Cold Denaturation of Yeast Phosphoglycerate Kinase: Kinetics of Changes in Secondary Structure and Compactness on Unfolding and Refolding†

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Received March 10, 1993; Revised Manuscript Received April 26, 1993

ABSTRACT: Under mildly destabilizing conditions (0.7 M GuHCl), phosphoglycerate kinase from yeast undergoes a reversible two-step equilibrium unfolding transition when the temperature is lowered from 30 to 1 °C (Griko, Y. V., Venyaminov, S. Y., & Privalov, P. L. (1989) *FEBS Lett. 244,* 276–278). The kinetics of the changes in compactness and secondary structure have been studied by means of dynamic light scattering and far-UV circular dichroism, respectively. It turned out that unfolding and refolding after an appropriate temperature jump (*T*-jump) was performed proceeded in substantially different ways. After a *T*-jump from 30 to 1 °C, a multiphasic unfolding behavior was observed, reflecting the independent unfolding of the N-terminal and C-terminal domains with time constants of about 7 and 45 min, respectively. A remarkable feature of the unfolding process is the simultaneous change of compactness and secondary structure. Refolding after a *T*-jump from 1 °C to higher temperatures occurs in two stages. At the first stage an appreciable amount of secondary structure is formed rapidly within the dead time of the *T*-jump, while the overall dimensions of the polypeptide chain remain essentially unchanged. Thus, an extended folding intermediate is formed at an early stage of folding. Further formation of secondary structure proceeds slowly within a time range of minutes in parallel with the increase of compactness. At 30 °C, both domains refold simultaneously, while at 15 °C, independent folding can be observed. These findings are discussed with respect to predictions of existing models of folding.

It has been unknown, up to now, how a protein finds its path from an unordered, statistical conformation to a unique compact structure. A simple estimation, known as Levinthal's paradox (Levinthal, 1968), shows that this pathway cannot be found by random search within the observed times of folding. On the contrary, folding must proceed on one or more defined pathways. Several plausible but in essential details contradictory hypotheses exist about this pathway of folding.

A well-known hypothesis is the framework model (Ptitsyn, 1973, 1991; Kim & Baldwin, 1982). According to this model, folding consists of the following steps: (i) formation of fluctuating embryos of regions with secondary structure in an otherwise unfolded chain; (ii) collapse of these regions into an intermediate, compact structure, called the molten globule state; and (iii) adjustment of this intermediate structure to the unique native structure. A detailed description of the features of the molten globule state and its role in protein folding was presented by Ptitsyn (1992).

Another model is the hydrophobic collapse model (Dill, 1985; Dill et al., 1989; Chan & Dill, 1991), where a rapid collapse of the polypeptide chain is assumed as an early stage in the folding transition. Leopold et al. (1992) have developed the concept of "protein folding funnels" to describe how the protein is guided from the randomly collapsed state to the native state. This concept follows from a few general considerations: (i) proteins fold from a random state by collapsing into a random dense state; (ii) reconfiguration occurs diffusively and follows a general drift from higher energy to lower energy conformations; (iii) reconfiguration occurs between conformations that are geometrically similar, i.e., global interconversions are energetically prohibitive after collapse, so local interconversions alone are considered.

