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Tests of the Simple Model of Lin and Brandts for the Folding Kinetics of Ribonuclease A[†]

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ABSTRACT: L.-N. Lin and J. F. Brandts recently proposed a simple model for the folding kinetics of ribonuclease A in which folding intermediates are not detectable. We have tested the basic assumption of the simple model for the major unfolded species, which is produced by a slow isomerization (the "$X \rightleftharpoons Y$ reaction" according to Lin and Brandts) after unfolding. The simple model assumes that in refolding the slow $Y \rightarrow X$ reaction must occur before any folding can take place. We have measured the $Y \rightarrow X$ reaction during folding. Tyrosine-detected folding occurs before the $Y \rightarrow X$ reaction; the difference in rate between the $Y \rightarrow X$ reaction and folding monitored by tyrosine absorbance becomes large when the stabilizing salt 0.56 M $(NH_4)_2SO_4$ is added. The simple model predicts that the kinetic properties of the $X \rightleftharpoons Y$ reaction in unfolded ribonuclease are the same as those of tyrosine-detected folding. We find, however, that the kinetics of the $X \rightleftharpoons Y$ reaction in unfolded ribonuclease are independent of urea concentration, whereas the rate of tyrosine-detected folding decreases almost 100-fold between 0.3 and 5 M urea, as reported by Lin and Brandts. We point out that the kinetic properties of the $X \rightleftharpoons Y$ reaction in unfolded ribonuclease are characteristic of proline isomerization.

Recently, L.-N. Lin and J. F. Brandts developed a new method, isomer-specific proteolysis, for studying the cis $\rightleftharpoons$ trans isomerization of proline residues after a protein unfolds. It can be used to test the hypothesis (Brandts et al., 1975) that proline isomerization accounts for the existence of both fast-folding (U_F)[1] and slow-folding (U_S) forms of an unfolded protein, a phenomenon first observed for RNase A (Garel &

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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; N, native RNase A; f_{CX}, fraction of RNase A molecules with native (C and X) isomers; Gdm, guanidinium.

Baldwin, 1973). Lin & Brandts (1983a,b) found that two isomerization reactions, termed the $X \rightleftharpoons Y$ and $C \rightleftharpoons T$ reactions, account for the $U_F \rightleftharpoons U_S$ equilibrium in unfolded RNase A. They showed that the kinetics of the $C \rightleftharpoons T$ reaction fitted those of the cis $\rightleftharpoons$ trans isomerization of Pro-93, and they left unspecified the nature of the $X \rightleftharpoons Y$ isomerization that produces the major U_S species of RNase A. They went on to propose a simple kinetic model (Lin & Brandts, 1983c) in which the U_F and U_S species account entirely for the kinetic complexity observed in the refolding of RNase A: in the simple model, no folding intermediates accumulate in measurable amounts during folding.

We reported earlier that structural intermediates are well populated when RNase A refolds at 0–10 °C [reviewed by Kim & Baldwin (1982); see also Schmid (1983)]. The point made by Lin and Brandts is that our experiments were made before a specific assay was available for analysis of the U_S species. Using their analysis of the U_S species, together with computer simulations of unfolding and refolding kinetics based on the simple model, they showed that they could match the observed kinetics. In particular, they were able to correlate the refolding kinetics with the unfolding kinetics measured at the same urea concentration (5 M), by measuring refolding at pH 5 and unfolding at pH 2 and assuming that the relevant rate constants were independent of pH. But to apply their model to refolding at a low urea concentration (such as 0.3 M), they had to make certain additional assumptions. The main purpose of our work is to test these assumptions for the kinetic process in which the major U_S species of RNase A refolds.

A second purpose to to review the evidence that both the $X \rightleftharpoons Y$ and $C \rightleftharpoons T$ reactions in unfolded RNase A have kinetic properties that are characteristic of proline isomerization. The simple model predicts that if the $Y \rightarrow X$ reaction is proline isomerization, then the folding rate of the major U_S species should have the kinetic properties of proline isomerization. But it is already known for this species that the activation enthalpy of the folding rate measured by tyrosine absorbance is much lower than that of proline isomerization (Lin & Brandts, 1983b; Nall et al., 1978).

