov, G. V. (1990) *Eur. J. Biochem. 189*, 575−579.
Vita, C., Fontana, A., & Jaenicke, R. (1989) *Eur. J. Biochem. 183*, 513−518.
Watson, H. C., Walker, N. P. C., Shaw, P. J., Bryant, T. N., Wendell, P. L., Fothergill, L. A., Perkins, R. E., Conroy, S. C., Dobson, M. J., Tuite, M. F., Kingsman, A. J., & Kingsman, S. M. (1982) *EMBO J. 1*, 1635−1640.
Wetlaufer D. B. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 697−701.
Wetlaufer, D. B. (1981) *Adv. Protein Chem. 34*, 61−92.
Williams, K. P., & Shoelson, S. E. (1993) *Biochemistry 32*, 11279−11284.
Wodak, S. J., & Janin, J. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 1736−1740.
Wu, L. C., Laub, P. B., Elöve, G. A., Carey, J., & Roder, H. (1993) *Biochemistry 32*, 10271−10276.
Zehfus, M. H., & Rose, G. D. (1986) *Biochemistry 25*, 5759−5765.

BI951973S