A decision between both models on the basis of theoretical arguments alone is obviously difficult and even impossible at the present time. Therefore, experiments are necessary which are suitable for monitoring the temporal changes of the protein conformation in the course of folding. The essential difference between the two models is as follows. In the case of the framework model, the secondary structure should be formed at first within an expanded structure, followed by a transition to the compact state. In the case of the hydrophobic collapse model, it should be expected that the collapse precedes the formation of secondary structure or that both processes are running in parallel. It has been shown for a number of proteins by far-UV CD[1] measurements that an essential part of secondary structure is formed rapidly within times less than 0.01 s after a change from unfolding conditions to folding conditions by an appropriate jump technique (Kuwajima et al., 1987; Goldberg et al., 1990; Sugawara et al., 1991; Elöve et al., 1992). However, the compactness of the observed intermediate state remained unclear. This situation is often met in the detection of intermediates, because the widely used spectroscopic techniques only reflect changes in local structures. Small-angle X-ray scattering and dynamic light scattering are methods of choice in determining the compactness of protein molecules directly. Recently, we described a DLS apparatus which is suitable for monitoring changes of the Stokes radius with time constants of a few seconds (Gast et al., 1992). Folding and refolding times of this order of magnitude and even longer have been reported in the literature, particularly in connection with cold denaturation of proteins. One of the first observations of this kind was made more than 20 years ago by Pace and Tanford (1968). Furthermore, this

† Supported by grants from the Deutsche Forschungsgemeinschaft (Da 292/1-1) and the Bundesministerium für Forschung und Technologie (0319682 A) and by a grant from the Fonds der chemischen Industrie to G.D.

[1] Abbreviations: ANS, anilinonaphthalene sulfonic acid; CD, circular dichroism; DLS, dynamic light scattering; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; GuHCL, guanidine hydrochloride; PGK, 3-phosphoglycerate kinase (EC 2.7.2.3); SAXS, small-angle X-ray scattering.

0006-2960/93/0432-7747$04.00/0

phenomenon became evident in kinetic studies of cold denaturation of metmyoglobin (Cho & Chan, 1984) and of T4 lysozyme and several of its mutants (Chen et al., 1989, 1992). GuHCl-induced unfolding and refolding reactions of phosphoglycerate kinase from different species also revealed a slow rate-limiting step (Betton et al., 1985, 1992; Semisotnov et al., 1991; Ballery et al., 1993). The nature of the rate-determining step, however, remained unclear until now.

In the present work, we investigate the kinetics of unfolding and refolding of PGK from yeast upon and after cold denaturation by using DLS and CD spectrometry. Combining these methods, one is able to monitor the compactness (DLS) and the amount of ordered secondary structure (CD) simultaneously. Accordingly, the results of these experiments are a good experimental test for the above mentioned folding models.

In a preceding paper (Damaschun et al., 1993), we have reported on the equilibrium unfolding and refolding transitions of PGK upon cold denaturation on the basis of SAXS, DLS, CD, and fluorescence spectrometry and calorimetric investigations. We have found that the cold-denatured form of PGK has a conformation like a random coil at the θ point. The radius of gyration amounts to 7.8 nm, corresponding to a mean end-to-end distance $\langle r_{ee}^2 \rangle^{0.5} = 19.1$ nm. The persistence length is 1.74 nm. The equilibrium transition proceeds in two steps. The fact that cold-denatured PGK adopts an unordered conformation and the observed slow relaxation times make this protein very suitable for kinetic investigations of the unfolding and refolding processes as reported in the following.

MATERIALS AND METHODS

Yeast 3-phosphoglycerate kinase (EC 2.7.2.3) was purchased from Boehringer Mannheim, FRG. All measurements were performed in 20 mM sodium phosphate buffer, pH 6.5, containing 0.7 M GuHCl, 10 mM EDTA, and 1 mM DTT. In the presence of 0.7 M GuHCl, PGK is destabilized in such a way that cold denaturation occurs predominantly at temperatures above 0 °C. Details concerning the preparation of the samples are described in the preceding paper (Damaschun et al., 1993). Kinetic traces of both the ellipticity at 220 nm and the Stokes radius, R_S, were recorded after temperature jumps (T-jumps) within the temperature range between 30 °C, the temperature of maximum stability, and 1 °C. At 30 °C, the protein is in a quasinative state with a Stokes radius of 3.1 nm. At 1 °C, the unfolding is not yet complete, but the protein molecules are already in an expanded, random-coil-like conformation with a Stokes radius of about 5 nm (Damaschun et al., 1993). For technical reasons, we did not use temperatures below 1 °C. Before a T-jump was performed, the samples were incubated at the initial temperature for some hours. Because of the two-step nature of the unfolding/refolding transitions observed in equilibrium studies (Griko et al., 1989; Damaschun et al., 1993), kinetic experiments were also carried out at intermediate temperatures between 30 and 1 °C, particularly at 5 and 15 °C.