A third purpose of this paper is to point out that the kinetic equations for the formation of the U_S species after unfolding take on a very simple form if the $X \rightleftharpoons Y$ and $C \rightleftharpoons T$ isomerizations are independent of each other in unfolded RNase A. This will be useful in future work on the U_S species of RNase A. Lin & Brandts (1983b,c) assumed that the $X \rightleftharpoons Y$ and $C \rightleftharpoons T$ reactions are independent in unfolded RNase A, but they did not give the kinetic equations because they used computer simulations based on numerical integration.

The basic assumption of the simple model, applied to the major U_S species, is that the slow $Y \rightarrow X$ isomerization must occur before any folding can take place. This has two consequences that we test here. (1) The simple model predicts that folding measured by tyrosine absorbance must coincide with the $Y \rightarrow X$ isomerization. Fortunately, the major U_S species folds (as measured by tyrosine absorbance) much more rapidly at low urea concentrations than do the minor U_S species. This is notable when a stabilizing salt such as 0.56 M $(NH_4)_2SO_4$ is added (Lin & Brandts, 1983b). Thus, the folding kinetics of the major U_S species can be studied almost in isolation, and we use the two-stage assay for refolding molecules that still contain nonnative isomers (Cook et al., 1979) to monitor the $Y \rightarrow X$ reaction. We find that folding measured by tyrosine absorbance precedes the $Y \rightarrow X$ isomerization. (2) The simple model predicts that the kinetic properties of the $Y \rightarrow X$ reaction are the same during folding as in unfolded RNase A in the same conditions and also that the $Y \rightarrow X$ reaction during folding can be monitored by tyrosine absorbance. The folding rate measured by tyrosine absorbance decreases almost 100-fold between 0.3 and 5 M urea (Lin & Brandts, 1983b). Therefore, we measured the kinetics of the $U_F \rightleftharpoons U_S$ reaction in unfolded RNase A to find out if its kinetics also depend strongly on urea concentration. The $U_F \rightleftharpoons U_S$ reaction results from the sum of the $C \rightleftharpoons T$ and $X \rightleftharpoons Y$ reactions, and the kinetics of the $C \rightleftharpoons T$ reaction in unfolded RNase A are independent of urea concentration within experimental error (Lin & Brandts, 1983b). We find that the kinetics of the overall $U_F \rightleftharpoons U_S$ reaction in unfolded RNase A are independent of urea concentration. Therefore, the kinetics of the $X \rightleftharpoons Y$ reaction, which dominate the $U_F \rightleftharpoons U_S$ kinetics, must also be independent of urea concentration.

Materials and Methods

Materials. RNase A (type XII A) was from Sigma (St. Louis, MO); urea (ultrapure) was from Schwarz/Mann (Orangeburg, NY). All other chemicals were purchased from Merck (Darmstadt, West Germany).

Methods. To monitor the $Y \rightarrow X$ and $T \rightarrow C$ isomerizations during a refolding experiment, we determined $1 - f_{CX}$ by the method of Cook et al. (1979); f_{CX} is the fraction of RNase A molecules that contain only native (C and X) isomers. An aliquot from the refolding experiment (10 mg/mL RNase A) was unfolded by a 3-fold dilution into 7.6 M GdmCl at 0 °C for 55 s; the final solution contained 5.1 M GdmCl, pH 2.2. Then, the U_S concentration was determined by a refolding assay. Folding was initiated by a 10-fold dilution into refolding buffer in the spectrophotometer cell at 25 °C. The final solution contained 1.5 M GdmCl and 0.4 M cacodylate, pH 6.3, and refolding was monitored by absorbance at 287 nm. Then, $1 - f_{CX} = 0.80\Delta\epsilon_{287}/1900$, based on $\Delta\epsilon_{287} = 1900\ M^{-1}\ cm^{-1}$ for complete folding of the U_S species, and U_F to $U_S = 0.20{:}0.80$. The data shown in Figure 2 ($\Delta\epsilon_{287}$ vs. time of refolding) are uncorrected, but a correction to f_{CX} has been made in Figure 3 for a minor conversion of U_F to U_S during the 55-s unfolding period: $(1 - f_{CX})_{corr} = (1 - f_{CX})_{obsd} - (0.06)[0.80 - (1 - f_{CX})_{obsd}]$. The factor 0.06 equals $1 - \exp(t/\tau)$, where $t = 55$ s and $\tau = 860$ s [Table I in Cook et al. (1979)].

