made the same observations with the epithelial dog kidney cell line MDCK (unpublished observations). One usually studies internalization of hormones, growth factors, or lipoproteins by following a reporter signal (often radioactive or fluorescent) placed on the ligand molecule. A particularly interesting application of ABD-ouabain would be to study the mechanism of internalization and recycling of the digitalis receptor by directly labeling it radioactively.

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Registry No. ATPase, 9000-83-3; ABD-ouabain, 83859-88-5; Boc-*p*-phenylenediamine, 71026-66-9; APh-ouabain, 86569-16-6; di-*tert*-butyl dicarbonate, 24424-99-5; *p*-phenylenediamine, 106-50-3; oxidized ouabain, 73165-88-5; *p*-aminophenol, 123-30-8.

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Mechanism of Folding of Ribonuclease A. Slow Refolding Is a Sequential Reaction via Structural Intermediates†

Franz X. Schmid

ABSTRACT: Two models have been proposed to explain the observed folding kinetics of small proteins. The sequential model assumes that folding proceeds on an ordered pathway via structural folding intermediates, whereas the simple model of folding involves only multiple unfolded forms of the protein and a single native state. In the latter model, refolding is limited by interconversion reactions in the unfolded protein; accumulation of structural intermediates during folding is excluded. Here, two experimental tests are presented to discriminate between these models for the major slow folding species of ribonuclease A. The first test shows that a nativelike intermediate accumulates during folding, which unfolds rapidly compared to native ribonuclease A, and the second test demonstrates that refolding is a sequential reaction, resulting in the transient accumulation of an intermediate and in a lag in the formation of fully native protein. Both results rule out the simple model of folding and agree with the sequential model via structural intermediates. The nativelike intermediate is stable toward unfolding and is on the pathway of refolding for denaturant concentrations up to 2 M guanidine hydrochloride at pH 6 and 10 °C.

Structural intermediates have been detected in the reversible folding reactions of ribonuclease A (RNase A)[1] [for a review, see Kim & Baldwin (1982)] and of other small protein molecules (Ko et al., 1977; Crisanti & Matthews, 1981; Desmadril & Yon, 1981; McPhie, 1982; Goto & Hamaguchi, 1982; Nall, 1983). The model of sequential folding (Kim & Baldwin, 1982) states that protein folding proceeds on an ordered pathway via a definite sequence of structural folding

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[1] Abbreviations: RNase A, bovine pancreatic ribonuclease A (EC 3.1.27.5) with disulfide bonds intact; U_S and U_F, slow- and fast-folding species of unfolded RNase A, respectively; Gdn·HCl, guanidine hydrochloride; τ, time constant of chemical reaction (reciprocal of the apparent rate constant, k^{-1}); I_1 and I_N, folding intermediates; 2′-CMP, cytidine 2′-phosphate; 2′,3′-CMP, cytidine 2′,3′-phosphate.

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intermediates. Depending on the final conditions, these intermediates may or may not be well populated. Folding is not blocked by incorrect proline isomers, but nativelike folded molecules may be formed before proline isomerization takes place. Experimental evidence for the sequential model of protein folding largely rests (i) on the observation of complex refolding kinetics (however, complex kinetics are also produced by multiple unfolded forms, which differ in their rates of refolding), (ii) on the finding that often substantial parts of the expected refolding amplitudes are missing due to fast steps in folding, and (iii) on noncoincident kinetics when folding is monitored by different physical probes.

Unfolded RNase A consists of a mixture of fast folding (U_F) and slow folding (U_S) molecules (Garel & Baldwin, 1973); U_F has all its essential proline residues in the native configuration, whereas U_S contains at least one incorrect proline isomer (Brandts et al., 1975; Schmid & Baldwin, 1978; Lin & Brandts, 1983a). At present, three different pathways of refolding have been detected, which originate from distinct unfolded states. They yield native RNase A in parallel reactions (Schmid & Blaschek, 1981) as outlined in eq 1–3.

$$U_F \xrightarrow{\text{fast}} N\ (20\%) \quad (1)$$

$$U_S^{II} \xrightarrow{\text{slow}} N\ (60\text{–}70\%) \quad (2)$$

$$U_S^{I} \xrightarrow{\text{very slow}} N\ (10\text{–}20\%) \quad (3)$$

The rates of all three refolding reactions depend on the folding conditions. The two slow folding reactions are only resolved at low temperature and low concentrations of denaturant (Cook et al., 1979; Schmid & Blaschek, 1981). A third minor phase has been resolved by Lin & Brandts (1983b) at high concentrations of urea.

Two structural folding intermediates have been detected on the major $U_S^{II} \rightarrow N$ refolding pathway (60–70% of all unfolded molecules, eq 2). An early hydrogen-bonded intermediate (I_1) is formed rapidly (Nall et al., 1978; Schmid & Baldwin, 1979a; Kim & Baldwin, 1980), and a folded intermediate (I_N) accumulates late in folding. I_N already shows many properties of the native protein, such as a folded globular structure, a binding site for the specific inhibitor 2′-CMP, and catalytic activity, but it still contains at least one incorrect proline isomer and fluorescence properties different from those of the native protein (Schmid, 1981; Schmid & Blaschek, 1981). I_1 and I_N are well populated under strongly native folding conditions. The minimal mechanism for the folding pathway of U_S^{II} is given by eq 4 (Kim & Baldwin, 1982).

$$U_S^{II} \rightarrow I_1 \rightarrow I_N \rightarrow N \quad (4)$$

Additional intermediates between I_1 and I_N may exist. Nothing can be said at present about intermediates on the minor $U_S^{I} \rightarrow N$ pathway.

