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Kinetic traps in lysozyme folding

(protein folding pathways/folding intermediates)

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ABSTRACT **Folding of lysozyme from hen egg white was investigated by using interrupted refolding experiments. This method makes use of a high energy barrier between the native state and transient folding intermediates, and, in contrast to conventional optical techniques, it enables one to specifically monitor the amount of native molecules during protein folding. The results show that under strongly native conditions lysozyme can refold on parallel pathways. The major part of the lysozyme molecules (86%) refold on a slow kinetic pathway with well-populated partially folded states. Additionally, 14% of the molecules fold faster. The rate constant of formation of native molecules on the fast pathway corresponds well to the rate constant expected for folding to occur by a two-state process without any detectable intermediates. The results suggest that formation of the native state for the major fraction of lysozyme molecules is retarded compared with the direct folding process. Partially structured intermediates that transiently populate seem to be kinetically trapped in a conformation that can only slowly reach the native structure.**

Experimental studies on the mechanism of protein folding have focused on the elucidation of kinetic pathways for unfolding and refolding reactions of small single-domain proteins. Refolding starting from the completely denatured state is often complex due to two major phenomena: (*i*) heterogeneity in the unfolded state leads to several distinct species of unfolded molecules that can refold on parallel pathways and (*ii*) partially folded states form during refolding and are subsequently converted into native molecules (for reviews, see refs. 1–6). Characterization of the structure and of the stability of partially folded states is believed to provide a key to understanding the mechanism of protein folding. However, recent results, mainly from theoretical studies, have questioned the importance of populated intermediate states for protein-folding pathways. These results, rather, suggest that partially folded states are trapped in nonproductive conformations and postulate the existence of faster folding channels (7–15).

One of the best characterized model systems for protein folding is lysozyme from hen egg white, where a large variety of experimental data is available. About 10% of unfolded lysozyme refolds very slowly in a reaction that is limited by cis → trans isomerization of one or both of the two trans Xaa-Pro peptide bonds (16)†. Refolding of the remaining molecules does not seem to involve prolyl isomerization reactions. Several partially folded states have been observed during refolding of these molecules. In an initial very rapid phase ($\tau < 1$ ms) hydrophobic collapse occurs, leading to a large change in the far-UV CD signal and to a change in the tryptophan fluorescence emission (17–20). Subsequently, well-defined partially folded structures populate during refolding. Comparison of amide hydrogen-exchange experiments analyzed by MS and by two-dimensional NMR spectroscopy revealed that in ≈80% of refolding lysozyme, part of the amide protons (those in the α domain) are forming hydrogen bonds on the time scale of 10–50 ms, whereas the amide protons in the β domain become protected from exchange in a slower folding reaction ($\tau \approx 400$ ms), concomitantly with the acquisition of the native tertiary structure (19–21). Interestingly, in the remaining 20% of the refolding molecules the entire hydrogen-bonding network forms fast ($\tau \approx 10$ ms). This result seemed to contradict results from fluorescence and near-UV CD experiments, which suggested that native lysozyme forms in a single slow kinetic step (19–21) and led to the conclusion that this rapidly formed structure with the full set of hydrogen bonds represents a partially folded intermediate still lacking the native tertiary interactions (20).

To gain additional insight into the mechanism of protein folding it would be important to find out whether all molecules become native in a single rate-limiting step or whether part of refolding lysozyme molecules actually reach the native state in a faster reaction. The latter would imply that the observed partially folded states are not obligatory for folding. Previous studies on the folding of lysozyme used either conventional spectroscopic techniques or hydrogen/deuterium-labeling methods to investigate the folding mechanism. These methods do not, however, necessarily allow specific monitoring for the formation of native molecules during refolding. An experimental problem with conventional spectroscopic measurements is the difficulty of determining the maximum expected signal change between native and unfolded molecules because the signal of the unfolded protein at high concentrations of denaturant has to be extrapolated over a wide concentration range back to refolding conditions. Thus, very fast reactions with only small amplitudes occurring within the dead time of mixing can easily be missed. Additionally, native-like partially active intermediate states, which have been observed to form transiently during refolding of several proteins, are often difficult to distinguish from native molecules by conventional spectroscopic methods. Hydrogen-exchange experiments, on the other hand, monitor the protection of amide protons as they become entrapped by developing secondary structure. Due to experimental restrictions the largest protection factors that can be measured in these experiments are usually 10^3, whereas protection factors in native proteins are often 10^7–10^8. This fact often makes it impossible to decide whether formation of a relatively stable folding intermediate or of completely native protein is being monitored.