The kinetics of the overall $U_F \rightleftharpoons U_S$ reaction in unfolded RNase A were measured at different urea concentrations by a refolding assay for U_S similar to the ones described by Schmid & Baldwin (1978) and Lin & Brandts (1983). (i) Unfolding step. Native RNase A in water (3 mM) was diluted with 4 volumes of concentrated urea, pH 1.9, 10 °C, to give final urea concentrations of 4–8 M, pH 1.9–2.0. (ii) Refolding assay. After varying times of unfolding, 50-μL samples were mixed with 950 μL of refolding buffer in the spectrophotometer cell to give a final sample of 30 μM RNase in 2.0 M urea and 0.1 M acetate, pH 5.2, 25 °C. The slow-refolding reaction was monitored by the increase in absorbance at 287 nm.

Kinetic Equations for Unfolding. The U_S species of RNase A are produced, according to the simple model of Lin & Brandts (1983c), by two isomerization reactions, $X \rightleftharpoons Y$ and $C \rightleftharpoons T$, that occur after RNase A unfolds:

$$\begin{tikzcd}
N_{CX} \arrow[r, shift left] & U_{F(CX)} \arrow[l, shift left] \arrow[r, shift left, "k_{CT}"] \arrow[d, shift right, "k_{XY}"'] & U_{S(TX)} \arrow[l, shift left, "k_{TC}"] \arrow[d, shift right, "k_{XY}"'] \\
 & U_{S(CY)} \arrow[u, shift right, "k_{YX}"'] \arrow[r, shift left, "k_{CT}"] & U_{S(TY)} \arrow[l, shift left, "k_{TC}"] \arrow[u, shift right, "k_{YX}"']
\end{tikzcd} \qquad (1)$$

The same box mechanism is used by Lin & Brandts (1983c) both for unfolding and for refolding. The $X \rightleftharpoons Y$ and $C \rightleftharpoons T$ reactions take place only in unfolded RNase A: the C and X isomers are frozen in N. Unfolding ($N \rightarrow U_F$) takes place before isomerization begins. Unfolding intermediates between N and U_F [cf. Hagerman & Baldwin (1976) and Hagerman et al. (1979)] are not included, and the $N \rightleftharpoons U_F$ reaction is assumed to be fast compared to the $U_F \rightleftharpoons U_S$ reaction, both in unfolding and refolding. Structural intermediates formed from any of the U_S species are explicitly excluded during refolding.

The box mechanism is easily understood for unfolding. It requires only that $N \rightarrow U_F$ be fast compared to the $U_F \rightleftharpoons U_S$ reactions and that the $X \rightleftharpoons Y$ and $C \rightleftharpoons T$ reactions be independent of each other. If the $X \rightleftharpoons Y$ and $C \rightleftharpoons T$ reactions are independent, each one should follow a single-exponential time course starting from $U_{F(CX)} = 1.00$ at zero time. Then one can obtain the concentrations of the different unfolded species by a simple probability analysis for independent reactions. The validity of this treatment is confirmed by an analytical solution of eq 1 for unfolding given in the Appendix. The fraction of unfolded molecules that contain the C isomer is denoted U_C, and so forth. Probability analysis gives

$$U_{F(CX)} = U_C U_X \tag{2a}$$

$$U_S = 1 - U_{F(CX)} \tag{2b}$$

$$U_S = U_T + U_Y - U_T U_Y \tag{2c}$$

If U_T and U_Y are formed in independent first-order reactions, these can be written as

$$U_T(t) = U_T(\infty)[1 - \exp(-t/\tau_{CT})] \tag{3a}$$

$$\tau_{CT} = 1/(k_{CT} + k_{TC}) \tag{3b}$$

$$U_Y(t) = U_Y(\infty)[1 - \exp(-t/\tau_{XY})] \tag{3c}$$

$$\tau_{XY} = 1/(k_{XY} + k_{YX}) \tag{3d}$$

Note that there are two kinetic phases in the formation of U_S even if $\tau_{XY} = \tau_{CT}$ (eq 2c). For unfolded RNase A, Lin & Brandts (1983b,c) give $U_T(\infty) = 0.270$, $U_Y(\infty) = 0.726$, $\tau_{CT} = 170$ s at 4.8 M urea and 180 s at 8.0 M urea, and $\tau_{XY} = 340$ s at 4.8 M urea.