Recently, Lin & Brandts (1983a–c) reinvestigated the slow refolding of RNase A. Extending the original two-state model of protein folding, they proposed a simple kinetic mechanism of folding of RNase A which involves only multiple unfolded forms of the protein (D_{ij}) and a single native state (N). They concluded that all unfolding and refolding kinetics, in the native and in the unfolded base-line regions, can be explained in terms of fully native and completely unfolded species only. In particular, they postulate that no structural intermediates are populated during folding under these conditions, i.e., that a sequential model is not necessary to explain the process of protein folding.

Here, two experimental tests are presented to discriminate between the *sequential folding model* for U_S^{II} (eq 4) via I_N and the *simple model* of Lin & Brandts (1983c), which excludes the existence of populated structural folding intermediates, such as I_N.

(1) The simple model requires that all nativelike molecules which are formed during folding are actually *fully native* RNase A, as only one folded species (N) exists in the simple model. In the first test, it is demonstrated that under strongly native folding conditions a folded species accumulates, which is catalytically active. However this species is less stable than the fully refolded protein; it unfolds about 15 times more rapidly than N.

(2) In the second test, the kinetic properties of sequential and of parallel reactions are used to discriminate the sequential $U_S^{II} \rightarrow I_N \rightarrow N$ mechanism and the simple mechanism, which involves the independent refolding of multiple unfolded states. The sequential mechanism requires that the concentration of I_N proceeds through a maximum and that the formation of N shows a lag phase (provided that the corresponding rate constants are similar). In the simple mechanism, formation of N does not display a lag phase [cf. Figure 1 of Lin & Brandts (1983c)]. Here, the distinct unfolding properties of I_N and N are used to design an unfolding assay which yields the concentrations of I_N and N molecules at any time point of folding. The results agree with the sequential model; a simple model without structural intermediates is ruled out. The unfolding assays for I_N and N are also used to test the stability of the intermediate I_N toward unfolding by Gdn·HCl.

Materials and Methods

Materials

Ribonuclease A (type XII A), 2′-CMP, and 2′,3′-CMP were purchased from Sigma, St. Louis, MO; Gdn·HCl (ultrapure) was from Schwarz/Mann, Orangeburg, NY; sodium cacodylate was from Serva, Heidelberg, West Germany. All other substances were purchased from Merck, Darmstadt, West Germany.

Methods

A Cary 118C spectrophotometer with jacketed cell holders was used. RNase concentrations were determined spectrophotometrically by using a molar absorbance at 278 nm of 9800 M^{-1} cm^{-1} (Sela & Anfinsen, 1957).

Unfolding Assays for I_N and N Molecules. After different times of refolding, 100-μL samples are withdrawn and mixed with 900 μL of a concentrated solution of Gdn·HCl to give final 4.6 M Gdn·HCl, pH 6. The resulting kinetics of unfolding of I_N and N are monitored by the decrease in A_{287} at 10 °C. The amplitudes of the two phases were obtained by analyzing the kinetics as the sum of two exponentials.

Experiments To Determine the Stability of I_N toward Unfolding by Gdn·HCl. (i) Prefolding Step To Populate I_N. Prefolding is initiated by mixing 10 μL of unfolded RNase A (in 4 M Gdn·HCl, 0.1 M glycine, pH 2.0, 0 °C, and 3 mM RNase A) with 90 μL of 0.9 M $(NH_4)_2SO_4$ and 0.05 M cacodylate, pH 6.2, at 0 °C to give prefolding conditions of 0.8 M $(NH_4)_2SO_4$ and 0.4 M Gdn·HCl, pH 6.0, 0 °C.

(ii) Stability Assays. Prefolding is interrupted after 15 s by addition of 90 μL of prefolding solution to 810 μL of the assay solution in the spectrophotometer cell (at 10 °C). The resulting changes in absorbance at 287 nm are recorded. Final assay conditions: 0.05 M cacodylate, pH 6.0, 30 μM RNase A, and concentrations of Gdn·HCl ranging from 0 to 5 M.

Folded and unfolded molecules with incorrect proline isomers were assayed by the two-step procedure developed by Cook et al. (1979).

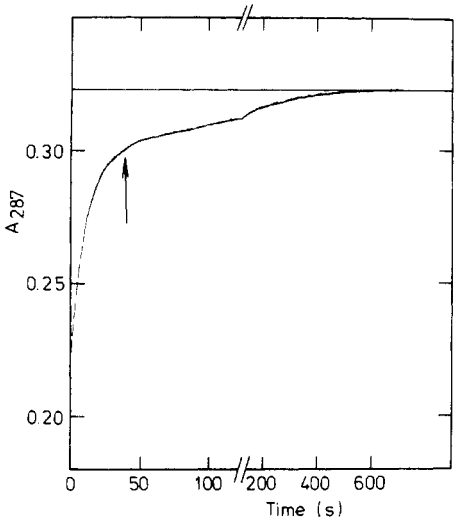

FIGURE 1: Slow refolding reaction of RNase A at 1 °C monitored by the change in tyrosine absorbance. Initial unfolding conditions: 1.04 mM RNase A in 4.0 M Gdn·HCl, pH 2. Refolding was initiated by a 20-fold dilution to 52 μM RNase A in 0.2 M Gdn·HCl, 0.4 M $(NH_4)_2SO_4$, and 0.05 M cacodylate, pH 6, 1 °C.