To detect possible faster channels in lysozyme refolding I used the technique of interrupted refolding experiments that monitor the absolute amount of native molecules formed at different times in the refolding process. The results show that most native lysozyme is formed in a kinetic reaction that is identical with the slowest process seen in previous kinetic studies. However, some of the molecules reach the native state

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†The following description of the observed folding reactions will only refer to molecules whose refolding is not limited by prolyl isomerization. Under strongly native conditions refolding of a small fraction of molecules is limited by cis → trans isomerization reactions at prolyl peptide bonds, giving rise to a very slow refolding pathway with a relaxation time in the range of 20 s.

in a significantly faster reaction. The formation rate of native molecules on this fast refolding channel corresponds to the rate expected for folding to occur in the absence of populated intermediate states.

MATERIALS AND METHODS

Materials. Hen egg white lysozyme and *N*-acetyltryptophanamide were purchased from Sigma. GdmCl (ultra pure) was from United States Biochemical and was used without further purification. All other chemicals were reagent grade and were purchased from Merck.

Methods. *Interrupted refolding experiments.* The formation of native protein was monitored by diluting completely unfolded lysozyme (in 3.6 M GdmCl/20 mM glycine·HCl, pH 1.8) 6-fold into final conditions of 0.6 M GdmCl/1.5 M GdmCl or 3.1 M GdmCl/20 mM NaOAc, pH 5.2 to initiate refolding. After various times (t_i) refolding was interrupted by transferring the solution into final conditions of 5.3 M GdmCl/20 mM glycine·HCl, pH 1.8. Under these conditions native lysozyme unfolds completely with a relaxation time of 20 s, while any partially folded intermediates unfold within a few milliseconds. The amplitude of the slow unfolding reaction is thus a measure for the amount of native protein present after time t_i, when refolding was interrupted. The observed unfolding amplitudes after various times of refolding (t_i) were normalized against the amplitude of completely refolded lysozyme to yield the fraction of native molecules that were present after t_i. The temperature was 20°C in all steps.

In control experiments a solution of *N*-acetyltryptophanamide in the same molar concentration as the tryptophan residues in the lysozyme solution (22.8 μM) was measured under the same experimental conditions as described above to detect possible mixing artifacts. Mixing was found not to interfere with the measurements. Completeness of unfolding in the initial unfolded lysozyme solution was checked by diluting unfolded lysozyme (in 3.6 M GdmCl/20 mM glycine·HCl, pH 1.8) into the same conditions used to detect unfolding of the native protein (5.3 M GdmCl/20 mM glycine·HCl, pH 1.8). In this case no unfolding reaction was observed, which rules out detectable amounts of native protein under the initial unfolding conditions.

Interrupted refolding experiments at 0.6 M and at 1.5 M GdmCl were done on an Applied Photophysics (Surrey, U.K.) model SX-17MV stopped-flow instrument equipped with double mixing facility. The final unfolding step was detected by the change at >300 nm in tryptophan fluorescence emission after excitation at 280 nm. The concentration of lysozyme in the final unfolding step was 3.8 μM. Kinetics for each time point were recorded at least four times with almost identical amplitudes and rate constants (±10% of the absolute values). The average amplitude of the kinetic runs was used. Unfolding assays at 3.1 M GdmCl were done by manual mixing, and the final unfolding step was detected by using a Hitachi F-4010 fluorimeter. Excitation was at 280 nm (2.5-nm band width), and fluorescence emission was monitored at 350 nm (10-nm band width).