Results

Kinetics of the Y → X Isomerization Reaction during Folding. We use folding conditions where $U_{S(CY)}$, the major U_S species, is the first U_S species to refold. In 0.3 M urea, 10 °C, pH 5, the folding kinetics of $U_{S(CY)}$ measured by tyrosine absorbance (287 nm) are well resolved from those of the other two, slower-folding, U_S species (Lin & Brandts, 1983b). When 0.56 M $(NH_4)_2SO_4$ is added to speed up the folding of $U_{S(CY)}$, its refolding kinetics measured by A_{287} are almost complete within the dead time of manual mixing. Figure 1 shows the spectrophotometer traces for folding in 0.3 M urea and in 0.3 M urea plus 0.56 M $(NH_4)_2SO_4$. The traces are extrapolated (dashed lines) back to $t = 0$, with the assumption that $\Delta\epsilon = 1900\ M^{-1}\ cm^{-1}$. Our results are similar to those reported by Lin & Brandts (1983b).

Figure 2 compares the isomerization kinetics measured in the two sets of folding conditions. The quantity measured is $\Delta\epsilon_{287}$ for folding of the U_S species: the aliquot from the refolding experiment is first unfolded to give a mixture of U_S + U_F, and then the concentration of U_S is determined. As Figure 2 shows, the isomerization kinetics are practically unaffected by 0.56 M $(NH_4)_2SO_4$. Consequently, the large difference between the folding kinetics measured by A_{287} in the presence and absence of 0.56 M $(NH_4)_2SO_4$ (Figure 1) is not caused by a change in the rate of the Y → X isomerization.

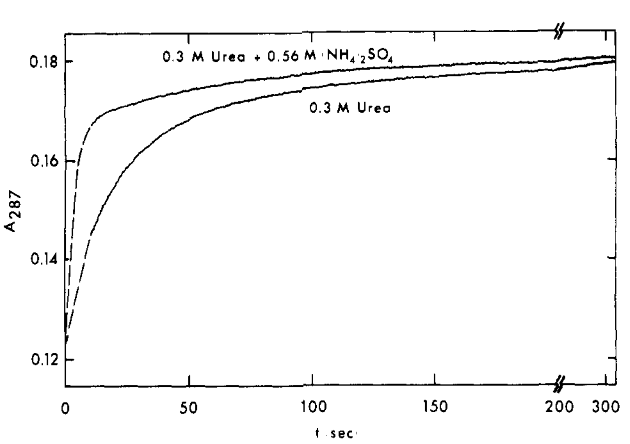

FIGURE 1: Spectrophotometer traces (287 nm) recording the folding of the U_S species of RNase A in 0.3 M urea (lower trace) and in 0.3 M urea plus 0.56 M $(NH_4)_2SO_4$ (upper trace), 10 °C, pH 5.1, 0.05 M acetate, after manual mixing in the spectrophotometer cell. The intercepts at zero time are computed from $\Delta\epsilon_{287} = 1900\ M^{-1}\ cm^{-1}$ for folding of the U_S species and $\epsilon_{287} = 6000\ M^{-1}\ cm^{-1}$ for the native protein.

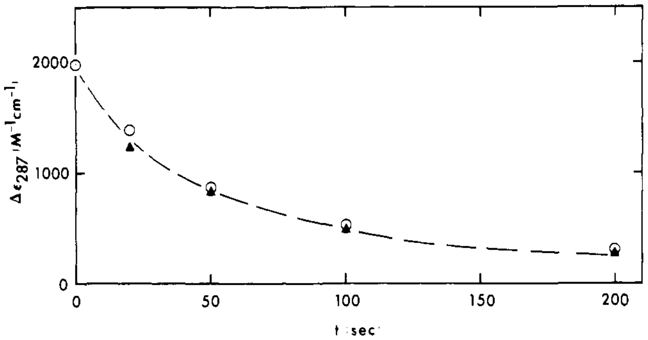

FIGURE 2: Measurement of the isomerization reactions (Y → X and T → C) during folding. The abscissa is proportional to $1 - f_{CX}$, where f_{CX} is the fraction of RNase molecules that contain only native (C and X) isomers. The measured quantity is $\Delta\epsilon_{287}$ for folding of the U_S species after an aliquot from the refolding experiment is unfolded at 0 °C (see Methods). Molecules that contain only native (C and X) isomers unfold to give U_F, and the remainder unfold to give U_S: see text and Cook et al. (1979). The value of $\Delta\epsilon_{287}$ at zero time of refolding is taken to be 1900 $M^{-1}\ cm^{-1}$ (see Figures 4 and 5). Refolding is measured under the same two conditions as in Figure 1: (○) 0.3 M urea; (▲) 0.3M urea + 0.56 M $(NH_4)_2SO_4$.

