Direct Measurement of Nucleation and Growth Rates in Lysozyme Folding[†]

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ABSTRACT: A kinetic folding intermediate of hen lysozyme is shown to form in a nucleation/growth type of mechanism. Under native solvent conditions, a nucleated state is formed slowly during refolding (τ = 14 ± 1 ms at 0 M GdmCl) and is rapidly converted to the folding intermediate (τ = 300 ± 150 μs at 0 M GdmCl). Under these conditions the nucleated state represents a high-energy state compared to the folding intermediate ($\Delta\Delta G^0$ = 13.7 ± 3 kJ/mol). At elevated concentrations of GdmCl, the nucleated state becomes more stable than the intermediate and it consequently becomes transiently populated during unfolding of the intermediate state. This allowed us to measure the rate constant of the growth step using stopped-flow double-jump experiments. At high concentrations of GdmCl (>5 M), the growth step becomes rate-limiting in unfolding, leading to the frequently observed rollover in the GdmCl dependence of the logarithm of the apparent rate constant of the unfolding reaction.

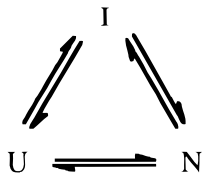
Recent theoretical (Bryngelson & Wolynes, 1989; Abkevich et al., 1994; Guo & Thirumalai, 1995; Wolynes et al., 1995) and experimental studies (Itzhaki et al., 1995; Kiefhaber, 1995) led to the revival of the nucleation/growth model for protein folding, popular in the 1960s and early 1970s (Tsong et al., 1972; Wetlaufer, 1973). According to this model, the rate-limiting step in protein folding is the formation of a nucleated species (Nu), which can rapidly proceed to the folded state (F) in a subsequent fast growth step.

$$\mathrm{U} \underset{\text{nucleation}}{\overset{\text{slow}}{\rightleftharpoons}} \mathrm{Nu} \underset{\text{growth}}{\overset{\text{fast}}{\rightleftharpoons}} \mathrm{F} \qquad (1)$$

In this mechanism, the growth reaction (Nu ⇌ F) may proceed through a number of rapidly interconverting intermediate states. These intermediates, as well as the nucleated state, will not become populated during refolding since their interconversion is faster than the formation of the nucleus. Evidence for this model comes from theoretical studies (Thirumalai, 1994) and from site-directed mutagenesis work on chymotrypsin inhibitor 2 (Itzhaki et al., 1995). Up to now, however, no direct experimental data on growth rates in protein folding are available.

The nucleation/growth model predicts that the nucleated state may become populated during unfolding starting from the folded state (Tsong et al., 1972). If the rapid equilibrium between the folded protein and the nucleated state (Nu ⇌ F) favors the folding nucleus under conditions where complete unfolding occurs, then the folded protein will rapidly be converted to the nucleated state at the beginning of the unfolding reaction. Unfolding of the nucleus, which is represented by the slow U ⇌ Nu equilibrium in eq 1, will be the final and rate-limiting step on the unfolding pathway. Consequently, the nucleated state will transiently populate during unfolding under these conditions. A similar kind of behavior was found by Pörschke and Eigen (1971) for formation and unfolding of RNA double helices (Pörschke, 1974). An alternative nucleation/condensation model for protein folding proposes the nucleated state to represent a high-energy state or a transition-state-like intermediate, which will not become populated under any conditions (Fersht, 1995).

To detect the possible formation of a nucleated state, we investigated folding of hen egg white lysozyme. Refolding of lysozyme starting from GdmCl-unfolded protein is a complex process (Tanford et al., 1973). In a first step, very rapid hydrophobic collapse occurs (τ < 1 ms) (Chaffotte et al., 1992; Itzhaki et al., 1994), which probably reflects the response of the unfolded polypeptide chain to altered solvent conditions rather than formation of a specific folding intermediate (Dill, 1990; Chan et al., 1996; A. Bachmann and T. Kiefhaber, unpublished results). From this state, the pathway branches and part of the unfolded molecules (U) can fold directly to the native state (N), while another part of the unfolded molecules form an intermediate (I) (Kiefhaber, 1995; Wildegger & Kiefhaber, 1997).