GdmCl dependence of folding rates. The GdmCl dependence of the refolding/unfolding rates was done by diluting completely unfolded protein or native protein into 20 mM NaOAc, pH 5.2, containing the appropriate concentration of GdmCl at 20°C. Fast reactions ($\tau < 10$ s) were measured by using stopped-flow mixing in an Applied Photophysics SX17-MV instrument, whereas slow kinetics were monitored by manual mixing in a Hitachi F-4500 fluorimeter. Under conditions where the reactions could be monitored both by manual and by stopped-flow mixing; both methods gave identical rate constants. The data were fitted according to the two-state model (6, 22, 23) in the region where rate constants both for unfolding and for refolding showed linear dependence on GdmCl concentration ($C_{GdmCl} > 2.5$ M).

$$N \underset{k_f}{\overset{k_u}{\rightleftharpoons}} U \qquad k_{app} = k_u + k_f \qquad [1]$$

$$\log(k_{app}) = \log[k_u^0 \times \exp(m_u \times C) + k_f^0 \times \exp(m_f \times C)], \qquad [2]$$

where k_{app} represents the measured apparent rate constant, and k_u and k_f are the unfolding and refolding rate constants, respectively. k_u^0 and k_f^0 are the respective rate constants at zero denaturant, and m_u and m_f are the slopes of the GdmCl dependence of the logarithm of the unfolding and folding rate constants, respectively. C is the concentration of GdmCl.

Data fitting. For data fitting the programs KALEIDAGRAPH (Synergy Software, Reading, PA), KINFIT (OLIS, Jefferson, GA), and the software provided with the Applied Photophysics stopped-flow instrument were used.

RESULTS

Refolding of lysozyme has previously been extensively studied by a variety of different methods (18–21). Fig. 1 shows refolding kinetics of lysozyme in 20 mM NaOAc, pH 5.2, containing either 0.6 M GdmCl or 1.5 M GdmCl monitored by the change in fluorescence emission. In a fast reaction ($\tau = 30$ ms at 0.6 M GdmCl and 90 ms at 1.5 M GdmCl) a major decrease in fluorescence emission intensity below the level of both the native and the denatured protein is observed. The final fluorescence intensity is reached in a slow step with a relaxation time of 360 ms at 0.6 M GdmCl and of 1000 ms at 1.5 M GdmCl. To measure specifically the formation of native molecules, interrupted refolding experiments were done (6, 24, 25). These experiments make use of the finding that the native state of a protein is separated from partially folded states on the refolding pathway by a high energy barrier and that it thus unfolds far more slowly than any nonnative structures (26). Interrupted refolding experiments start from completely unfolded protein, which is diluted into native solvent conditions to initiate refolding. Folding is stopped after various times by transferring the solution into conditions where the native protein unfolds slowly but any partially folded intermediates unfold rapidly. The amplitude of the slow unfolding reaction is thus a measure for the amount of native protein that was present when refolding was interrupted. Plotting the amplitude of the slow unfolding reaction versus the time when refolding

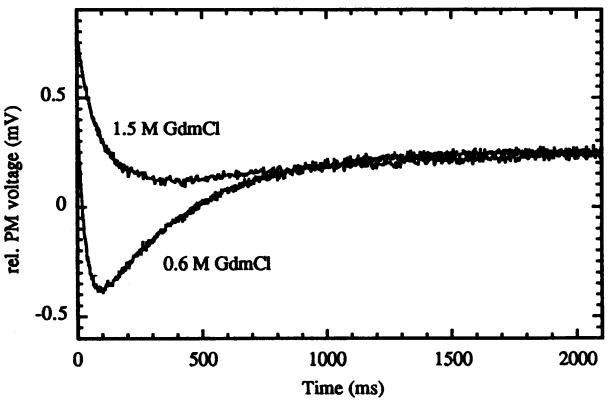

FIG. 1. Refolding lysozyme in the presence of 0.6 M and 1.5 M GdmCl detected by the change in tryptophan fluorescence emission >300 nm after excitation at 280 nm. Refolding conditions were 20 mM NaOAc, pH 5.2 at 20°C. A double-exponential fit of the data gave relaxation times of 30 ± 5 ms and of 380 ± 10 ms for refolding at 0.6 M and of 90 ± 5 ms and 1000 ± 50 ms at 1.5 M GdmCl. PM, photomultiplier.

was interrupted gives the time course of formation of native molecules during refolding. The advantage of interrupted refolding experiments over conventional spectroscopic methods is the ability to (*i*) solely detect native molecules and (*ii*) unambiguously assign the signal of completely unfolded protein; namely, in the absence of native molecules, no unfolding reaction will be observed that has the same unfolding rate as native protein.