Results

Nativelike Intermediate Is Populated during Folding, Which Unfolds Rapidly Compared to the Native Protein. Under strongly native conditions, such as 0.4 M $(NH_4)_2SO_4$ and 0.2 M Gdn·HCl, pH 6, 1 °C, the slow refolding reactions of RNase A are considerably accelerated. Figure 1 shows the time course of refolding as monitored by the increase in tyrosine absorbance. Only the two slow-folding reactions are resolved; the fast $U_F \rightarrow N$ reaction is complete within the dead time of mixing. After 40 s of refolding, about 85% of the protein appears to be native RNase A by virtue of its tyrosine absorbance properties. Also under these conditions, about 80% of the enzymatic activity has been regained after 40 s (Schmid & Blaschek, 1981).

The simple model of RNase A folding (Lin & Brandts, 1983c) requires that all the material that appears to be native RNase A as judged by absorbance is actually native RNase A; i.e., after 40 s of refolding under the conditions of Figure 1, about 85% of the protein present is required to be native RNase A. The actual amount of native enzyme after this time of folding is determined here by using an unfolding assay for N molecules (Schmid, 1982), which is based on the observation that native RNase A unfolds at 10 °C, pH 6, and 4.6 M Gdn·HCl in a single slow phase with an absorbance change of $\Delta\epsilon_{287} = 2800\ M^{-1}\ cm^{-1}$ and a time constant of $\tau = 260$ s. Such an unfolding assay, carried out after 40 s of refolding under the conditions of Figure 1 (cf. the arrow in Figure 1), is shown in Figure 2a. Instead of a single unfolding curve with 85% amplitude, as expected from the simple model, biphasic unfolding kinetics are observed. Only 1020/2800 = 36% of all RNase molecules unfold slowly like fully native protein. The remaining 50% nativelike molecules unfold 15× faster ($\tau = 19$ s) than the native protein. In a control experiment, folding under the conditions of Figure 1 is allowed to go to completion. The resulting unfolding assay (Figure 2c,d) yields a single unfolding reaction with $\tau = 261$ s and $\Delta\epsilon = 2850\ M^{-1}\ cm^{-1}$ as expected for completely refolded RNase A.

The unfolding assays (Figure 2) demonstrate that less than half of the nativelike and active species which accumulate after 40 s of folding are actually fully native protein. The major part consists of the nativelike folding intermediate I_N, which resembles N in many respects (Schmid & Blaschek, 1981) but

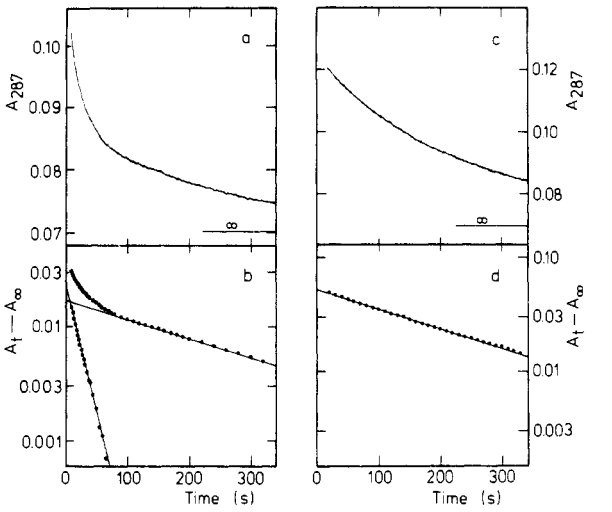

FIGURE 2: Unfolding assays of RNase A samples. (a) Refolding at 0.2 M Gdn·HCl and 0.4 M $(NH_4)_2SO_4$, pH 6, 1 °C, was interrupted after 40 s of folding, and the sample was transferred to unfolding conditions of eventually 16.8 μM RNase A in 4.6 M Gdn·HCl and 0.04 M $(NH_4)_2SO_4$, pH 6 at 10 °C, in the spectrophotometer cell. The kinetics of the resulting decrease in tyrosine absorbance are shown. (b) Semilogarithmic plot of the data. The curve was analyzed as a sum of two exponentials. (c) Refolding under the conditions of (a) was allowed to go to completion for 1 h, and then the sample was transferred to unfolding conditions as in (a). (d) Semilogarithmic plot as in (b).

which unfolds much faster than N.

Nativelike Intermediate Unfolds to U_S. The product of the rapid unfolding reaction of Figure 2a,b is the slow-folding species U_S. The two-step assay for species with incorrect isomers of essential prolines (Cook et al., 1979), carried out after 40 s of refolding under the conditions of Figure 1, shows that at this time of refolding about 55% of all RNase A molecules still contain incorrect proline isomers; i.e., upon unfolding, they yield slow-folding species, U_S. This is less than the expected 100 − 36 = 64% U_S species. The origin of this discrepancy is unclear; it may arise from the combined uncertainties of both the unfolding assays to determine N (see above) and the two-step assay for slow-folding molecules. If all the nativelike molecules which are present after 40 s of folding were fully native RNase A, molecules with incorrect prolines would be expected to be present at only 15%.