Circular Dichroism. Kinetic CD measurements were done in a Jasco J-720 instrument operating in the time-scan mode at a wavelength of 220 nm. HELLMA Suprasil cuvettes equipped with a flow-through jacket for temperature control (type 165-QS) with 0.5-mm or 1-mm optical path length were used. Temperature jumps were performed by switching the flow from a thermostat adjusted to the initial temperature, T_i, to a thermostat adjusted to the final temperature, T_f. Using this technique, the sample approaches the final temperature within times between 10 and 20 s. The ellipticity was recorded in constant time intervals of 5 or 10 s. Typically, one kinetic record consisted of up to 1000 data points. Additionally, we have recorded the CD spectra within the wavelength range between 210 and 250 nm before and after each kinetic experiment. Protein concentrations between 0.3 and 0.75 g/L were used.

Dynamic Light Scattering. The DLS apparatus was basically the same as for the equilibrium investigations (Damaschun et al., 1993). The particular modifications for kinetic investigations and the attainable time resolution in the measured Stokes radius, R_S, have been described in detail in a previous paper (Gast et al., 1992). An argon laser operating at the 514.5-nm wavelength and at a power of about 0.5 W was used. All measurements were done at a scattering angle of 90°, where it is possible to use rectangular flow-through microcells with a sample volume of 100 μL (Hellma, FRG). The T-jump was performed in a somewhat different way compared to the CD measurements. The sample cell was carefully flushed with filtered buffer. Either 20- (Biotage Europe, U.K.) or 100-nm-pore-size filters (Sartorius, FRG) were used. The sample cell was then mounted in the thermostated cell holder, the temperature of which was adjusted to T_f. About 1 mL of the protein solution was incubated in a syringe at the initial temperature, T_i. Protein concentrations ranged from 1.0 to 2.2 g/L. At the beginning of the kinetic experiment, the solution was rapidly injected through the filter into the scattering cell. The protein solution adopted the final temperature, T_f, within times of less than 20 s. Time-autocorrelation functions of the scattered light intensity and the total scattered intensity, I, were then recorded in constant time intervals, t_A, the data acquisition time. Data acquisition times between 5 and 40 s were suitable for getting sufficient time resolution and a good signal-to-noise ratio for the present purpose. The translational diffusion coefficient, D_T, and the Stokes radius, R_S, which is related to D_T by the Stokes–Einstein equation, were determined from the autocorrelation functions either by using the cumulant method (Koppel, 1972) or by performing the inverse Laplace transformation with the program CONTIN (Provencher, 1982).

Treatment of Kinetic Data. The absolute values of $\theta(t)$ and $R_S(t)$, respectively, are important for direct comparison with equilibrium data. To compare the kinetic CD data with the corresponding DLS results, it is more useful to transform the measured time dependences of θ and R_S into a normalized form. The ellipticities at 220 nm, measured at time t after the T-jump, were transformed into $\mathrm{CD}(t) = (\theta(t) - \theta(\infty))/(\theta(0) - \theta(\infty))$, where $\theta(0)$ and $\theta(\infty)$ are the ellipticities at $t = 0$ and at long times, when a steady-state value is reached, respectively. An equivalent form, $r(t) = (R_S(t) - R_S(\infty))/(R_S(0) - R_S(\infty))$, was used for the Stokes radius. $R_S(0)$ was taken from corresponding equilibrium measurements because it is not directly measurable during a kinetic experiment. The advantage of these transformations is that both $\mathrm{CD}(t)$ and $r(t)$ are always positive and decaying functions of time irrespective of whether an increase or a decrease in θ or R_S is obtained during the experiment.