(2)

Of the unfolded molecules, 20% fold directly to the native state in the presence of 0.6 M residual concentration of GdmCl at 20 °C, pH 5.2. The remaining 80% of the unfolded molecules take the pathway through the folding intermediate (Kiefhaber, 1995). In the intermediate, the α-helical part of the protein is already formed, whereas the β-sheet regions are largely unordered (Radford et al., 1992). This intermediate is converted to native protein in a slow reaction (τ = 400 ms).

Our results show that the partially folded intermediate, which transiently accumulates during lysozyme folding, is formed in a nucleation/growth type of mechanism. During

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unfolding of the intermediate, a fast kinetic reaction is observed, which is shown to represent the growth step. The extrapolated time constant for this reaction at 0 M GdmCl is 300 ± 150 μs. The time constant of the slower unfolding reaction of the intermediate is 14 ± 1 ms at 0 M GdmCl, which is identical with the time constant of formation of the intermediate measured in refolding experiments. This reaction represents the nucleation step. Under native solvent conditions, the nucleated state is energetically located between the unfolded state and the folding intermediate. No indication for a nucleation/growth type of folding mechanism could be detected for the native state.

MATERIALS AND METHODS

Materials

Lysozyme from hen egg white was from Sigma and was used without further purification. Ultrapure GdmCl (AA-grade) was from Nigu (Waldkraiburg, Germany). All other chemicals were from Merck (Darmstadt, Germany) and were of reagent grade.

Methods

Unfolding Kinetics of the Intermediate. Double-jump experiments to measure unfolding kinetics of the intermediate were performed by diluting completely unfolded lysozyme (in 3.6 M GdmCl, 20 mM Gly/HCl, pH 1.8) 6-fold with 20 mM NaOAc, pH 5.4. This initiated the refolding reaction under final conditions of 0.6 M GdmCl, pH 5.2. After 100 ms of refolding, the intermediate is maximally populated. At this time, a second mixing step was applied which transfers the protein to final conditions of 20 mM NaOAc, pH 5.2, 2.6 M GdmCl. Under these conditions, the intermediate unfolds within 300 ms. In a subsequent very slow reaction (τ = 35 s), the unfolded protein is converted to native protein. Unfolding of the intermediate was measured for 500 ms. These data were fit to double-exponential kinetics. The influence of the subsequent slow folding reaction was checked by measuring the kinetics for 500 s and fitting the data to triple-exponential kinetics. In all cases, the rate constants and the amplitudes of the fast unfolding reactions were independent of the detection time. The experiments were repeated 5 to 10 times, and the average of the kinetic traces was used for data analysis. The errors in the rate constants and their respective amplitudes were usually between 5 and 10% of the absolute values. However, under conditions where the amplitude of the faster unfolding reaction was small (between 1.7 and 2.5 M GdmCl), the errors of the rate constants and the amplitude of the fast unfolding reaction were between 10 and 25%.

The presence of mixing artifacts interfering with the kinetic measurements was checked over the complete range of final GdmCl concentrations by performing the same double-mixing experiments as described above (a) with *N*-acetyl-tryptophanamide at the same molar tryptophan concentration as in lysozyme used in the experiments and (b) by starting with native protein in 0 M GdmCl, pH 5.2, which does not unfold up to 3 M GdmCl and which unfolds very slowly (in the minutes to hours time range) between 3.2 and 7.2 M GdmCl. In no event could a fast reaction be detetected ruling out mixing artifacts as the origin of the observed unfolding reactions of the intermediate.

All experiments were carried out in an Applied Photophysics SV17 stopped-flow instrument with double-mixing setup. The final reaction was monitored by the change in Trp fluorescence above 320 nm after excitation at 280 nm. The final protein concentration was 3.6 μM. All experiments were carried out at 20 °C.