Unfolding Assays for I_N and N Molecules. The distinct unfolding behavior of I_N and N (Figure 2) can be exploited to design an unfolding assay for the simultaneous detection of the amounts of I_N and of N independent of each other at any time point of refolding. After different times of folding, samples are transferred to 4.6 M Gdn·HCl, pH 6 at 10 °C, and the unfolding reactions of I_N and N are monitored by the decrease in tyrosine absorbance. Because of the good separation in rate, the amplitudes of both reactions can be determined accurately and can be related to the amounts of I_N and N present at the time the sample is transferred to unfolding conditions. I_N unfolds to U_S; N unfolds to U_F. I_N and N are similar in absorbance (Cook et al., 1979; Schmid & Blaschek, 1981), as are U_F and U_S (Garel & Baldwin, 1973; Rehage & Schmid, 1982); therefore, the relative unfolding amplitudes are proportional to the amounts of I_N and N present at the time when folding was interrupted.

Refolding of U_S Is a Sequential Reaction via the Intermediate I_N. The unfolding assays for I_N and N were used to examine whether refolding of U_S is a sequential reaction. These assays are specific for I_N and N molecules, in contrast to spectroscopic probes which generally monitor the sum of

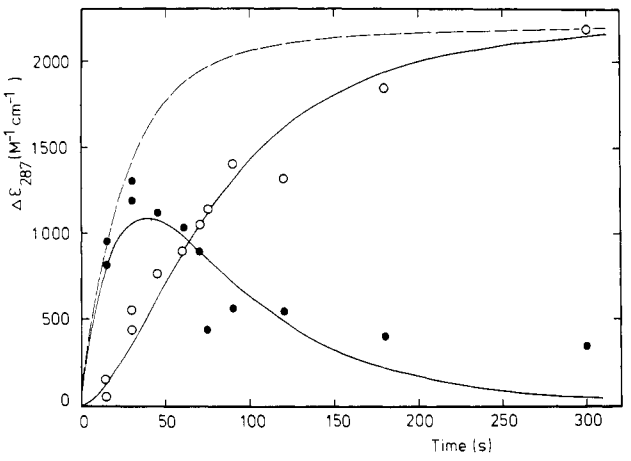

FIGURE 3: Concentration vs. time curves for I_N (●) and N (○) during refolding at 0.5 M Gdn·HCl and 0.05 M cacodylate, pH 6, 10 °C. The amplitudes of the unfolding assays for I_N and N (expressed as molar changes) are shown as a function of the time of sample withdrawal from the refolding solution. A value of $\Delta\epsilon$ = 560 M^{-1} cm^{-1} has been subtracted from all N values to account for 20% N formed rapidly in the $U_F \rightarrow N$ reaction. The continuous lines represent concentration vs. time curves calculated on the basis of eq 5 and 6. The dashed line is the time course of absorbance (287 nm) detected refolding.

the properties of all species that participate in refolding.

Refolding at 10 °C, 0.5 M Gdn·HCl, pH 6, was investigated by unfolding assays after different times of folding, and the amplitudes of the unfolding of I_N and N are shown in Figure 3. Note that a constant value of $\Delta\epsilon$ = 560 M^{-1} cm^{-1} (=20% of 2800 M^{-1} cm^{-1}) has been subtracted from the N amplitudes to account for native RNase A formed during the fast $U_F \rightarrow$ N reaction. The concentration of I_N proceeds through a maximum (reached after 30–40 s of refolding), and formation of N is characterized by an initial lag phase. This result suggests that under the given conditions I_N is on a sequential $U_S \rightarrow I_N \rightarrow N$ pathway.

The rates of the $U_S \rightarrow I_N$ and the $I_N \rightarrow N$ reactions were determined independently from absorbance- and fluorescence-detected refolding kinetics (Cook et al., 1979; Schmid & Blaschek, 1981). Equations 5 and 6 list the time constants derived under the conditions of Figure 3.

$$U_S^{II} \xrightarrow{\tau = 25\ s} I_N \xrightarrow{\tau = 60\ s} N\ (70\%) \qquad (5)$$

$$U_S^{I} \xrightarrow{\tau = 150\ s} I_N \xrightarrow{\tau = 60\ s} N\ (10\%) \qquad (6)$$

For the calculation, it is assumed that I_N is an intermediate on both pathways. With eq 5 and 6, the reaction profiles for I_N and N were calculated and are represented by the continuous lines in Figure 3. The experimental points, derived from the unfolding assays, agree with the computed progress curves for a sequential mechanism. The formation of native protein, N, is considerably slower than the absorbance-detected refolding kinetics (cf. the dashed line in Figure 3).

Panels a and b of Figure 4 show analogous reaction profiles for I_N and N during refolding at 1.5 and 2.0 M Gdn·HCl respectively. At 1.5 M Gdn·HCl, refolding is still a sequential process via the intermediate I_N, and the experimental points agree with the computed progress curves.[2] As the rate of the

[2] At concentrations of Gdn·HCl higher than 0.5 M, the rate of the $I_N \rightarrow N$ step can no longer be determined from simple fluorescence-detected refolding experiments, as the fluorescence changes become limited in rate by the $U_S \rightarrow I_N$ process. Instead, the rate of $I_N \rightarrow N$ is determined in a jump experiment: I_N is populated by an interrupted folding experiment under strongly native conditions (where $U_S \rightarrow I_N$ is very fast) and then the $I_N \rightarrow N$ reaction is measured by fluorescence under the final folding conditions.