The time constants for the unfolding and refolding processes, τ_i, were estimated by fitting $\mathrm{CD}(t)$ and $r(t)$ by a sum of exponential functions, e.g., $\mathrm{CD}(t) = \sum_{i=1}^{N} A_i \exp(-t/\tau_i)$, where up to three components were used. In some cases, particularly when long time constants, τ_i, were observed, a preaveraging over individual data points was performed prior to data evaluation.

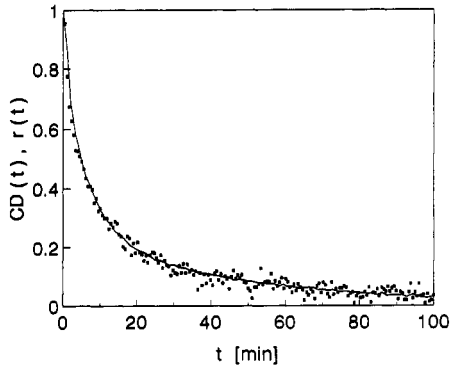

FIGURE 1: Time dependence of the normalized changes in CD measured at 220 nm, CD(t) (—), and in the Stokes radius, $r(t)$ (■), upon unfolding after a T-jump from 30 to 1 °C. Protein concentrations of 0.73 and 1.7 g/L were used for the CD and DLS measurements, respectively.

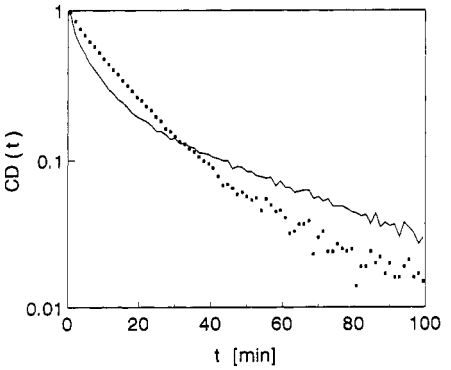

FIGURE 2: Normalized changes in CD at 220 nm measured after T-jumps from 30 to 1 °C (—) and from 30 to 5 °C (■). The protein concentration was 0.73 g/L in both cases.

Table I: Kinetic Parameters for Unfolding and Refolding of PGK[a]

expt	T-jump (°C)	probe	A_1	τ_1 (min)	A_2	τ_2 (min)	A_3	τ_3 (min)
unfolding	30 → 1	CD	0.16	<0.5	0.62	6.6 ± 0.7	0.22	46 ± 5
	30 → 1	DLS	0.21	<0.5	0.55	7.2 ± 0.7	0.24	45 ± 10
	30 → 5	CD	0.05	<0.5	0.86	13.8 ± 1.4	0.10	53 ± 10
	30 → 5	DLS			1	18 ± 2		
	5 → 1	CD	0.16	<0.5			0.84	42 ± 10
refolding	1 → 30	CD	0.40	≤0.3	0.60	4.4 ± 0.5		
	1 → 30	DLS			1	4.3 ± 0.5		
	1 → 15	DLS			0.25	2.0 ± 0.3	0.69	40 ± 0.5
	1 → 5	CD	0.48	<0.5	0.52	14 ± 2		

[a] τ_i and A_i are results of fitting both CD(t) and $r(t)$ by a sum of exponentials, $A_i \exp(-t/\tau_i)$; the error in the amplitudes of the A_i values is about ±0.05.