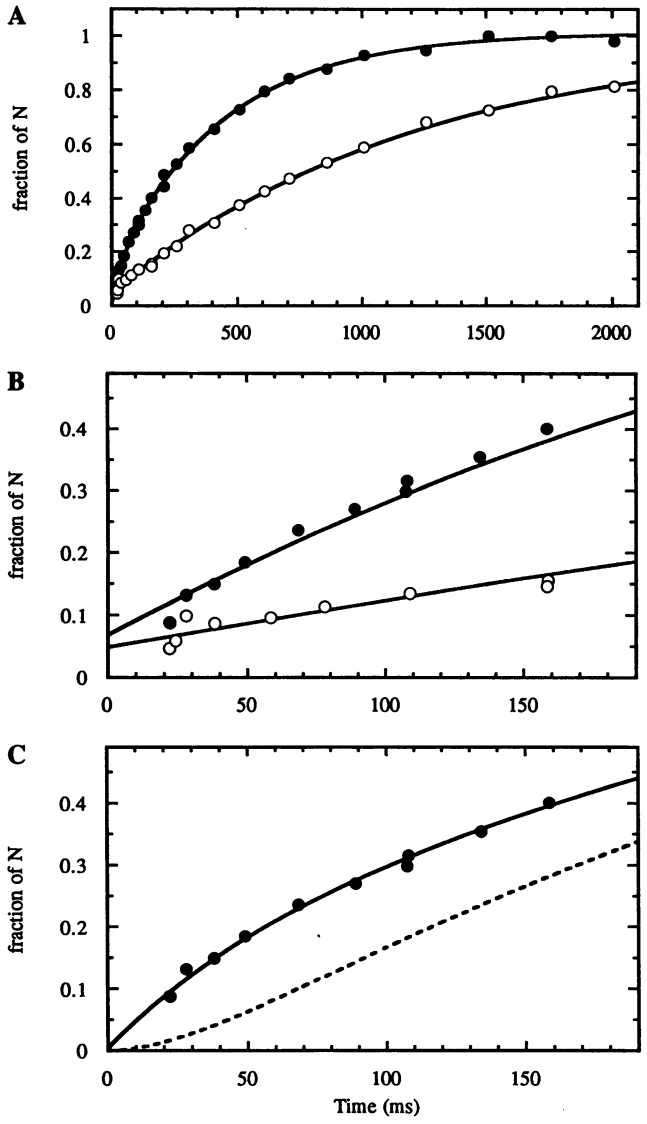

FIG. 2. (*A*) Time course of appearance of native molecules during refolding in 0.6 M (●) and 1.5 M (○) GdmCl measured by interrupted refolding experiments. Single-exponential fits of the data give values of τ = 385 (±20) ms (amplitude = 93 ± 2%) at 0.6 M GdmCl and of τ = 1100 (±20) ms (amplitude = 95 ± 2%) at 1.5 M GdmCl. Formation of native molecules at 0.6 M GdmCl is better described by a double-exponential fit with τ_1 = 420 (±10) ms (amplitude$_1$ = 86 ± 1%) and τ_2 = 50 (±10) ms (amplitude$_2$ = 14 ± 1%). The solid lines represent the double-exponential fit of the data at 0.6 M GdmCl and a single-exponential fit of the data at 1.5 M GdmCl. Refolding conditions were 20 mM NaOAc, pH 5.2 at 20°C. (*B*) Early-time region of *A* with the results from the single-exponential fits shown as solid lines. (*C*) Early-time region for formation of native molecules at 0.6 M GdmCl. The solid line represents the double-exponential fit of the data; the dotted line represents a simulated curve for the sequential formation of native molecules ($U \rightarrow I \rightarrow N$), with relaxation times of 30 ms for formation of the intermediate state and of 380 ms for formation of *N*, as seen in conventional spectroscopic measurements (compare with Fig. 1).