Figure 3 compares the folding kinetics with the isomerization kinetics for the two sets of folding conditions. In both cases, folding measured by tyrosine absorbance precedes the isomerization reaction. The effect is particularly striking in 0.56 M $(NH_4)_2SO_4$ (Figure 3b). The fraction of the total absorbance change is compared with f_{CX}, the fraction of RNase molecules that contain only native (C and X) isomers. In the model of Lin & Brandts (1983c), the absorbance change occurs as the native protein is formed. If the model correctly described these experiments, then the fraction of the absorbance change would coincide with f_{CX}. Figure 3 shows that folding precedes the Y → X isomerization in 0.3 M urea (Figure 3a) as well as in 0.3 M urea plus 0.56 M $(NH_4)_2SO_4$ (Figure 3b). The assumption by Lin & Brandts (1983b,c) that tyrosine absorbance monitors the isomerization reactions is not correct.

Dependence on Urea Concentration of the Kinetics of the $X \rightleftharpoons Y$ and $C \rightleftharpoons T$ Reactions in Unfolded RNase A. We measure the kinetics of the overall $U_F \rightleftharpoons U_S$ reaction that

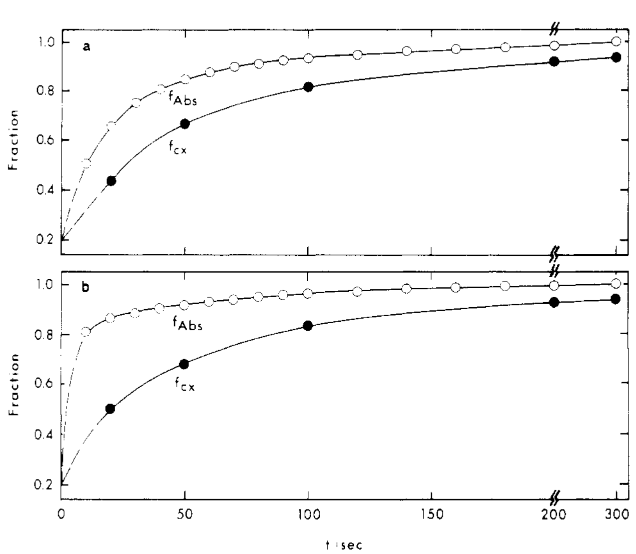

FIGURE 3: Comparison of the folding kinetics measured by tyrosine absorbance (Figure 1) with the isomerization kinetics given by measuring $1 - f_{CX}$ (figure 2). The fraction of the total absorbance change (○) for the refolding of both the U_F and U_S species is given; f_{CX} (●) is the fraction of all RNase A molecules that contain only native (C and X) isomers. Refolding is shown only for the slow-refolding reaction: the fast-folding species U_F refolds instantly on this time scale. According to the model of Lin & Brandts (1983c), the ordinate should in each case equal the fraction of native protein formed by refolding, and the two curves should coincide. Folding conditions were (a) 0.3 M urea (b) 0.3 M urea plus 0.56 M $(NH_4)_2SO_4$, 10 °C, pH 5.1. The values at zero time of refolding are computed by assuming that U_F to U_S = 0.20:0.80 at zero time and that $\Delta\epsilon_{287}$ = 1900 M^{-1} cm^{-1} for folding of the U_S species. The values of f_{CX} have been corrected for a small conversion of U_F to U_S during the 55-s period used for unfolding: see Methods.