RESULTS

Unfolding at 1 and 5 °C. The traces of the normalized changes of ellipticity, CD(t), and of the Stokes radius, $r(t)$, upon unfolding at 1 °C are shown in Figure 1. CD(t) and $r(t)$ exhibit a remarkable coincidence. This indicates that the rupture of the secondary structure proceeds in parallel with the expansion of the entire protein molecule. Both curves deviate considerably from a single-exponential function (see also Figure 2). Thus, processes with rather different time constants are involved. Fitting these curves by a sum of exponentials yielded three components. The time constants and the corresponding amplitudes are shown in Table I. Both CD and DLS data provide a minor fast component decaying within the dead time of the T-jump technique. The values of τ_1 in Table I are upper limits. The essential changes proceed slowly with two time constants within the time range of minutes. According to the data shown in Table I, we could not detect significant differences in the time constants of the changes in the Stokes radius and in the ellipticities within experimental error. At 5 °C, a different unfolding behavior was observed. To demonstrate the differences in the unfolding processes at 5 and 1 °C, we have plotted the logarithm of CD(t) versus time for both temperatures in Figure 2. In contrast to the pronounced multiexponential behavior at 1 °C, CD(t) at 5 °C decayed predominantly with a time constant of 13.8 min (Table I). Additionally, a weak component with a time constant of 53 min was obtained. Again, a few percent of the signal decayed in times comparable with the dead time. A similar behavior was found for $r(t)$ at 5 °C (data not shown). Because the plot of $\ln(r(t))$ versus time was essentially linear, the data were fitted by a single exponential with a time constant of 18 min (Table I).

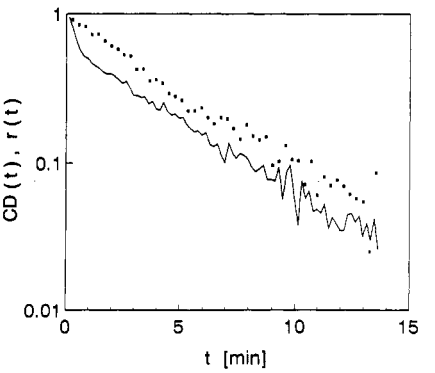

FIGURE 3: Kinetics of refolding monitored by measuring CD(t) (—) and $r(t)$ (■) after a T-jump from 1 to 30 °C. Protein concentrations of 0.73 and 1.7 g/L were used for the CD and DLS measurements, respectively.

Refolding at 30 and 15 °C. The time dependences of CD(t) and $r(t)$ upon refolding at 30 °C are shown in a semilogarithmic plot in Figure 3. In contrast to unfolding, there is a remarkable difference between CD(t) and $r(t)$, particularly at short times. $r(t)$ changed essentially with only one time constant of 4.3 min. It is noteworthy that $r(t)$ in Figure 3 was normalized using $R_S(0) = 5.0$ nm according to the equilibrium data. An extrapolation of $r(t)$ to 0 time yields $r(0) \approx 1$. Thus, there is no indication for a fast component. In contrast to this, nearly 40% of CD(t) decayed rapidly within the dead time of the T-jump. At long times, CD(t) changed nearly parallel with $r(t)$ with a time constant of 4.4 min. The kinetic parameters for refolding at 30 °C are summarized in Table I.

Having in mind the two-step nature of the equilibrium unfolding/refolding transition, we at first did not expect the appearance of only one time constant for the changes of the Stokes radius upon refolding at 30 °C. Therefore, we have studied refolding at 15 °C, too. At this temperature, where the equilibrium transition, appearing at lower temperatures, is complete and the other one is still proceeding, we expected most pronounced differences in the refolding kinetics. Indeed, a biphasic behavior was observed at 15 °C (Table I).

T-Jump Experiments between 1 and 5 °C. In the experiments described above, the kinetic traces were predominantly effected by the first stage of unfolding. To get further results that come from the second stage alone, we have performed T-jump experiments between 5 and 1 °C. After a T-jump from 5 to 1 °C, the ellipticity decreased with a time constant of 42 min. This is practically the same value as for the second component after a T-jump from 30 to 1 °C. After a T-jump from 1 to 5 °C, a biphasic increase of the ellipticity was

observed. Nearly 50% of the change in the CD signal occurred within the dead time. The final value was approached slowly with a time constant of 14 min (Table I).