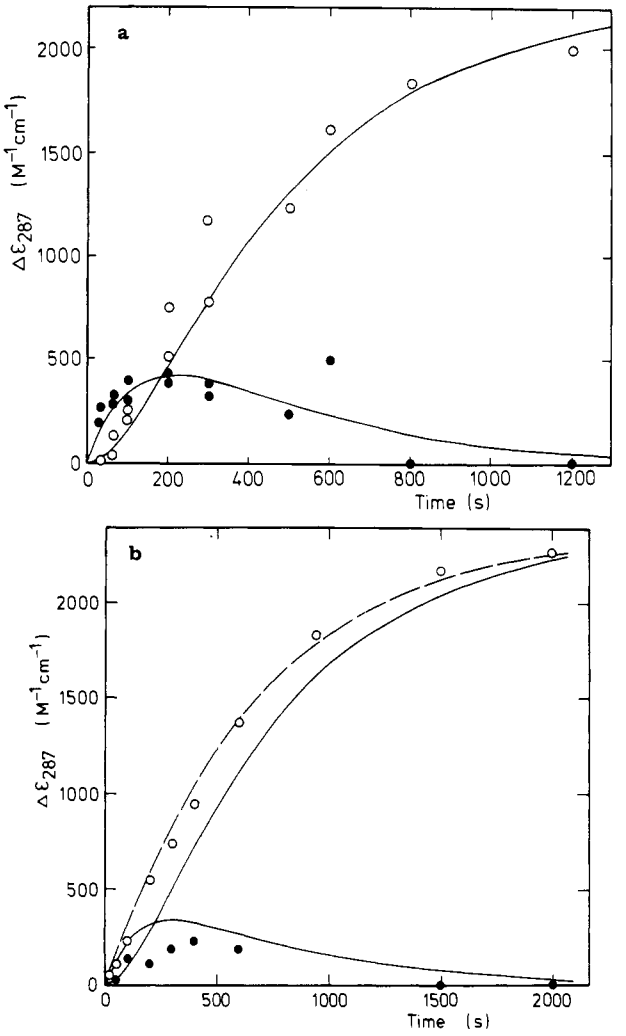

FIGURE 4: Concentration vs. time curves for I_N (●) and N (○) during refolding. (a) At 1.5 M Gdn·HCl and 0.05 M cacodylate, pH 6, 10 °C. The amounts of I_N and N were determined by unfolding assays. The continuous lines were calculated for a $U_S \xrightarrow{\tau = 400\ s} I_N \xrightarrow{\tau = 130\ s}$ N mechanism. (b) At 2.0 M Gdn·HCl and 0.05 M cacodylate, pH 6, 10 °C. The continuous lines were calculated for a $U_S \xrightarrow{\tau = 700\ s} I_N \xrightarrow{\tau = 150\ s} N$ mechanism; the dashed line represents the kinetics of absorbance-detected refolding.

$U_S \rightarrow I_N$ reaction is strongly decreased by increasing concentrations of Gdn·HCl, I_N is less populated during folding, compared to the results obtained at 0.5 M Gdn·HCl (Figure 3). At 2.0 M Gdn·HCl, less I_N is detected by the unfolding assays than would be expected from a strictly sequential model, and consequently, the reaction profile of N shows only a small lag phase, if any. Possibly at 2.0 M Gdn·HCl alternative pathways of refolding compete with the pathway via I_N.

Stability of the Nativelike Intermediate I_N. The two-step assay for molecules with incorrect proline isomers (Cook et al., 1979) as well as the fluorescence-detected kinetics (Schmid, 1981) detect I_N only, when it is well populated, i.e., under strongly native conditions. When these probes are used, nothing can be deduced regarding the stability of I_N and the pathway of refolding at higher concentrations of Gdn·HCl. The results of the unfolding assays for I_N, however, indicate that I_N is quite stable: it is even on the pathway of refolding—at least partially—at 2.0 M Gdn·HCl. Information about the stability of I_N toward Gdn·HCl-induced unfolding was obtained by a two-step procedure. (i) I_N is populated by an interrupted folding experiment under strongly native folding conditions, where the $U_S^{II} \rightarrow I_N$ step is fast compared to the subsequent $I_N \rightarrow N$ reaction. (ii) The transiently formed I_N

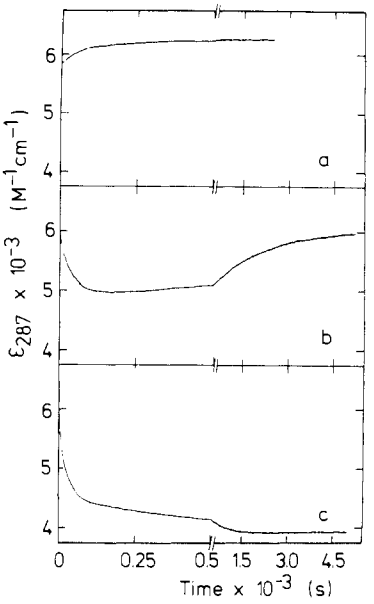

FIGURE 5: Representative folding and unfolding assays to determine the stability of I_N and N. Unfolded RNase A was exposed to prefolding for 15 s and then transferred to the different assay conditions. The change in absorbance at 287 nm under the final assay conditions is shown as a function of time. Unfolded state: 3 mM RNase A in 4.0 M Gdn·HCl and 0.1 M glycine, pH 2, 0 °C. Prefolding conditions: 15 s at 0.8 M $(NH_4)_2SO_4$, 0.4 M Gdn·HCl, 0.05 M cacodylate, pH 6, and 0.3 mM RNase A. Stability assays were performed at 10 °C, 0.05 M cacodylate, pH 6, and 30 μM RNase A in the presence of (a) 0.2 M Gdn·HCl, (b) 3.0 M Gdn·HCl, and (c) 5.0 M Gdn·HCl.

is then exposed to varying concentrations of Gdn·HCl (0–5 M) to determine its stability toward unfolding by Gdn·HCl.

