Occurrence of Transient Multimeric Species during the Refolding of a Monomeric Protein*

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Recent advances in rapid methods of isotope exchange monitored by NMR, site-directed mutagenesis, or stopped-flow circular dichroism have led to important developments in protein folding research (1). Until recently, the protein folding process was often described as a sequential series of steps (2), with secondary structure being formed in early steps, then the monomer globule (3) and, in a late rate determining event, the native structure. In contrast to other possible models (4), this model supposes a single folding pathway rather than several parallel pathways, and it implies that the interactions existing in folding intermediates still persist in the native structure, i.e., that nonnative interactions are not usually present in the folding intermediates. However, it is widely accepted that this scheme is further complicated by the possible, although not general, occurrence of slow and fast folding species related to proline isomerization in the unfolded forms (5).

It is important to keep in mind that methods commonly used to monitor folding, such as circular dichroism or fluorescence, give an average picture of the molecular species present at a given folding time. Few methodologies are able to resolve heterogeneous populations. A classical example is the determination of disulfide bond formation that, many years ago, had suggested that early folding events in bovine pancreatic trypsin inhibitor could lead to a set of diverse one-disulfide intermediates (6). These rapidly formed a limited set of two-disulfide intermediates, including a low proportion of molecules (7) containing a nonnative disulfide bond.

Recent results obtained by isotope exchange and mass spectrometry have also demonstrated that protein folding can give rise to a heterogeneous population of intermediates or proceeds via multiple pathways (8). Similarly, labeling kinetics of cysteinyl residues genetically introduced in phosphoglycerate kinase revealed that an "intermediate" produced in the fast folding phases is in fact a population of two distinct molecular species (9).

Folding studies are often focused on the folding process of small proteins (2). It is, however, clear that the folding process of larger ones is frequently associated with unproductive refolding pathways leading to nonnative multimeric species (10, 11). These species are generated through wrong intermolecular pairing of local structures present in folding intermediates; the association acts as a kinetic trap and results in irreversibly aggregated forms. These side reactions are frequently, but not exclusively, observed with oligomeric proteins and are greatly favored at high protein concentrations.

This paper describes experiments conducted with yeast phosphoglycerate kinase. Phosphoglycerate kinase is a classic example of a two-domain monomeric protein, whose folding process has been intensively studied by several groups (9, 12–16). Phosphoglycerate kinase unfolded by high concentration of urea or guanidinium chloride can completely refold in vitro into its original monomeric active form. The isolated domains have been produced by genetic engineering and shown to refold independently both in vivo and in vitro (17, 18). The two folded isolated domains do not reassociate upon mixing and therefore do not regenerate a complemented and active protein. Two other pairs of complementary fragments were produced by chemical cleavage of two mutant phosphoglycerate kinases, in which a unique cysteinyl residue was previously introduced by site-directed mutagenesis (19). For these two pairs of fragments, the cleavage point is located within the N- or the C-domain. These studies have shown that for these two pairs of fragments, the fragment smaller than a domain has a rather low degree of structure and cooperativity. These two pairs of fragments did regenerate a partial enzymatic activity upon mixing. One could intuitively expect that complementation is related to the folding autonomy of the isolated fragments. In contrast, these observations actually suggest that correctly folded domains do not necessarily reassociate and that it is not necessary for protein fragments to correspond to protein domains, or to fold autonomously, to be able to reassociate functionally.

We have recently extended this study to other pairs of complementary fragments derived from phosphoglycerate kinase. This paper shows that the associated multimeric species ob-
served with one of the previously studied fragments (19) are also observed with a set of other fragments corresponding to incomplete N-domain. Furthermore, this paper shows that transient multimeric species are present during the folding of this monomeric protein.

EXPERIMENTAL PROCEDURES

Materials—Urea and EGS1 were obtained from Pierce; GdnHCl was from Life Technologies, Inc.; and NbSe2 was from Sigma. All other reagents were of analytical grade.

Mutagenesis and Protein Purification—Mutants from yeast phosphoglycerate kinase (PKG), C97A/124C, C97A/140C, C97A/S153C, C97A/V281C, were obtained from a first mutant in which the unique cysteine was replaced by an alanine residue. Site-directed mutagenesis was performed as described previously (20). The coding sequences of the mutated genes were completely sequenced in order to check the absence of other unwanted mutations. Each mutated gene was then inserted in the expression vector (20) and over-expressed in pgk Saccharomyces cerevisiae strain BC3 (21), and purified according to previously published procedures (22). Horse muscle PKG was purified as reported previously (23).