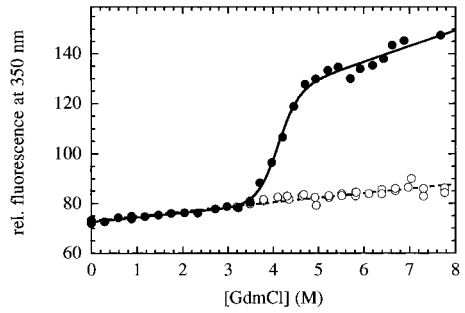

FIGURE 1: GdmCl-induced unfolding transition of lysozyme detected by the change in intrinsic tryptophan fluorescence at 350 nm (●). Additionally, the initial fluorescence at the beginning of the slow unfolding reaction is displayed above 3 M GdmCl (○). The solid line represents the fit of the transition curve according to a two-state model (Santoro & Bolen, 1988) with $\Delta G^0 = -42.9 \pm 4.5$ kJ/mol and $m = d\Delta G^0/d[GdmCl] = 10.5 \pm 1.5$ (kJ/mol)/M. The dashed line is a linear extrapolation of the fluorescence of the native protein to high concentrations of GdmCl.

To investigate the time dependence of the amplitudes of the faster and the slower unfolding reaction of the intermediate, the same experiments as described above were carried out with varying length of the refolding pulse (between 36 ms and 2 s) and unfolding in final conditions of 3.2 M GdmCl, pH 5.2, 20 °C.

GdmCl Dependence of the Unfolding Reactions of the Intermediate. The GdmCl dependences of the unfolding kinetics of the intermediate were also measured as described above with a 100 ms refolding pulse and varying concentrations of GdmCl in the final step (between 0.7 and 7.2 M).

Unfolding of Native Lysozyme. Unfolding of the native protein was measured by unfolding native lysozyme (in 20 mM NaOAc, pH 5.2) at various concentrations of GdmCl between 0 and 7.8 M. Unfolding was carried out by stopped-flow mixing and in parallel by manual mixing, since global unfolding of lysozyme is extremely slow under theses conditions (Kiefhaber, 1995). Additionally, an equilibrium unfolding transition was measured under identical conditions and monitored by the change in tryptophan fluorescence at 350 nm.

RESULTS AND DISCUSSION

Unfolding Kinetics of Lysozyme. To detect the possible population of a nucleated state during unfolding of lysozyme, we started from native lysozyme and unfolded it at various concentrations of GdmCl between 3.2 and 7.8 M. Under these conditions, global unfolding of lysozyme occurs in the minutes to hours time range (Kiefhaber, 1995). No faster reaction was observed in fluorescence-detected stopped-flow mixing experiments, and the amplitude of the slow unfolding reaction corresponds to the complete amplitude expected from equilibrium transitions at all concentrations of GdmCl (Figure 1), indicating that no partially folded states become populated during unfolding of native lysozyme under these experimental conditions.

We then investigated unfolding of the partially folded intermediate. Since the intermediate accumulates transiently during refolding, we had to use double-mixing experiments to monitor its unfolding reaction (Schmid, 1983). In these experiments, refolding is initiated in a first mixing step and