(*i*) *Population of I_N.* So that a maximal transient concentration of I_N could be obtained, the prefolding step was carried out at 0.8 M $(NH_4)_2SO_4$ and 0.4 M Gdn·HCl, pH 6, 0 °C. Folding was interrupted after 15 s. Under these conditions, the fast-folding reaction ($U_F \rightarrow N$) is complete within manual mixing. The $U_S^{II} \rightarrow I_N$ step is governed by a time constant of about τ = 5 s, while the subsequent $I_N \rightarrow N$ reaction is much slower, with τ = 130 s. The $U_S^I \rightarrow N$ reaction proceeds with τ = 300 s. Based on these numbers and on the relative amounts of the different unfolded species present initially (cf. eq 1–3), the expected distribution of unfolded, partly folded, and fully native molecules after the 15-s prefolding step can be calculated. Of the refolding molecules, 59% are expected to be in the I_N state, 28% have folded entirely to N, and only small amounts of U_S^I and U_S^{II} (9% and 4%, respectively) remain unfolded.

(*ii*) *Test of the Stability of I_N.* After the 15-s prefolding step to populate I_N, the resulting mixture of N, I_N, U_S^{II}, and U_S^I molecules is assayed by transfer to varying folding and unfolding conditions and subsequent recording of the kinetic changes that occur in the tyrosine absorbance at 287 nm. The absorbance properties of N, I_N, U_S^I, and U_S^{II} are well-known, and therefore, the observed kinetics in the assay conditions are readily interpretable in terms of folding and unfolding reactions of the species present after prefolding. Figure 5 presents three kinetic traces which were obtained under different final assay conditions.

After 15 s of prefolding, the resulting mixture is transferred to 0.2 M Gdn·HCl, pH 6, 10 °C (Figure 5a). Under these conditions, N and I_N both remain folded. I_N is converted to N; U_S^I and U_S^{II} refold to native RNase A. At the end, only fully native molecules, N, are present. Figure 5a shows the small refolding reactions of U_S^I and U_S^{II}, which are observed.

Figure 5b shows the kinetic trace observed after transfer of the initial mixture of N, I_N, U_S^I, and U_S^{II} to 3 M Gdn·HCl, pH 6. Under these conditions, N remains essentially folded (3 M Gdn·HCl is at the beginning of the unfolding transition of native RNase A, which shows a midpoint at 3.4 M Gdn·HCl). I_N, however, is no longer stable at 3 M Gdn·HCl; it unfolds rapidly (τ = 45 s) under the assay conditions, resulting in an initial decrease in absorbance of $\Delta\epsilon_{287} = -1000\ M^{-1}\ cm^{-1}$. After this fast unfolding step ($I_N \rightarrow U_S^{II}$), U_S^{II} refolds again (together with the small amounts of U_S^I and U_S^{II} which were present initially) to the stable native state. The late time region of Figure 5b shows this slow refolding reaction with an amplitude of $\Delta\epsilon_{287} = +1300\ M^{-1}\ cm^{-1}$ and a time constant of τ = 1700 s. As expected, the time constant is identical with values observed for the $U_S \rightarrow N$ reaction in simple refolding experiments under the same conditions.

In a third experiment, the prefolded mixture was transferred to 5 M Gdn·HCl, pH 6 (Figure 5c). Under these conditions, only unfolded forms of RNase A are present at equilibrium. Hence, U_S^I and U_S^{II} remain unfolded; the folded species I_N and N unfold completely, giving rise to two well-separated reactions, which both result in a decrease in absorbance. I_N unfolds fast (τ = 12 s, $\Delta\epsilon_{287} = -1610\ M^{-1}\ cm^{-1}$), and N unfolds slowly (τ = 160 s, $\Delta\epsilon_{287} = -740\ M^{-1}\ cm^{-1}$). On the basis of a total change in absorbance of 2800 $M^{-1}\ cm^{-1}$ upon unfolding, the relative amounts of I_N and N present after the prefolding step are determined from the above amplitudes to be 57% and 26%, respectively. These numbers are close to the expected fractions of 28% N and 59% I_N.

In further experiments such as shown in Figure 5, the Gdn·HCl concentration in the folding/unfolding assays was varied from 0 to 5 M. (i) From 0 to 2 M Gdn·HCl, N as well as I_N is stable to unfolding; only small reactions, originating from residual U_S^I and U_S^{II}, are monitored. (ii) From 2.0 to 3.3 M Gdn·HCl, two kinetic phases with opposite signs are observed in the assays. First, the absorbance rapidly decreases because of progressive unfolding of the intermediate I_N. Then, in the second kinetic phase (see also Figure 5b) the absorbance increases again in the course of refolding of U_S^I and U_S^{II} (initially present, plus U_S^{II}, which was formed from I_N in the first phase). At 2.8–3.0 M Gdn·HCl, about half of the maximal unfolding amplitude of I_N is observed. (iii) Beyond 3.3 M Gdn·HCl, again two kinetic phases are present, but both result in a decrease of absorbance; i.e., they are unfolding reactions. The first phase is caused by the rapid complete unfolding of I_N; the second phase originates from the slow unfolding of N.