Fig. 2 shows the time course of formation of native lysozyme in 0.6 M GdmCl and in 1.5 M GdmCl measured by interrupted refolding experiments. Single-exponential fits of the data give relaxation times of 385 ms at 0.6 M GdmCl and of 1100 ms at 1.5 M GdmCl. These values correspond to the values observed for the slowest refolding reactions in direct optical measurements (compare with Fig. 1). However, a single-exponential fit does not account for the complete amplitude of formation of native molecules. Extrapolation of the fitted curves to zero time gives 7% and 5% of native molecules present at the beginning of refolding at 0.6 M and at 1.5 M GdmCl, respectively (Fig. 2*B*), suggesting that part of native lysozyme is formed in a faster reaction. Fitting the data for refolding at 0.6 M GdmCl to a double-exponential curve with relaxation times of 50 ms (amplitude = 14%) and of 420 ms (amplitude = 86%) increases the quality of the fit significantly and accounts for the complete amplitude of the formation of native molecules (Fig. 2*C*). This result shows that in addition to the previously observed slow step in formation of native lysozyme a second, faster refolding channel exists. Fig. 2*C* also shows a simulated time course of formation of native molecules, for the case that the observed partially folded states are part of a sequential folding pathway with a single rate-limiting step for the formation of native molecules, as postulated in previous studies (20). The large deviation of the experimental data from the simulated curve shows that this model is not in agreement with experimental data.

The data for refolding at 1.5 M GdmCl could not be fitted to a double-exponential curve, probably because the rate constants of the two reactions are too similar to be separated under these conditions (see *Discussion* below).

In control experiments completely unfolded protein was unfolded directly without a refolding step. In this case no unfolding reaction was observed, ruling out the existence of residual amounts of native protein in the starting solution.

The rate of the observed slow unfolding reaction of the native protein is independent of the time when refolding was interrupted and is identical to the rate observed for unfolding of native lysozyme (data not shown), confirming that the experiments monitor solely the formation of native molecules during lysozyme refolding.

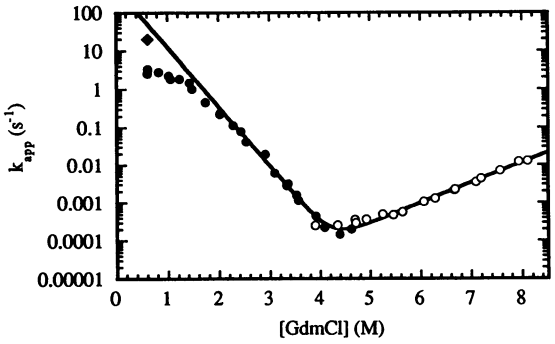

FIG. 3. GdmCl dependence of the observed unfolding (○) and refolding (●) rate (k_{app}) of lysozyme in 20 mM NaOAc, pH 5.2 at 20°C. (The transition region is between 3.6 M and 5 M GdmCl; data not shown.) Additionally, the apparent rate constant of the faster reaction in the formation of native molecules measured by interrupted refolding experiments (compare Figs. 1 and 2) at 0.6 M GdmCl is shown (◆). At low concentrations of GdmCl (< 2.5 M) an additional fast reaction corresponding to the formation of partially folded intermediates and a very slow, GdmCl-independent phase (τ = 23 ± 2 s, amplitude = 10–15%), probably caused by prolyl isomerization, is observed (16). The rate constants of these reactions are not displayed. The solid curve represents a fit of the data at GdmCl concentrations > 2.5 M according to Eqs. **1** and **2**. The fits gave values of (k_u^0) = 6.2 × 10^{-7} s^{-1}, m_u = 1.23 M^{-1}; (k_f^0) = 447 s^{-1}; m_f = −3.60 M^{-1}.

The unfolding and refolding rates of lysozyme depend linearly on GdmCl concentration over a wide concentration range (Fig. 3). Deviations from linearity are only observed at very low denaturant concentrations (<2.5 M GdmCl), where partially folded states are seen during refolding. To monitor the formation of native molecules under conditions where no partially folded intermediates can be detected, interrupted refolding experiments were done at 3.1 M GdmCl (data not shown). Under these conditions the formation of native molecules occurs in a single kinetic reaction with a relaxation time of 130 s and 100% amplitude.