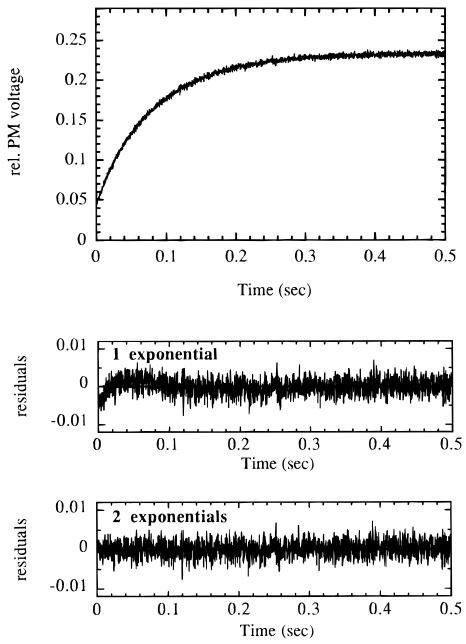

FIGURE 2: Fluorescence-detected unfolding of the lysozyme folding intermediate measured by stopped-flow double-jump experiments. The intermediate was first populated by a 100 ms refolding pulse in 0.6 M GdmCl, pH 5.2 and 20 °C and subsequently transferred to 2.6 M GdmCl, pH 5.2 and 20 °C. The resulting unfolding reaction is displayed. Additionally, the residuals of single- and double-exponential fits to the kinetic trace are shown. The data are best described by double-exponential kinetics with $\tau_1 = 86 \pm 4$ ms (90 ± 2% amplitude) and $\tau_2 = 20 \pm 2$ ms (10 ± 2% amplitude).

is allowed to proceed for a certain time to populate the intermediate. Then, a second mixing step is applied to transfer the protein into conditions where the intermediate unfolds. The resulting unfolding reaction is monitored by the change in tryptophan fluorescence. In our experiments, lysozyme was refolded in 0.6 M GdmCl, pH 5.2, in a first step. After 100 ms, the intermediate is maximally populated (Kiefhaber, 1995; Wildegger & Kiefhaber, 1997) and the protein was transferred into final conditions of 2.6 M GdmCl, pH 5.2. Figure 2 shows that under these conditions two unfolding reactions are observed. In refolding experiments starting from the unfolded state, single-exponential kinetics are measured for formation of the intermediate under all conditions (Kiefhaber, 1995; Wildegger & Kiefhaber, 1997). Under equilibrium conditions, lysozyme is still native in the presence of 2.6 M GdmCl (Figure 1). Consequently, a slow refolding reaction to the native state ($\tau = 35 \pm 2$ s) is observed after unfolding of the intermediate. This slow reaction does not influence the data analysis shown in figure 2. Measuring the unfolding reaction of the intermediate and the subsequent refolding reaction to completion and fitting the data to three exponentials gives identical rate constants and amplitudes for the two unfolding reactions as the double exponential fit of the data shown in Figure 2. This is due to the largely different time scales of the unfolding and refolding reaction.

To investigate the origin of the faster unfolding reaction, we used the same double-jump experiments as shown in Figure 2 but varied the length of the refolding pulse. Monitoring the time dependence of the amplitudes of the two unfolding reactions should give information on whether both unfolding reactions are caused by the same intermediate or whether a second intermediate gives rise to the kinetic heterogeneity in unfolding. Figure 3 shows that the amplitudes of both unfolding reactions increase with the same time constant ($\tau \approx 30$ ms), which is identical to the time constant for formation of the intermediate observed in direct-refolding experiments (Kiefhaber, 1995; cf. Figure 4A). The subsequent decrease of both amplitudes also occurs at identical rates ($\tau \approx 400$ ms) and represents the interconversion of the intermediate to the native state (Kiefhaber, 1995; Wildegger & Kiefhaber, 1997). Consequently, the ratio of the amplitudes (A_1/A_2) is independent of the refolding time. These results suggest that the two unfolding reactions reflect unfolding steps starting from a single kinetic species. Both reactions must therefore be part of a sequential unfolding pathway. On parallel pathways, starting from the same initial state, the ratio of amplitudes of two reactions reflects the ratio of the respective rate constants (Moore & Pearson, 1981). The majority of the signal change would thus occur in the faster reaction, which is not observed (Figure 3). The results also rule out that the faster reaction monitors unfolding of the collapsed state. Formation of the collapsed state could be detected up to 3.05 M GdmCl. It is complete in the dead time of stopped-flow mixing (1 ms) under all conditions (A. Bachmann and T. Kiefhaber, unpublished results).