Production of Yeast PKG Fragments—Fragments were obtained by cleavage at the introduced cysteinyl residue. The method of cleavage used was adapted from Vanaman and Stark (24) and has been described previously (19). Fragment 1–172 was obtained by CNBr cleavage of wild-type PKG, as described by Adams et al. (25). Detailed purification procedures will be published elsewhere. Purified fragments were controlled for the absence of complementary fragments or uncleaved protein by SDS-PAGE.

Production of Soluble Aggregated Form of Horse Muscle PKG—Horse muscle PKG (42 μM) was incubated for 1 h in 10 mM phosphate buffer, pH 7.5, containing 0.75 mM GdnHCl and 1 mM dithiothreitol. A sample was chromatographed on a Superose 12 column eluted with a 10 mM phosphate buffer, containing 1 mM dithiothreitol. Flow rate was 0.13 cm min⁻¹. The peak corresponding to the excluded material was collected and used to check the efficiency of the cross-linking method.

Cross-linking Experiments—The oligomerization of fragments and uncleaved PKG was studied by cross-linking with glutaraldehyde or EGS. Proteins in a 10 mM phosphate buffer, pH 7.5, were cross-linked with glutaraldehyde, according to Hermann et al. (26). The glutaraldehyde (25% v/v) was added to samples at 1% (v/v) of the final mixture. The reaction was allowed to occur at 20°C, typically for 30 s unless otherwise indicated, and was quenched by the addition of a freshly prepared 2 M NaBH₄ in 0.1 M NaOH.

The EGS was dissolved in Me₂SO at 5 mM and added to the sample to give the final concentration of 200 μM. After 30 s, the reaction was quenched by the addition of 0.5 M Tris-HCl buffer (final concentration, 100 mM), pH 8.

Polyacrylamide gel electrophoresis was performed by procedures at room temperature with a 12% polyacrylamide slab gel. The gels were stained with Coomassie Blue and scanned with a Desaga CD60 densitometer.

All of the refolding and cross-linking experiments were performed at 20°C.

Sedimentation Equilibrium—Equilibrium ultracentrifugation analysis were conducted with two PGK fragments. The N-terminal domain (fragment 1–184), used as a control for a monomeric species, was dissolved in a 10 mM phosphate buffer, pH 7.5. The fragment concentration was 2 μM. Fragment 1–172 was previously refolded in the same phosphate buffer. Protein and residual denaturant concentrations were 10 μM and 0.1 M GdnHCl, respectively. These samples were centrifuged at 20°C for 11 h at 25,000 rpm in a Beckman XL-A analytical ultracentrifuge. After reaching the equilibrium, the concentration profiles were recorded by monitoring the absorbance at 276 nm. The partial specific volumes of fragments at 20°C were calculated to be 0.728 ml/g for fragment 1–280 and 0.743 ml/g for fragment 1–184, according to Perkins (27). Experimental data were analyzed by nonlinear least-squares fitting, using the SEDEQ1B program (28).

RESULTS

The Presence of Stable Multimeric Forms of PGK Fragments Is Mainly Associated with the N-terminal Part of the Sequence—Previous studies (19) on subdomain fragments of PGK have shown that a fragment (1–96), corresponding to the first half of the N-terminal domain, forms a series of oligomeric species even at relatively low protein concentration. These species were clearly observed using cross-linking experiments followed by SDS-PAGE analysis. The electrophoretic mobility of the species was shown to correspond exactly to the mobility expected for dimer, trimer, and tetramer.

Using the same methods, we produced and studied a more extensive set of fragments as described previously (19). These fragments were obtained by chemical cleavage at the unique cysteinyl residue previously introduced in several mutants by site-directed mutagenesis. The results of cross-linking analysis are shown in Fig. 1. A distribution of oligomeric species, as previously observed with fragment 1–96, is also clearly present with fragments 1–123, 1–139, and 1–152. All of these fragments correspond to the N region relative to cleavage points located within the N-terminal domain, and therefore, these fragments correspond to incomplete N-terminal domains. This behavior was no longer observed when the cleavage point approaches the limit between the domains; neither fragment 1–172 nor fragment 1–184, which corresponds exactly to the complete N-domain, gives rise to the pattern observed with the shorter N fragments. When the cleavage point was located beyond the limit of the domain, the N-side fragments 1–280 (Fig. 1) recovered a tendency to form dimers, but in a much lower proportion than the incomplete N domain fragments.