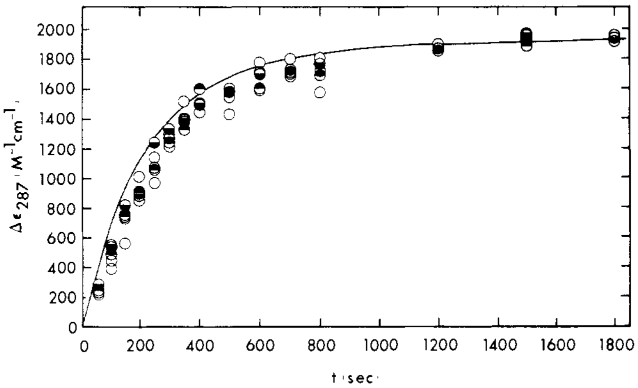

FIGURE 4: Kinetics of formation of the slow-folding (U_S) species of RNase A at different urea concentrations as a function of time after the start of unfolding at 10 °C, pH 2: (◒) 4, (○) 5, 6, and 7, and (◓) 8 M urea. The U_S species were measured by refolding assays as described under Methods. The line shows the curve predicted from $U_S = U_T + U_Y - U_TU_Y$ with eq 2 and 3 and the data of Lin & Brandts (1983b) for the relative amplitudes and time constants (τ_{CT} = 175 s and τ_{XY} = 340 s) of the C $\rightleftharpoons$ T and X $\rightleftharpoons$ Y reactions and with $\Delta\epsilon_{287}(\infty)$ = 1930.

occurs after RNase A unfolds at different urea concentrations. The assay again gives f_{CX}, the fraction of molecules that contain only native (C and X) isomers. In unfolded RNase A, $f_{CX} = U_F$. The kinetic measurements have been made over a range of urea concentrations where RNase A is completely unfolded. At pH 2, 10 °C, where the unfolding measurements of Lin & Brandts (1983b) have been made, RNase A is completely unfolded above 4 M urea. We have also made measurements at 25 °C, pH 1.9, where RNase A is unfolded above 3 M urea.

The results are shown in Figures 4 and 5. There is no significant change in the kinetics with urea concentration, either at 10 °C (4–8 M urea) or at 25 °C (3–8 M urea). The kinetics measured by Lin & Brandts (1983b) at 10.5 °C, 4.8 M urea, are shown as a solid line, calculated from their values of $U_T(\infty)$, $U_Y(\infty)$, τ_{XY}, and τ_{CT} and our eq 2c, 3a, and 3c.

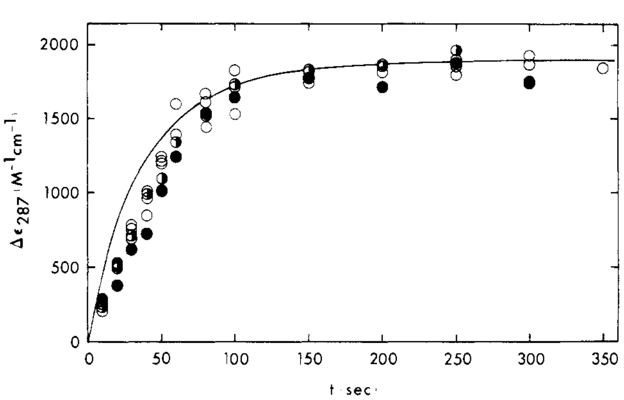

FIGURE 5: Kinetics of formation of the U_S species of RNase A at 25 °C, pH 1.9 ± 0.1, at different urea concentrations: (●) 3, (○) 3.5, 4, and 6, and (◑) 8 M. The line is predicted for the same case shown in Figure 4 but with both reactions 6-fold faster at 25 than at 10 °C, corresponding to $\Delta H^{\ddagger}$ = 20 kcal/mol for each reaction: τ_{CT} = 29.2 s, τ_{XY} = 56.7 s, and $\Delta\epsilon_{287}(\infty)$ = 1900.

Lin & Brandts (1983b) report values of τ_{CT} at 4.8 M urea (170 s) and at 8.0 M urea (180 s) that are the same within experimental error, indicating that the kinetics of the C $\rightleftharpoons$ T reaction in unfolded RNase A are independent of urea concentration. Since we now find that the kinetics of the overall $U_F \rightleftharpoons U_S$ reaction are also independent of urea concentration, it follows that the kinetics of the X $\rightleftharpoons$ Y reaction must be independent of urea concentration in unfolded RNase A.

The apparent activation enthalpy for the overall $U_F \rightleftharpoons U_S$ reaction (10–25 °C) is 20 ± 2 kcal/mol at 4 and 6 M urea. Lin and Brandts report an activation enthalpy of 21 kcal/mol for the C $\rightleftharpoons$ T reaction in unfolded RNase A. It follows that the activation enthalpy of the major X $\rightleftharpoons$ Y reaction must be close to 20 kcal/mol for RNase A unfolded in urea.