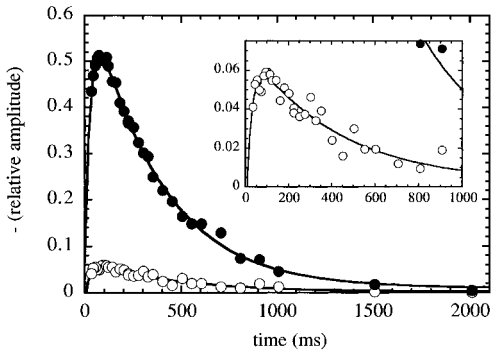

FIGURE 3: Time dependence of the formation and the decay of the amplitudes of the slow (●) and the fast unfolding reaction (○) of the lysozyme folding intermediate. The experimental procedure was as in Figure 2 with a varying length of the refolding pulse (from 36 ms to 2 s) and a final unfolding concentration of 3.2 M GdmCl. The inset shows a zoom of the early time region of the amplitude of the faster reaction (A_2). Fitting the time course and exponential rise and fall mechanism (solid lines) gives relaxation times of 30 ± 3 and 360 ± 15 ms for formation and decay of the slower unfolding reaction and of 26 ± 7 and 415 ± 60 ms for the faster unfolding reaction.

GdmCl Dependence of the Unfolding Reactions. The GdmCl dependence of unfolding of the intermediate (Figure 4) was measured by populating the intermediate maximally by a 100 ms refolding pulse in 0.6 M GdmCl followed by unfolding at various concentrations of GdmCl between 0.7 and 7.2 M. The rate constants of the two reactions are well-separated below 5 M GdmCl. The apparent rate constants (λ_i) of both reactions display the commonly observed V-shaped profile (Figure 4A). At GdmCl concentrations, which allow measurements of both refolding and unfolding kinetics of the intermediate, only the slower reaction is observed in refolding experiments whereas two reactions are visible in unfolding. The amplitudes of the two unfolding reactions and their ratio (A_1/A_2) depend strongly on the final concentrations of GdmCl (Figure 4B). At low concentrations of GdmCl, the largest part of the signal change occurs in the slower reaction. Below 1.7 M GdmCl the faster reaction has no amplitude and cannot be observed experimentally. The amplitude of the faster reactions increases with the concentration of GdmCl. Above 5 M GdmCl, a single

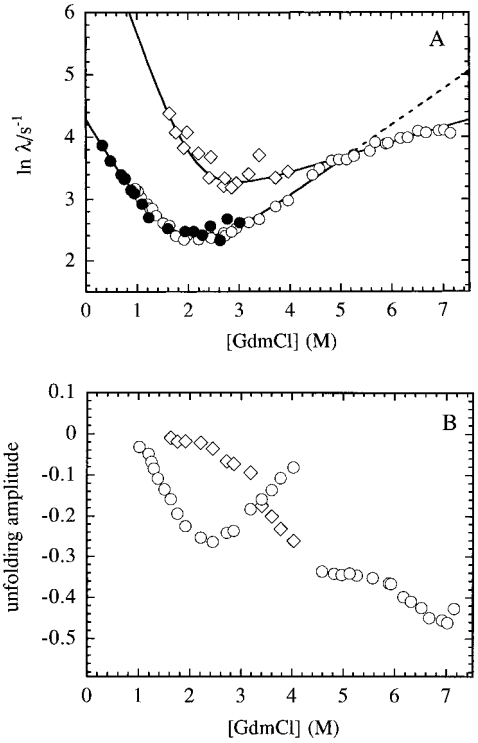

FIGURE 4: (A) GdmCl dependence of the apparent rate constants (λ) of the faster (λ_2; ◇) and of the slower (λ_1; ○) unfolding reaction of the lysozyme folding intermediate. The data were obtained as described in Figure 2 with varying concentrations of GdmCl in the final unfolding step. Additionally, the GdmCl dependence of the rate constant for formation of the intermediate starting from completely unfolded protein is shown (●). The lines represent nonlinear least-squares fits to the analytical solution of the linear three-state model (Moore & Pearson, 1981; Kiefhaber et al., 1992)

$$U \underset{k_{NuU}}{\overset{k_{UNu}}{\rightleftharpoons}} Nu \underset{k_{NuI}}{\overset{k_{NuI}}{\rightleftharpoons}} I$$