DISCUSSION

Yeast PGK consists of two well-separated structural domains of nearly equal size (Watson et al., 1982). Folding/unfolding transitions of multidomain proteins are often characterized by the appearance of partially folded equilibrium intermediates (Privalov, 1982; Jaenicke, 1987, 1991). The existence of such folding intermediates (Murphy et al., 1992) depends on the stability and the mutual interactions of the individual domains. From equilibrium unfolding investigations (Griko et al., 1989; Damaschun et al., 1993), there is clear evidence for a two-step conformational transition upon cold denaturation of PGK, pointing to differences in the stability of the N-terminal and C-terminal domains at low temperatures. The N-terminal domain unfolds in the temperature range between 20 and about 7 °C, whereas unfolding of the C-terminal domain is observed at temperatures below 7 °C. These differences in the stability of the two domains are also clearly manifested in our kinetic unfolding and refolding studies.

Unfolding upon Cold Denaturation. After a *T*-jump from 30 to 1 °C, the unfolding process revealed three distinct phases, according to the changes of both CD and R_S (Figure 1; Table I). In the following we will show that the dominant phase, the time constant of which is about 7 min, can be attributed to the unfolding of the N-terminal domain, while the slowest phase ($\tau \approx 45$ min) represents the unfolding of the C-terminal domain. This distinction is based on the fact that the less stable N-terminal domain unfolds at higher temperatures than the C-terminal domain, as is known from equilibrium unfolding experiments. The different time constants, τ, or rate constants of unfolding, $k_u = 1/\tau$, result from the different unfolding conditions for both domains at this temperature. The unfolding conditions are strong for the N-terminal domain, but considerably weaker for the C-terminal domain, which is still within the unfolding transition region at 1 °C. These findings are also supported by the unfolding experiments at 5 °C (Figure 2; Table I), where partial unfolding of the C-terminal domain could only be detected as an insignificant slow phase in CD experiments. At 5 °C, the time constant of unfolding of the N-terminal domain is enlarged and amounts to 13.8 and 18 min for the changes in CD and R_S, respectively. Furthermore, kinetic CD experiments after a *T*-jump from 5 to 1 °C yielded essentially only one slow phase with a time constant of 42 min. This gives further evidence that the slowest phase, observed upon unfolding at 1 °C, is caused by structural changes of the C-terminal domain alone. The origin of the weakly pronounced fast phase decaying within the dead time of the *T*-jump is not yet clear. For reasons discussed below, we believe that it is connected with conformational changes within the hinge region.

A remarkable result of the unfolding experiments is the coincidence of the changes in the Stokes radius and the ellipticity, as is particularly shown for unfolding at 1 °C (Figure 1). In interpreting this result, we will disregard the fastest component at first. According to the observed coincidence of CD(t) and $r(t)$, the disruption of elements of local (secondary) structure is strongly coupled with the loosening of the global structure. Obviously, the secondary structure is protected as long as particular long-range interactions exist. This applies to both domains, since coincidence of CD(t) and $r(t)$ was observed within the time range of both slow phases. According to our results, unfolding of the two domains proceeds independently. The ability of both domains to unfold and refold independently has been shown by Adams et al. (1985) in GuHCl-induced unfolding/refolding experiments.

The interpretation of the fastest phase, the amplitude of which is only small, is more difficult and less straightforward. As already mentioned above, it probably reflects structural changes of the hinge region, e.g., of interdomain helix V (Watson et al., 1982). Helix V is less involved in long-range interactions with the two domains than other secondary structure elements and therefore may fold and unfold rapidly and mostly independently of the two domains. This could further result in a rapid change of the interdomain distance.