Discussion

Folding of $U_{S(CY)}$ Precedes the Y → X Isomerization. The evidence that folding of $U_{S(CY)}$ precedes the Y → X isomerization is shown in Figures 1–3. The results are clear-cut, especially when 0.56 M $(NH_4)_2SO_4$ is added to speed up folding: then folding of $U_{S(CY)}$ measured by tyrosine absorbance is complete at a time when the isomerization reactions have barely begun. The folding kinetics measured by tyrosine absorbance agree satisfactorily with those reported by Lin & Brandts (1983b), who did not measure the isomerization reactions.

The fact that folding of $U_{S(CY)}$ precedes the Y → X isomerization also explains the difference between the urea-independent kinetics of the X $\rightleftharpoons$ Y reaction in unfolded RNase A, reported here, and the strongly urea-dependent folding kinetics of $U_{S(CY)}$, reported by Lin & Brandts (1983b). The basic point is that the Y → X reaction is not rate limiting in the folding of $U_{S(CY)}$. Then it is not surprising that the folding of $U_{S(CY)}$, which precedes the Y → X reaction, shows strongly urea-dependent kinetics. The folding rate of RNase A (U_F → N) is known to be decreased dramatically by denaturants. For example, it is decreased 800-fold when folding takes place in 1 M GdmSCN (Tsong & Baldwin, 1978). The fact that the kinetics of the X $\rightleftharpoons$ Y reaction in urea-unfolded RNase A are urea independent is also not surprising: see The X $\rightleftharpoons$ Y Reaction in Unfolded RNase A Has Kinetic Properties

Characteristic of Proline Isomerization below.

The Box Mechanism of Lin and Brandts Is Not Valid for the Refolding Kinetics of the Major U_S Species of RNase A. The box mechanism of Lin & Brandts (1983c) makes two critical assumptions about the folding of a U_S species such as $U_{S(CY)}$: (1) the rate of the Y → X isomerization precedes any folding and (2) the rate constant for folding is closely approximated by k_{YX} for the Y → X reaction as it occurs in completely unfolded RNase A. Both assumptions have been tested here and are found to be invalid for the major U_S species.

Lin & Brandts (1983c) were able to use the simple model to correlate unfolding kinetics (at pH 2) with refolding kinetics (at pH 5) at the high urea concentration of 5 M. It is likely that folding intermediates are not populated in 5 M urea and that the simple model is more applicable in 5 M urea for this reason [cf. Nall et al. (1978)].

The $X \rightleftharpoons Y$ Reaction in Unfolded RNase A Has Kinetic Properties Characteristic of Proline Isomerization. The isomerization reactions that produce the U_S species of RNase A are known to be catalyzed by strong acid ($[HClO_4] > 5$ M; Schmid & Baldwin, 1978). This is apparent from the data given for the overall $U_F \rightleftharpoons U_S$ reaction. No catalysis is observed at or below 3.3 M $HClO_4$. In 10.6 M $HClO_4$, there is a 100-fold increase in average rate. If either the $X \rightleftharpoons Y$ or $C \rightleftharpoons T$ reaction were not acid catalyzed, it would appear in these experiments as a well-resolved, separate kinetic phase at 10.6 M $HClO_4$. Acid catalysis of cis–trans isomerization in amides can be observed directly by NMR [cf. Redfield & Waelder (1979)]. It probably results from N-protonation, which gives nearly free rotation about the N–C bond (Perrin, 1974; Redfield & Waelder, 1979).

Measurements of the apparent activation enthalpy have also been made for the overall $U_F \rightleftharpoons U_S$ reaction of unfolded RNase A (Schmid & Baldwin, 1978). The measured activation enthalpy represents an average for the $C \rightleftharpoons T$ and $X \rightleftharpoons Y$ reactions. The observed value is 21 kcal/mol (both in 3.3 M $HClO_4$ and in 5.0 M GdmCl; Schmid & Baldwin, 1978), which agrees with the expected value for cis–trans isomerization in amides. This also agrees with the value we now find for either 4 or 6 M urea: 20 ± 2 kcal/mol. NMR studies show an activation enthalpy of 21 kcal/mol for cis–trans isomerization both of X–Pro peptide bonds (Maia et al., 1971, 1976; Cheng & Bovey, 1977; Roques et al., 1977) and of N-methylated peptide bonds (Portnova et al., 1970).