with values of $k_{UNu}(0) = 14 \pm 1$ ms, $m_{UNu} = -1.47 \pm 0.09$, $k_{NuU}(0) = 376 \pm 50$ ms, $m_{NuU} = 0.50 \pm 0.05$, $k_{NuI}(0) = 300 \pm 150$ μs, $m_{NuI} = -2.61 \pm 0.5$, $k_{INu}(0) = 63 \pm 10$ ms, and $m_{INu} = 0.20 \pm 0.03$. The m_{ij}'s denote the GdmCl dependence of the logarithm of the respective rate constants (d ln k_{ij}/d[GdmCl]). The dashed line represents the expected faster apparent rate constant (λ_2) at high concentrations of GdmCl, which has no amplitude in unfolding experiments. (B) GdmCl dependence of the amplitudes of the faster (◇) and of the slower (○) unfolding reaction of the lysozyme folding intermediate. The same symbols as in panel A are used for the individual reactions.

unfolding reaction is observed. The strong GdmCl dependence of the unfolding amplitudes is further evidence for consecutive reactions on a linear unfolding pathway starting from the same state (eq 1). If two populated refolding intermediates on parallel pathways were to give rise to the observed double-exponential unfolding kinetics, the ratio of the unfolding amplitudes should only depend on the initial conditions and thus be independent of the final GdmCl concentration.

Kinetic Model for Formation of the Intermediate. The presented results show that both unfolding reactions are produced by the same intermediate state indicating that the faster reaction monitors the growth step in a nucleation/growth type of refolding mechanism. The slower reaction is identical to the formation of the intermediate monitored in refolding, and it represents the nucleation step (Figure 5). The observation of a nucleation and a growth reaction leads to a three-state model as a minimal mechanism for formation of the intermediate.

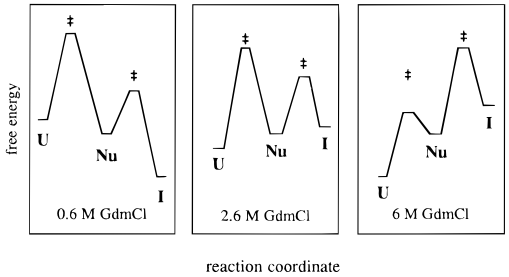

FIGURE 5: Schematic energy diagrams at different concentrations of GdmCl for the relative energies of the unfolded state (U), the nucleated state (Nu), the helical intermediate (I), and of the respective transition states (‡). To emphasize the energy differences under the respective conditions the absolute free energy values are not drawn up to scale.

$$U \underset{\text{nucleation}}{\rightleftharpoons} Nu \underset{\text{growth}}{\rightleftharpoons} I \qquad (3)$$

Linear extrapolation of the rate constant of the growth step to 0 M GdmCl gives a value of $\tau = 1/\lambda = 300 \pm 150$ μs. The two rate constants have very different GdmCl dependences. At 5 M GdmCl, they are identical, and above 5 M GdmCl only a single unfolding reaction is observed. Under these conditions, the growth step is actually slower than the nucleation step and it consequently becomes rate-limiting for the unfolding reaction (Figure 5). This change in the unfolding mechanism gives rise to a pronounced kink in the GdmCl dependence of the unfolding reaction, which is observed for the unfolding reactions of several proteins such as cytochrome c (Ikai et al., 1973; Sosnick et al., 1996) and arc repressor (Jonsson et al., 1996). Our results suggest that this behavior is indicative for a nucleation/growth mechanism in protein folding. In cases like the lysozyme intermediate, the rapid Nu ⇌ I equilibrium allows population of the nucleated state under some unfolding conditions (between 1.7 and 5 M GdmCl; Figure 5). This gives rise to double-exponential unfolding kinetics under these conditions. Above 5 M GdmCl, the nucleated state is still formed in a first unfolding reaction, but now the consecutive U ⇌ Nu equilibrium is faster than the Nu ⇌ I equilibrium and the intermediate does not become populated during unfolding. Formation of the nucleated state starting from the intermediate is the rate-limiting step in the unfolding process under these conditions.