A particular feature of the cold denaturation-induced unfolding of PGK is its slowness. Similar long time constants have been observed upon cold denaturation of metmyoglobin (Cho & Chan, 1984) and mutants of phage T4 lysozyme (Chen et al., 1989, 1992). Kinetic experiments on the GuHCl-induced unfolding of yeast PGK and of the isolated domains have been reported by Missiakas et al. (1992). The time constants were also within the time range of minutes and were strongly dependent on denaturant concentration. In the present case, the time constants of unfolding and refolding are strongly dependent on temperature. For the kinetic phase, which is caused by folding of the N-terminal domain, a maximum value of about 50 min (data not included in Table I) was obtained in the vicinity of 12 °C. Our data are consistent with a V-shaped dependence of the relaxation rate on the reaction coordinate (temperature in this case), as is frequently observed in unfolding/refolding reactions. But we have to complete the data before calculating activation free energies of the transition state existing between the native state and the intermediate state with the N-terminal domain unfolded and the C-terminal domain folded. Slow rates and a biphasic transition have also been observed upon urea-induced unfolding and refolding of the two-domain protein γII-crystallin (Rudolph et al., 1990).

Refolding after Cold Denaturation. Refolding of PGK from an unordered, random-coil-like conformation to a compact, quasinative state can be monitored best after a *T*-jump from 1 to 30 °C (Figure 3). By inspection of Figure 3, it becomes evident that refolding of PGK after cold denaturation occurs in a substantially different way compared to unfolding. According to the observed change of the Stokes radius, the increase in compactness proceeds essentially with only one time constant of 4.3 min. Particularly, no fast phase reflecting a rapid collapse was observed. In contrast to this, there is a rapid increase in the ellipticity reflecting the formation of an essential part of ordered secondary structure in times comparable with or shorter than the dead time of the *T*-jump. The fast phase amounts to about 40% of the total increase observed during the refolding process. The remaining 60% of the ellipticity is regained with a time constant of 4.4 min, in parallel with the decrease of the Stokes radius. Fast formation of secondary structure was also observed after a *T*-jump from 1 to 5 °C (Table I), where half of the total increase was reached within the dead time. Thus, a transient intermediate state containing an appreciable amount of secondary structure is formed at an early stage of folding. Such fast recovery of secondary structure, as revealed by far-UV CD, has been observed in refolding studies with different proteins (Kuwajima et al., 1985, 1987, 1988; Sugawara et al., 1991; Elöve et al., 1992). Sugawara et al. (1991) have summarized data on the restoration of secondary structure in various proteins at the first stage of refolding measured by

far-UV CD. Percentages from 30% up to even more than 100% (e.g., for β-lactoglobulin) compared to the CD of the native protein have been observed, showing that the extent of secondary structure formation varies strongly with protein species. But the understanding of how such elements of secondary structure fit into the overall conformation of the polypeptide chain is essentially incomplete. Information about this problem is becoming available from hydrogen-exchange labeling techniques (Udgaonkar & Baldwin, 1988; Roder et al., 1988; Radford et al., 1992). In particular, it is unclear whether these elements exist within a still rather expanded or an already collapsed conformation of the protein. As the amount of rapidly formed secondary structure varies considerably with protein species, the global structure of the intermediate may also be rather different for various proteins. Thus, we consider our results typical of a particular class of proteins.

The transient intermediate state found in refolding studies at 30 °C is far from being comparable to that with the C-terminal domain folded and the N-terminal domain unfolded, as observed in kinetic unfolding and equilibrium unfolding experiments. The Stokes radius of the intermediate state during refolding at 30 °C is nearly as large as that of the cold-denatured protein at 1 °C. Hence, the rapidly formed secondary structure elements exist within an essentially extended conformation. This state resembles a particular equilibrium intermediate defined by Murphy et al. (1992), namely, the so-called extended folding intermediate, which is characterized by the existence of secondary structure elements exposed to solvent. Further secondary structure formation toward native conformation proceeds with the same time constant as the compactness increases. At 30 °C, this process turned out to be monophasic. Thus, we could detect neither independent folding of the two structural domains nor further intermediate states upon refolding. On the other hand, independent refolding of the two domains was observed at 15 °C (Table I) as monitored by the biphasic change of R_S. The refolding time constant of the C-terminal domain was estimated to be 2 min. According to the final Stokes radius of 3.5 nm, refolding of the N-terminal domain appeared to be not yet complete and proceeded slowly with a time constant of 40 min. These results indicate the existence of a second transient intermediate with a folded C-terminal domain and an unfolded N-terminal domain upon refolding at 15 °C.