C-terminal fragments or complete C-domain (185–415) formed a low fraction of dimeric species in this range of concentrations. The fragments corresponding to the whole C-domain plus an extra N segment 124–415, 140–415, 153–415 were also essentially monomeric, although a very faint band indicated that a small fraction of fragments 124–415 was dimeric.

From these results, it is clear that the tendency to form a set

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1 The abbreviations used are: EGS, ethylene glycol-bis(succinimide acid N-hydroxysuccinimide ester); GdnHCl, guanidine hydrochloride; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; PKG, phosphoglycerate kinase.
of multimeric species is mainly due to a subset of the sequence located in the N-terminal part of the polypeptide chain. The shortest N-fragment, and therefore the minimal known region susceptible to give rise to associations, is located between residues 1 and 96. This phenomenon appears to be correlated with the folding autonomy of the fragments. When the sequence is long enough to form the stable tertiary structure of the N domain, the associated forms are not observed. Indeed, the N fragments 1-184 and 1-172 have been shown to form a relatively stable structure, while fragment 1-96 was shown to be not as well structured and to unfold with a lower cooperativity (18, 19, 25).

A much weaker tendency to produce associated forms was also observed with some C-terminal fragments, including the whole folded C-domain. This suggests that a secondary, but weaker, contribution to association could be related to a stretch of the C-terminal sequence.

A Similar Distribution of Oligomeric Species Is Observed Transiently during the Refolding of the Whole Protein—Previous studies on PGK have shown that the refolding process involves at least two kinetic phases (9, 14). At least one fast phase occurs during the dead time of manual mixing experiments. This phase is followed by a slow phase, which leads to the fully refolded and active protein. This later phase has been clearly observed using different signals such as far UV CD, tryptophan fluorescence, titration of engineered thiol groups (9), fluorescence of 8-anilino-1-naphthalenesulfonic acid expulsion (14), susceptibility to proteolysis (30), binding of monoclonal antibodies, and enzyme activity (9). This phase is not related to proline isomerization (9, 31) but rather corresponds to the transformation of a compact but fluctuating state in the unique and relatively rigid native structure. An important point, established by thiol titration experiments, is that the fast folding phase leads to a distribution of at least two different molecular populations more flexible than the native state (9). Furthermore, the results described above indicate that the associated fragments species are formed rapidly and at low protein concentrations. These two observations suggest that the associated states observed with the fragments might also be accessible to the whole protein as long as the structure is not locked in the native conformation. In other words, the associated forms might be apparent during the slow refolding phase. The experiments reported below show that such associated forms are indeed observed during the slow refolding phase.

Cross-linking experiments can be conducted in a sufficiently short time to freeze the states of association during the refolding process (26). The refolding kinetics monitored by cross-linking are reported in Fig. 2. In addition to the bands corresponding to the monomeric form, three bands corresponding to a distribution of dimeric, trimeric, and tetrameric forms are present. These multimeric forms are formed rapidly and disappear progressively when the refolding time, prior to the cross-linking step, increases; they were not observed when the refolding time was long enough to produce mainly refolded protein. The disappearance of the multimeric forms is concomitant with the formation of the native protein during the slow refolding phase. Upon cross-linking, the monomeric protein displays an electrophoretic pattern that is dependent on its conformational state. When the protein is internally cross-linked in its native form, the band corresponding to the monomeric protein appears sharper and higher in the gel than the protein internally cross-linked in its unfolded form. This unexpected peculiarity provides a kind of internal control, supporting the observation that the dissociation of the multimeric forms occurs in parallel with the refolding of the monomeric form. Furthermore, the approximate rates of disappearance of the "unfolded monomeric" species and of formation of the "native monomeric" species (Fig. 2B), were estimated from the scanning of the SDS-PAGE gel (Fig. 2A). These two processes were found to have rate constants close to the rate constant of the slow folding phase under the same conditions (k ~ 4.6 ± 10^-3 s^-1) as determined by other signals (see Fig. 5 and Ref. 28). In control experiments (Fig. 3), the positions of the "monomeric unfolded" and "monomeric native" bands were also observed for the unfolded protein cross-linked in the denaturant and for the native protein cross-linked without denaturant, respectively.