Under strongly native solvent conditions (below 1.7 M GdmCl), the Nu ⇌ I equilibrium strongly favors the intermediate state and the nucleated state does not become significantly populated during unfolding. Under these conditions, the nucleated state represents a high-energy structure with respect to the intermediate state. Comparison of the extrapolated folding and unfolding rate constants of the Nu ⇌ I equilibrium (Figure 4A) shows that the intermediate state is 13.7 ± 3 kJ/mol more stable than the nucleated state at 0 M GdmCl.

In other proteins the folded state seems to be more stable than the nucleated state under all experimental conditions. In this case, single-exponential kinetics will be observed under all unfolding conditions, but the rate-limiting step in unfolding may still change when the growth step becomes slower than the nucleation reaction at high concentrations of GdmCl (Figure 5). This will produce the frequently observed kink in the GdmCl dependence of the logarithm of the unfolding reaction (Figure 4A).

Comparison of the slopes of the GdmCl dependences of the rate constants for nucleation and growth allows the

determination of the degree of solvent exposure in the nucleated state (Tanford, 1970). In formation of the lysozyme intermediate, 42% of the change in solvent accessibility occurs between the unfolded state and the nucleated state, showing that a considerable amount of structure formation has occurred in the nucleated state. The term "growth" process might therefore be misleading. The late steps in the formation of the intermediate rather seem to represent a final collapse into a folded conformation as proposed by Thirumalai (1995) in theoretical studies or a condensation process similar to that proposed by Fersht and co-workers for folding of chymotrypsin inhibitor 2 (Itzhaki et al., 1995) . The term "growth" is used here in the sense of the classical kinetic phenomenon of a slow initial seeding process followed by fast steps leading to the product (eq 1).

CONCLUSIONS

The presented results show that the formation of a partially folded intermediate in lysozyme folding follows the nucleation/growth model. The extrapolated time constants for the nucleation and the growth steps at 0 M GdmCl are 14 ± 1 ms and 300 ± 150 μs, respectively, assuming a linear dependence of the logarithm of the rate constants on the concentration of GdmCl (Tanford, 1970). Growth rates for α-helix formation in polypeptides (Gruenewald et al., 1979) and in short model peptides (Williams et al., 1996) were shown to be in the sub-microsecond time range. In the intermediate formed during lysozyme folding, three α-helices are present (Radford et al., 1992). Our results show that growth rates for more complex systems such as intact proteins are slower than the intrinsic growth rates for helix formation. At present, we do not know whether the growth process represents a pathway of several rapidly interconverting species or whether it reflects a single molecular event.

The presented results show that, in apparent two-state folding reactions, like the formation of the lysozyme intermediate, partially folded nucleated states exist, which are located after the rate-limiting step of the refolding process on the reaction coordinate (Figure 5). The observation of pronounced kinks in the GdmCl dependence of the logarithm of the apparent rate constant for unfolding reactions of several proteins suggests that similar intermediates are located between the transition state and the native state in folding of many proteins. The formation of these intermediates starting from the unfolded polypeptide chain is associated with the highest energy barrier in the folding process. Their subsequent conversion to the folded state is very fast. These partially folded nucleated states might play an essential role in directing the folding polypeptide chain to the correctly folded conformation. Similar kinds of intermediates located between the native state and the transition state of folding were postulated for cytochrome *c* (Bai et al., 1995) and for ribonuclease H (Chamberlain et al., 1996) on the basis of native-state hydrogen exchange. As in the case of the nucleated state in formation of the lysozyme intermediate, these partially folded states were shown to represent high-energy structures under native solvent conditions. Interestingly, the GdmCl dependence of the logarithm of the unfolding reaction in cytochrome c also shows a pronounced rollover at high concentrations of denaturant (Ikai et al., 1973; Sosnick et al., 1996).

The lower stability of the lysozyme folding intermediate compared to native protein structures obviously allows the transient population of the high-energy nucleated state during unfolding at elevated concentrations of GdmCl. In unfolding reactions of native proteins, high-energy nucleated states will be more difficult to observe since the native state is more stable than the nucleated state under all solvent conditions. This might explain the failure to detect a high-energy state in unfolding of native lysozyme.

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