Kinetic refolding studies of PGK after cold denaturation are not reported in the literature. Therefore, we will compare our results with refolding studies after denaturation by GuHCl (Semisotnov et al., 1991; Betton et al., 1992; Missiakas et al., 1992, Ballery et al., 1993). The refolding process resembles in many aspects that observed after cold denaturation. A rapid and a slow phase with time constants in the range of minutes have been observed by means of far-UV CD measurements (Betton et al., 1992; Missiakas et al., 1992; Ballery et al., 1993), pointing to the existence of an early intermediate state. It is reasonable to assume that the intermediate state observed by us is very similar to that observed in refolding investigations after unfolding by GuHCl. Additionally, Missiakas et al. (1992) have studied refolding of the isolated domains. Half-times of refolding within the time range of minutes were also found by Semisotnov et al. (1991) following the changes of the absorbance at 292 nm, of the tryptophan fluorescence, and of the binding affinity of the hydrophobic fluorescence probe ANS toward protein molecules. Ptitsyn et al. (1990) have shown for a number of proteins that ANS binding is indicative of the formation of the molten globule state. Accordingly, Semisotnov et al. (1991) concluded that an early intermediate is accumulating during refolding of PGK, which has a pronounced secondary structure and high compactness but no rigid tertiary structure. A similar conclusion on the nature of the kinetic intermediate has also been drawn by Betton et al. (1992). The assumption of an early intermediate state with high compactness is not consistent with our data. Ballery et al. (1993) have studied the accessibility of naturally occurring and genetically introduced cysteinyl residues during refolding. They also postulate that the observed intermediate has characteristics of the molten globule state. But an essential result of their work is that the intermediate is in equilibrium with an unfolded form which remains highly populated until the protein adopts its fully folded form. This emphasizes the importance of direct measurements of the compactness of intermediates on the folding pathway.

Double-jump experiments performed according to Brandts et al. (1975), Semisotnov et al. (1991), and Missiakas et al. (1992) have shown that proline cis–trans isomerization can essentially be ruled out as a reason for the long time constants of refolding.

Implication for Existing Models of Protein Folding. In summary, our results are consistent with the following picture. The starting point of folding is a random-coil-like structure of the polypeptide chain. At the first stage, secondary structure elements are formed rapidly that are fully accessible to solvent. The dimensions of the polypeptide chain remain essentially unchanged. Thus, we have to assume that the contraction of the chain caused by the formation of secondary structure is just compensated by the stiffening of the chain. This behavior is consistent with the predictions of the framework model. Further formation of secondary structure proceeds at a second stage entirely parallel to the condensation of the protein. This process is slow compared to the formation of the initial secondary structure elements. However, it is useless to speculate on whether formation of secondary structure at the second stage effects the condensation or hydrophobic collapse-driven condensation causes secondary structure formation due to the reduced volume available to the polypeptide chain. The second alternative is consistent with particular predictions of the hydrophobic collapse model. The hydrophobic collapse model postulates the existence of a contracting disordered globule, intermediate between the expanded random-coil structure and the native structure. In any case, the formation of a molten globule-like state is only likely at the end of the second stage when a sufficiently high degree of compactness is reached. More evidence is expected from experiments with different proteins, including single-domain proteins, using the techniques described in this paper.

ACKNOWLEDGMENT

The authors thank Mrs. D. Otto for skillful technical assistance.

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