Several control experiments were conducted in order to demonstrate that the presence of transient multimeric forms is not an artifactual observation. The most convincing evidence against cross-linking of unassociated species subsequently to intermolecular collision events is that both denatured and native protein appeared as perfectly monomeric (Fig. 3) after the same cross-linking treatment. A more difficult question is whether or not the distribution observed after cross-linking quantitatively reflects the distribution of associated forms originally present in the solution. Indeed, higher aggregates, which were incompletely trapped, could give rise to this type of electrophoretic pattern. The following experiments indicate that the cross-linking step is likely to be complete. Control experiments show that the apparent species distribution is not modified either by an increase of the cross-linker concentration or by an increase of the time-length of the cross-linking step. Other experiments

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2 P. Minard, unpublished observations.
linked by glutaraldehyde for 30 s. The formed at the end of the fast folding phase (Fig. 2). Furthermore, there are about 35% of multimeric species of thiol groups in different laboratories (9, 16, 18, 23, 25). The permanent cross-linked forms.

The permanently associated forms observed with the fragments by cross-linking were also observed by analytical equilibrium ultracentrifugation. A sample of the monomeric N-terminal domain (1–184), which was analyzed in parallel with fragment 1–280 gave rise, as assessed by cross-linking, to a small fraction of dimer. This fragment was chosen in spite of its less pronounced susceptibility to oligomerization relative to other shorter N fragments, because it allows a more sensitive detection and therefore an analysis at a lower protein concentration than smaller fragments. These fragments were not cross-linked before the ultracentrifugation analysis. The results presented in Fig. 4 show that the N domain behaves as a monomeric protein, while the sample of fragment 1–280 contains both monomeric and dimeric species.

Recent dynamic light-scattering experiments have shown that species with large Stokes radius are observed transiently during the refolding of yeast PGK (15). This observation was originally interpreted as resulting from expanded monomeric species but may be related to the presence of transient oligomeric species.

A further indication that multimeric species do not result from some unknown cross-linking artifact is provided by measurements of light scattering during the folding process (Fig. 5). The scattered light intensity monitored during the refolding of the protein decreases with a rate corresponding to that of the slow folding phase under the same conditions.

Properties of the Transient Multimeric Species—The refolding process of PGK from yeast or from horse muscle displays similarities but also a difference relative to the stability of the two domains, the C domain being more stable than the N domain in the horse muscle protein (14). The results of the cross-linking experiments conducted during the refolding process of the horse muscle enzyme, reported in Fig. 3, indicate that the formation of multimeric species also occurs during the refolding process of this protein. This suggests that the production of oligomeric species is not a peculiarity of yeast PGK, but rather a conserved property between PGK from two different species, in spite of the differences observed in the domain stability.

“Double jump” experiments have indicated that the two phases observed during the refolding of PGK did not result from the presence of different unfolded forms due to proline isomerization (9, 31). The same observation also holds for the presence of oligomeric forms. The associated forms are observed even when the denaturation step was too short (31) to allow proline isomerization in the unfolded states (data not shown). The multimeric forms are no more related to cysteine pairing since they were observed, even in the presence of diethiothreitol, with the wild-type protein, which has only one cysteine, and with fragments, which have no cysteine. The same distribution of transient oligomeric forms can also be observed if the protein previously denatured by urea is refolded.
by dilution (data not shown) indicating that this phenomenon was not dependent upon the nature of the denaturant. In order to evaluate the affinity between the protomers in the transient oligomers, a set of cross-linking experiments was conducted with different protein concentrations. The results are reported in Fig. 6. The important, but completely unexpected result of this experiment is that the distribution of the multimeric species is not affected by the protein concentration in a range of more than 2 orders of magnitude. If this is not due to incomplete cross-linking, as suggested by the control experiments reported above, the inescapable conclusion is that the different species, monomers, dimers, trimers, and tetramers, are not in equilibrium during the lifetime of the transient species.