Discussion

Existence of I_N Has Been Confirmed. The intermediate I_N has been located on the major slow refolding pathway $U_S^{II} \rightarrow N$, which accounts for 60–70% of all unfolded molecules. I_N is a nativelike intermediate which still contains at least one incorrect proline isomer. The properties of I_N have been deduced from comparative kinetic folding experiments, using absorbance, fluorescence, inhibitor binding, and enzymatic activity as probes for the recovery of native properties. The final step of refolding, $I_N \rightarrow N$, was shown to be almost independent of the residual denaturant concentration, and its activation enthalpy was determined as 17.5 kcal/mol. These results supported the conclusion that the $I_N \rightarrow N$ step is rate limited by a proline isomerization reaction in the folded protein (Cook et al., 1979; Schmid, 1981; Schmid & Blaschek, 1981). The existence of I_N was questioned (Lin & Brandts, 1983b,c) because the evidence for I_N stems largely from the analysis of the complex slow refolding kinetics, which consist of a sum of parallel and of sequential reaction steps.

In this work, different evidence for the existence of the intermediate I_N was presented. After kinetic accumulation of I_N in an interrupted folding experiment under suitable conditions, I_N could be detected directly by its distinct unfolding properties. Although almost identical with N by absorbance, I_N unfolds much more rapidly than completely refolded RNase A (Figure 2). This constitutes an independent proof that during the slow refolding reaction a nativelike intermediate accumulates which is kinetically less stable than the native protein. It corroborates the analysis of the refolding kinetics, which originally led to the discovery of I_N (Cook et al., 1979).

Slow Refolding of RNase A Is a Sequential Reaction. The distinct kinetic unfolding properties of I_N and of N allow the quantitative determination of these two folded species at any time point of folding, independent of each other and independent of residual unfolded species or earlier intermediates. The results of such analyses (Figures 3 and 4) show (i) that at low concentrations of denaturant I_N is strongly populated and (ii) that up to 2 M Gdn·HCl folding is a sequential process via I_N. In the course of the slow refolding reaction, the concentration of I_N goes through a maximum, and the formation of N displays a lag at the beginning as expected for a sequential process. These results disagree with the simple model of folding (Lin & Brandts, 1983c), which excludes the population of structural folding intermediates under any conditions.

Previously, I_N could be detected by spectroscopic techniques only when it was well populated during folding. Therefore, it was assumed that I_N is an intermediate of folding only under conditions which effectively stabilize folded protein structures [i.e., low temperature, low concentration of denaturant, addition of $(NH_4)_2SO_4$]. The results of the unfolding assays show that I_N is on the pathway of folding under a wide variety of folding conditions; even at Gdn·HCl concentrations as high as 2 M. I_N is a considerably stable intermediate. Once populated kinetically, I_N is stable toward unfolding by Gdn·HCl up to 2–2.5 M Gdn·HCl. However, I_N unfolds rapidly in the range of the equilibrium unfolding transition of native RNase A (3–4 M Gdn·HCl), which indicates that I_N is not an equilibrium intermediate. This can be expected, as the unfolding transition of RNase A is adequately described by the two-state approximation (Brandts & Hunt, 1967; Salahuddin & Tanford, 1970; Privalov & Khechinasvili, 1974). As I_N is rapidly destabilized above 2.5 M Gdn·HCl, the pathway of refolding may be goverened by different rate-limiting steps under such marginally stable conditions.

Relation to the Simple Model of Folding. A different notation has been introduced by Lin & Brandts when they proposed their simple model of folding (Lin & Brandts, 1983c). The major slow refolding reaction, $U_S^{II} \rightarrow N$, relates to their XY process; the minor $U_S^{I} \rightarrow N$ reaction is correlated with their CT process.[3]

The first basic postulate of the simple model is that all complexities of the slow refolding kinetics of RNase A are due to slow chain equilibration reactions (e.g., proline isomerization) in the denatured protein. The population of structural folding intermediates during refolding is excluded. However, the intermediate I_N (and also, earlier in refolding, the intermediate I_1, Schmid & Baldwin, 1979a; Kim & Baldwin, 1980) accumulates to a high level on the $U_S^{II} \rightarrow N$ pathway prior to the rate-limiting formation of native RNase A. Furthermore, refolding to I_N is not limited by a slow isomerization of the unfolded chain but by a reaction, the rate and activation enthalpy of which are strongly dependent on the folding conditions (Nall et al., 1978; Cook et al., 1979; Schmid, 1981; Schmid & Blaschek, 1981).

The second important postulate of Lin & Brandts' model states that unfolding and refolding are counterpart processes and that the unfolding kinetics can be predicted quantitatively from the refolding kinetics and vice versa. The rate of the slow $U_S^{II} \rightarrow N$ refolding reaction (the XY process) is strongly decreased by increasing concentrations of Gdn·HCl (Nall et al., 1978) as well as of urea (Lin & Brandts, 1983b). Application of the simple model requires that the XY process is strongly dependent on denaturant concentration in unfolding as well. However, this isomerization reaction is completely independent of Gdn·HCl (Schmid & Baldwin, 1979b) and of urea (F. X. Schmid, M. H. Buonocore, and R. L. Baldwin, unpublished results). These experimental results, together with the previously published kinetic properties of the slow refolding reaction of RNase A, demonstrate that the simple model (Lin & Brandts, 1983c) is not adequate to describe the major slow refolding reaction of RNase A.