DISCUSSION

Existence of a Fast Pathway for Formation of Native Molecules. Interrupted refolding experiments were done with lysozyme to specifically monitor the time course of formation of native molecules during refolding. The experiments show clearly that under strongly native conditions, where partially folded states populate during refolding, lysozyme can reach the native state via two different kinetic channels (Fig. 2). At 0.6 M GdmCl a significant fraction of the molecules (14%) become native in a fast reaction ($\tau = 50$ ms). This kinetic phase may correspond to a fast reaction seen in hydrogen-exchange experiments (19–21). Here, ≈20% of refolding lysozyme molecules become protected from amide proton exchange in both the α and the β domain very quickly ($\tau \approx 10$ ms). The data from the interrupted refolding experiments show that native molecules are formed in a kinetic reaction that occurs on a similar time scale and with a similar amplitude and suggest that both methods see the same process—namely, the formation of native molecules. Recent findings by the group of Dobson for refolding at lower temperatures are also in agreement with the presence of a second kinetic phase in the formation of native lysozyme. The predicted rate of this reaction at 20°C agrees well with the observed rate of the faster reaction from the interrupted refolding experiments (S. Radford, personal communication). The reason for the absence of a faster phase in inhibitor binding at 20°C might be that it is partially masked by a lag phase caused by binding of the inhibitor to native protein, which is a rather slow process (τ = 5–10 ms) under the given experimental conditions (20).

The major part of unfolded lysozyme (86%) reaches the native state on a slower pathway (τ = 420 ms). This reaction has previously been observed with a variety of different methods (20). Data from the interrupted refolding experiments confirm that this process actually leads to native protein and that no slower reactions are seen in the formation of native molecules on this pathway. On this slower folding channel partially structured states have been characterized (18–20). A major folding intermediate is formed with a relaxation time of 30 ms (Fig. 1) and is subsequently converted to native molecules. Hydrogen-exchange experiments show that on this pathway the α domain of lysozyme can fold very rapidly, whereas the formation of the β domain does not occur before the rate-limiting step (19, 27). This result suggests that the kinetic block for refolding on the slow pathway might be located within the β domain or at the contact site between the two domains.

If the partially folded state that is observed on the slower pathway were part of a sequential folding mechanism, a pronounced lag phase would be expected in the formation of native molecules (Fig. 2*C*). This lag phase is clearly not detected by unfolding assays or by inhibitor-binding studies (20). It is probably masked by the faster pathway for the formation of native molecules. The presence of a lag phase in the formation of native molecules on the slower pathway would, however, influence the kinetic analysis of the experimental data. In this case the faster phase is described by a relaxation time of 40 ms and an amplitude of 20%.

Fig. 3 shows that the rate of formation of native molecules on the faster pathway corresponds well to the rate expected from the linear extrapolation of the rate constants for unfolding and refolding at higher GdmCl concentrations. This result suggests that under strongly native conditions part of the molecules can refold by the two-state mechanism without any detectable intermediates, whereas other molecules become trapped on a slower pathway and have to refold on an alternative slower pathway. On the slower pathway partially folded structures can accumulate. It has recently been pointed out by Creighton (28) that folding for many proteins seems to be slowed down under conditions where partially folded intermediates form. Interestingly, for chymotrypsin inhibitor 2 a linear relationship between refolding rates and GdmCl concentration was found even at very low GdmCl concentrations (29). Under all refolding conditions the observed rate constants agree with values predicted by the two-state model. Furthermore, no partially folded states could be detected during refolding of chymotrypsin inhibitor 2, suggesting that all unfolded chymotrypsin inhibitor 2 molecules refold fast and according to the two-state mechanism. In lysozyme refolding only part of the molecules seem to be able to fold fast, whereas most of the unfolded molecules become trapped on slower pathways and form partially folded states.

Fig. 3 provides an explanation for why the formation of native molecules during refolding at 1.5 M GdmCl could not be fitted to a double-exponential curve. The rates of the two reactions that lead to native protein are predicted to differ by only a factor of ≈2 under these conditions, which makes it difficult to separate them. The missing amplitude in the single-exponential fit of the data suggests, however, that a faster reaction is present.

Origin of the Kinetic Heterogeneity. Two general models are feasible for the kinetic partitioning into a fast and a slow refolding channel. In the first model the species of unfolded molecules is a heterogeneous mixture of fast and slow refolding molecules (U_1 and U_2). As long as refolding of the fast molecules is rapid compared with the interconversion between these two species, folding will occur on parallel pathways:

$$\begin{array}{ccc} U_1 & \xrightarrow{\text{fast}} & N \\ {\scriptstyle\text{slow}}\uparrow\downarrow & & \\ U_2 & \xrightarrow{\text{slow}} & N \end{array} \quad [3]$$

A well-described example of heterogeneous unfolded states is cis–trans isomerization at prolyl peptide bonds, giving rise to very slow refolding steps (5, 30). A possible origin for a heterogeneity in the unfolded state of lysozyme could be cis–trans equilibria at non-prolyl peptide bonds. Theoretical studies predict very low cis contents at regular peptide bonds under equilibrium conditions, but the high number of these bonds in a protein leads to a high probability of molecules with at least one non-prolyl cis peptide bond. Recently a trans → cis isomerization reaction of a non-prolyl cis peptide bond was identified to be the rate-limiting step in refolding of a variant form of ribonuclease T1 (31).