**DISCUSSION**

The results described in this paper demonstrate that a set of oligomeric species is rapidly formed during the folding of a monomeric protein. The tendency of incorrectly or incompletely folded species to aggregate is a well documented property of proteins (11, 33). We have observed transient oligomeric species that have several features not previously reported, indicating that these oligomers are distinct from the frequently observed “wrong aggregates.” These species are formed at a very low protein concentration, and the extent of oligomerization does not depend on protein concentration. Wrong aggregation is frequently related to illicit hydrophobic interactions and is therefore strongly temperature-dependent, while the transient oligomeric species described here are also observed to the same extent at 4°C (data not shown). Finally, the most unusual property of these transient oligomers is precisely that they are transient, while wrong aggregation is generally irreversible.

The oligomers were first observed as stable species with the fragments corresponding to the incomplete N-domain and later as transient species during the folding of the whole protein. These observations suggest that these oligomers are due to somewhat sequence-specific interactions originating in the N-domain sequence. The illicit interactions between monomers is certainly related to the formation of partial or incorrect structure in the fragments. Indeed, the stable oligomers produced by the N-fragments are not observed in the presence of denaturant. Moreover, they are no longer observed when the sequence is long enough to fold cooperatively as a “correct” domain.

**Fig. 4. Ultracentrifugation analysis of fragments 1–280 (A) and 1–184 (B).** The fragments were in native conditions and were not cross-linked before the experiments. For fragment 1–280 (molecular mass expected, 30.7 kDa), the best fit was obtained using a model where the sample is a mixture of monomer and dimer (molecular mass observed, 30.8 and 62.1 kDa, respectively), the fraction of higher multimeric species being negligible. For fragment 1–184 (molecular mass expected, 20.3 kDa), the best fit was obtained using a model where the protein is essentially monomeric (molecular mass observed, 19.5 kDa) and the fraction of multimeric species is negligible. Semilogarithmic plots (Ln Abs. versus r^2) are reported in insets.

**Fig. 5. Refolding kinetics monitored by light scattering at 310 nm.** The kinetics were initiated by dilution in phosphate buffer of a solution of yeast PGK previously unfolded in guanidium chloride. The scattered intensity is plotted against the refolding time. The continuous line corresponds to a monoexponential fit (k = 4.6 × 10^{-3} ± 0.3 × 10^{-3} s^{-1}).

**Fig. 6. Effect of protein concentration on the distribution of the transient multimeric forms.** SDS-PAGE of yeast PGK cross-linked after 20 s of refolding. The protein concentrations during the refolding step are indicated at the top of the gel.
Finally, the formation of the native-like structure of the protein during the refolding process dissociates the oligomers.

These results directly demonstrate that during the folding of phosphoglycerate kinase, an heterogeneous population of intermediates is present and that, in these intermediates, interactions are involved that do not persist in the final native structure.

The disappearance of transient multimeric species is obviously not due to an irreversible aggregation or adhesion to the reaction vessels; these transient species are converted into native monomeric species. A possible mechanism that could explain the paradoxical absence of a concentration dependence of a reaction leading to multimeric species is suggested in Fig. 7. Although several aspects of this scheme are purely hypothetical, its essential feature is that the oligomer distribution is explained by the existence of a kinetic competition between two monomolecular steps (or between two bimolecular steps). In this qualitative model, the observed oligomer distribution results from the relative rates of two processes. One process leading to a monomeric state able to initiate the formation of oligomers or to extend the already formed oligomers by a unidirectional, fast, and tight association, and the other process leading to a different monomeric state incompetent to produce an expandable association. The relative distribution of oligomeric forms actually observed indicates that the formation of these two types of states must have rate constants of the same order of magnitude. The overall mechanism must take place during the fast folding phase.

The scheme proposed in Fig. 7 is formally analogous to an irreversible reaction of copolymerization between two types of monomers. The incorporation of the first type of monomers produces an association that will be further extended, while the incorporation of the second one produces a dead-end association. A simulation of this model shows that the distribution finally obtained is directly dependent on the ratio between the two types of monomers, but does not depend on the total concentration of the two species. The initial ratio of the two species is directly dependent on the ratio of the rate constants $k_1/k_2$ (Fig. 7).

This model supposes that the association step is essentially irreversible during the fast folding phase. It is, however, clear that during the time range of the slow folding phase, the tight association observed between the monomers is reversed by a folding step producing a monomeric folded protein. Alternative mechanisms are probably possible for the formation of these oligomeric species, such as the formation of cyclic oligomers, but should explain the following experimental observations: (i) the oligomer distribution does not depend on protein concentration; (ii) the association is sufficiently tight to take place for protein concentrations as low as 0.05 M (and may be lower); and (iii) the association distribution is established at the end of the fast folding phase.