Correlation of Fluorescence Changes and Proline Isomerization. The fast-folding (U_F) and the slow-folding (U_S) species of unfolded RNase A differ in tyrosine fluorescence. This effect was ascribed to Tyr-92, which in the unfolded chain is sensitive to the isomeric state of the neighboring Pro-93 (Rehage & Schmid, 1982). By analogy, we tentatively concluded that the fluorescence changes during the final $I_N \rightarrow N$ step are probably also caused by the isomerization of the Tyr-92–Pro-93 peptide bond. By using isomer-specific proteolysis, Lin & Brandts (1983a) were able to monitor directly the isomerization of Pro-93 during unfolding and refolding and to correlate it to the fluorescence changes. Surprisingly, they found that Pro-93, which is cis in native RNase A, isomerizes only to 30% to the incorrect trans state in the unfolded protein. From their data, they concluded that isomerization of Pro-93 is not related to the major $U_S^{II} \rightarrow N$ (XY) process but that the minor $U_S^{I} \rightarrow N$ reaction (CT) is limited by Pro-93. As I_N is populated on the major $U_S^{II} \rightarrow N$ pathway, Lin and Brandts' results suggest that the difference in fluorescence between I_N and N is not caused by isomerization of Pro-93 but by some yet unidentified process. This could be the isomerization of another proline peptide bond.

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[3] The relative amplitudes of the XY process (50%) and the CT process (30%) derived by Lin & Brandts (1983b) from the analysis of absorbance- and fluorescence-detected folding kinetics do not agree very well with the relative amounts of U_S^{II} (60–70%) and U_S^{I} (10–20%) as determined from folding kinetics in the presence of 2′-CMP (Schmid & Blaschek, 1981). It is not clear, however, whether this difference is significant, or whether it arises from a different kinetic analysis [e.g., choice of base lines, cf. Lin & Brandts (1983b)] of the slow folding reactions, the rates of which are not very well separated in most of the employed folding conditions.

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Förster Energy Transfer Measurements of Thiol 1 to Thiol 2 Distances in Myosin Subfragment 1†

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ABSTRACT: Förster energy transfer was used to measure the distance between reporter groups on the two reactive thiols of myosin, SH_1 and SH_2, and to detect changes in this distance upon binding of nucleotide. SH_1 was labeled with the fluorophore 5-[[2-[(iodoacetyl)amino]ethyl]amino]naphthalene-1-sulfonic acid (1,5-IAEDANS) and SH_2 with the chromophoric acceptor *N*-[4-(dimethylamino)-3,5-dinitrophenyl]-maleimide (DDPM). Peptide studies verified that [^{3}H]-1,5-IAEDANS reacted specifically with SH_1, while [^{14}C]DDPM labeled both SH_2 and the alkali light chains. The [^{14}C]-DDPM-modified alkali light chains were replaced with unmodified light chains by the exchange procedure of Wagner and Weeds [Wagner, P. D., & Weeds, A. G. (1977) *J. Mol. Biol. 109*, 455-473]. Subfragment 1 labeled with 1,5-IAEDANS and then with DDPM exhibited two fluorescence lifetimes, 20.6 (AEDANS-SF_1, unquenched) and 9.3 ns (AEDANS-SF_1, quenched by DDPM). The latter lifetime decreased to an average of 2.85 ns after the addition of MgAMP-PNP, MgADP, or $MgPP_i$ (no change with MgAMP), indicating that the distance between the donor and acceptor decreased. An R_0 of 29 Å was calculated for the AEDANS/DDPM system assuming random orientation of the donor/acceptor pair. The decrease in the observed lifetimes upon the addition of Mg nucleotide corresponds to a change in the donor–acceptor distance from 28 to 21–22 Å. This observation is consistent with the proposal that nucleotide binding juxtaposes SH_1 and SH_2 to enhance their cross-linking with various bifunctional reagents [Burke, M., & Reisler, E. (1977) *Biochemistry 16*, 5559-5563; Wells, J. A., & Yount, R. G. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4966-4970].

It is well established that myosin and its proteolytic fragment, myosin subfragment 1 (SF_1),[1] undergo spectroscopically sensitive conformational changes during ATP binding, hydrolysis, and product release [Morita, 1967; Seidel et al., 1970; Werber et al., 1972; Murphy, 1974; Mendelson et al., 1975; for reviews see Trentham et al. (1976) and Highsmith & Cooke (1983)]. Extrinsic spectroscopic probes used to monitor these changes are generally introduced by modification of SH_1, the most reactive cysteine in the heavy chain of myosin [see Reisler (1982) for a recent review]. A second activity critical thiol, SH_2, may also be modified rapidly if MgADP (or related compounds) is added (Sekine & Yamaguchi, 1963; Yama-

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[1] Abbreviations: SF_1, myosin chymotryptic subfragment 1; AL1, alkali light chain 1; AL2, alkali light chain 2; AMP-PNP, 5′-adenylyl imidodiphosphate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; 1,5-IAEDANS, 5-[[2-[(iodoacetyl)amino]ethyl]amino]-naphthalene-1-sulfonic acid; DDPM, *N*-[4-(dimethylamino)-3,5-dinitrophenyl]maleimide; IAA, iodoacetamide; MalNET, *N*-ethylmaleimide; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tes, 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid; Tris, tris(hydroxymethyl)-aminomethane; $NaDodSO_4$, sodium dodecyl sulfate; 9-AA, 9-aminoacridine; DPH, 1,6-diphenyl-1,3,5-hexatriene; POPOP, 1,4-bis(5-phenyloxazol-2-yl)benzene; AEDANS-SF_1, subfragment 1 modified at SH_1; SF_1-DDPM, subfragment 1 modified at SH_2; ATPase, adenosinetriphosphatase; DTE, dithioerythritol; Gdn·HCl, guanidine hydrochloride.

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