It cannot be ruled out completely that one of the two trans prolyl peptide bonds in lysozyme causes the separation in fast and slow refolding molecules, although they are believed to give rise to the 10% very slow-folding molecules. However, the refolding rate of the slower folding reaction is much faster (τ = 400 ms) than rate constants observed for proline isomerization reactions (τ = 10–100 s at 20°C), and the amplitude of the fast reaction (14%) is much smaller than predicted for a prolyl residue which is trans in the native state (5).

The second possible mechanism leading to parallel refolding channels is kinetic partitioning during refolding. In this case refolding starts from a homogenous species, but part of the molecules can refold directly to the native state, while in a

competing reaction other molecules become trapped in partially folded states, which decelerates their rate of interconversion to the native state:

$$\begin{array}{ccc} & I & \\ \text{fast} \nearrow & & \searrow \text{slow} \\ U & \xrightarrow[\text{fast}]{} & N \end{array} \quad [4]$$

Under conditions where partially folded states are not stable (e.g., at elevated concentrations of GdmCl) all the molecules will refold on a single kinetic pathway as observed for lysozyme refolding.

A mechanism that has been discussed to give rise to kinetic traps in protein refolding is the threading of the folding protein chain through disulfide loops in polypeptide chains that contain overlapping disulfide bonds (32). Different possible isomeric arrangements of the loops in the unfolded chain could become trapped in the respective configuration in collapsed intermediate states. The interconversion between these isomers will involve reactions that are equivalent to untying knots in the chain, which might be a slow process. Lysozyme contains four disulfide bonds that form two pairs of overlapping loops.

It cannot be decided at this stage what the molecular origin of the kinetic heterogeneity in lysozyme refolding is. Examples for both models proposed have been described recently. In the case of ribonuclease T1 refolding a specific tertiary interaction between a tryptophan side chain and a proline residue in a folding intermediate slows down a proline-limited folding reaction. This effect can be relieved by replacing the bulky tryptophan by the smaller tyrosine side chain (33). In the oxidative refolding pathway of bovine pancreatic trypsin inhibitor, a native-like folding intermediate containing two disulfide bonds decelerates the formation of native protein with three disulfide bonds drastically (34–36). Both ribonuclease T1 and bovine pancreatic trypsin inhibitor refolding can be accelerated by destabilizing the partially folded states (33, 35). Assembly of collagen triple helices leads to mismatched chains and low refolding rates under strongly native refolding conditions (37). These examples show that nonnative interactions in folding intermediates can have adverse effects on the rates of protein folding.

In refolding of horse cytochrome *c* a slow refolding pathway with well-populated partially folded states was shown to be caused by nonnative heme ligands in the unfolded state. Under conditions that prevent these nonnative interactions in the unfolded chain folding is accelerated and does not seem to involve partially folded states (38).

Implications for the Folding Mechanism. The existence of a fast kinetic pathway for lysozyme folding fits well into the "new view" of protein folding that has recently been discussed in detail by Baldwin (39). On the basis of statistical mechanical considerations and on Monte Carlo simulations, theoreticians have been pointing out that the experimentally observed partially folded states might be partly misfolded structures caught in kinetic traps (7–15). According to these considerations folding can occur very fast and without any populated partially folded states. An argument that has been put forward against this theory is the finding that in the case of some proteins the same partially folded states are observed during refolding and at equilibrium under extreme solvent conditions (39). A simple explanation for this phenomenon might be that these structures can form in the absence of specific interactions and that they thus also populate under conditions where folding is slowed down by some additional processes. It will be important to find out whether kinetic traps are a general phenomenon in protein folding and whether the observed intermediate states in lysozyme refolding accumulate due to a heterogeneity in the unfolded chain or whether nonnative interactions are introduced by the formation of the partially folded states.

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