When a kinetic intermediate is detected, it is important to know whether this intermediate is on or off pathway. The observation that the species are not in equilibrium rules out the possibility that these species result from a side reaction such as a reversible association of an intermediate preceding the rate-limiting step. In other words, the step that leads to the dissociation of the multimeric species is not simply the reverse of the step that leads to their association. Their dissociation is probably induced by a folding step occurring in the multimeric species. These oligomers are formed rapidly and disappear with a rate similar to the slow folding phase. From these criteria, the oligomeric species are kinetically productive and could be considered to be on the pathway, or more correctly, on some of the pathways.

However, it is essential to note that a large fraction of the protein remains monomeric and is not in equilibrium with the oligomeric forms. This result suggests that this fraction of protein does fold without going through any oligomeric forms. Therefore, these intermolecular nonnative interactions cannot be considered as necessary for the protein to fold correctly.

Previous kinetic studies on PGK folding have shown that the rate constant of the slow refolding phase increases, as expected, when the denaturant concentration decreases. However, for denaturant concentration lower than 0.2 M, the folding rate constant decreases with the denaturant concentration (31). We suggest that this unusual effect could be related to the presence of oligomeric species. The proportion of transient multimeric species decreases progressively when the denaturant concen-
transient increases to a value higher than 0.2–0.3 M. It seems possible that for very low denaturant concentrations the oligomeric forms are sufficiently stable to slow down the folding process. Under these conditions, GdnHCl in destabilizing too stable intermediates, accelerates the overall folding process. The effects of low denaturant concentrations to destabilize nonnative interactions have already been shown to increase the refolding yield of proteins difficult to refold (11) or to speed-up the formation of a disulfide bond in native-like bovine pancreatic trypsin inhibitor species (7).

The fundamental observation reported in this paper, i.e. the presence of transient multimeric species during the folding of a monomeric protein, was certainly not anticipated, and it is currently difficult to evaluate whether or not PGK should be considered as a rare exception. There are very few examples of related observations. A transient absorbance at 320 nm (where the difference spectrum is zero) was observed during the refolding of α-tryptophan synthase (34). This absorbance was attributed to transient aggregates. Transient oligomeric forms of bovine growth hormone have also been indirectly observed by Brems (35). It seems therefore that such species could occur at least for a few other proteins, although for protein concentration higher than the very low concentration range used in the present work. However, there are no reported direct observation of such transient multimeric species.

The process leading to irreversible aggregation has been studied in few model systems such as bovine carboxic anhydrase (36) or P22 tailspike protein (37). It has recently been shown with P22 tailspike that during in vitro refolding experiments, a distribution of small oligomers is rapidly formed and is the first step toward irreversible aggregation.

The irreversible formation of aggregates is more common in large multidomain proteins than in small proteins. Furthermore, it has been reported for some multidomain proteins, including horse muscle PGK, that stable species observed in intermediate concentrations of denaturant can induce some degree of irreversibility (32). These observations have been interpreted as the consequence of the wrong intramolecular pairing of folded but unpaired domains (11). This idea was nicely illustrated by the recent demonstration that domain swapping can indeed be experimentally observed (38). However, in the case of the transient multimeric forms of PGK, fragments with only a fraction of a domain, in which wrong pairing of associated domains is not possible, do form multimeric species. Therefore, the associated forms are produced by the wrong pairing of structural units smaller than a domain.

An interesting aspect of these results is that the formation of multimeric species is under kinetic control. The multimeric species are formed in the fast folding steps by kinetic partitioning and are not in equilibrium. It is only on a longer time scale that these different states converge to form the unique native protein.

To summarize, this study shows that a sequence located within the N-domain of PGK confers on protein fragments smaller than the N-domain the ability to form stable multimeric species. These multimeric forms are not observed as stable species with the whole protein and with the complete, stabilized, and folded N domain. However, they were shown to occur during the folding of the whole protein as transient species.

Thus, the distribution of multimeric species probably results from a kinetic competition taking place during the fast folding phase. The dissocation of the multimeric forms occurs during the unique slow folding phase, giving rise to the native monomeric protein. In contrast to folding models where the folding process is described as a strictly sequential and hierarchical process, these results provide direct evidence the polypeptide chain can explore transiently nonnative interactions during the folding process.

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