The kinetics of the overall $U_F \rightleftharpoons U_S$ reaction in unfolded RNase A, including the major $X \rightleftharpoons Y$ reaction, are independent of the concentration of GdmCl (Schmid & Baldwin, 1979) and of urea (this work). The kinetics of proline isomerization in the dipeptide Ala–Pro are independent of GdmCl concentration in the range measured (0–2 M) (Nall et al., 1978). These three sets of results (acid catalysis, but only by strong acid; characteristic activation enthalpy; rate almost unaffected by denaturants) indicate a close correspondence between the kinetic properties of the $X \rightleftharpoons Y$ reaction in RNase A and the cis–trans isomerization of a proline or other peptide bond.

It is probable that cis–trans isomerization about a peptide bond (M–N) is too fast to account for the $X \rightleftharpoons Y$ reaction of RNase A unless N is proline [cf. Brandts et al. (1977) and Lin & Brandts (1978)]. Isomerization about X-Pro bonds (Brandts et al., 1975) occurs in the right time range for the $X \rightleftharpoons Y$ reaction. Isomerization rates are not available for ordinary peptide bonds because it is difficult to populate the cis isomer. N-Methylation of a peptide bond increases the fraction of the cis isomer, and kinetic data are available for some N-methylated alanine dipeptides (Portnova et al., 1970). Their isomerization rates are considerably faster than those of X–Pro peptide bonds, and probably the isomerization rates of nonmethylated peptide bonds are still faster.

Concluding Comments. The main point of this paper is that structural intermediates are populated during the folding at 10 °C of the major U_S species of RNase A, in contradiction to the model of Lin & Brandts (1983c). This disagreement should not obscure our appreciation of the major advance made by them in introducing isomer-specific proteolysis as a method for studying the cis–trans isomerization of proline residues in proteins.

Appendix

The box mechanism in eq 1 shows the C–T and X–Y reactions as independent in that the X–Y rate constants do not depend on the C–T states, and vice versa. The kinetics of unfolding by this box mechanism have been simulated by using a digital computer (Lin & Brandts, 1983c). Exact closed-form kinetic equations for the box mechanism are obtained below by solving explicitly the set of four coupled linear differential equations for the box mechanism. In matrix form, the differential equations of the box mechanism are given by (·Å denotes the time derivative)

$$\begin{bmatrix} \dot{U}_{CX}(t) \\ \dot{U}_{TX}(t) \\ \dot{U}_{CY}(t) \\ \dot{U}_{TY}(t) \end{bmatrix} = \begin{bmatrix} -(k_{CT}+k_{XY}) & k_{TC} & k_{YX} & 0 \\ k_{CT} & -(k_{TC}+k_{XY}) & 0 & k_{YX} \\ k_{XY} & 0 & -(k_{YX}+k_{CT}) & k_{TC} \\ 0 & k_{XY} & k_{CT} & -(k_{YX}+k_{TC}) \end{bmatrix} \begin{bmatrix} U_{CX}(t) \\ U_{TX}(t) \\ U_{CY}(t) \\ U_{TY}(t) \end{bmatrix} \tag{A-1}$$

This 4 × 4 matrix equation is of the standard form $\dot{U} = AU$, where $U = [U_{CX}(t)U_{TX}(t)U_{CY}(t)U_{TY}(t)]'$ (′ denotes transpose) and $\mathbf{A}$ is the 4 × 4 matrix.

This 4 × 4 matrix equation can be solved by using an eigenvalue–eigenvector decomposition of the matrix $\mathbf{A}$, as described for general equations by Strang (1980). With the initial condition $U(t) = U_0$, the formal solution to $\dot{U} = AU$ is written

$$U(t) = U_0 e^{At} \tag{A-2}$$

Although the meaning of e^{At} is not immediately clear, the matrix $\mathbf{A}$ can be written as a matrix product if it can be diagonalized:

$$\mathbf{A} = \mathbf{S \Lambda S^{-1}}$$

Here $\mathbf{S}$ is the so-called modal matrix (whose columns are the eigenvectors of a matrix $\mathbf{A}$) and Λ is a diagonal matrix whose diagonal elements are the eigenvalues of the matrix $\mathbf{A}$. In terms of the eigenvalue–eigenvector decomposition of $\mathbf{A}$, eq A-2 can be written in a meaningful matrix form:

$$U(t) = \mathbf{S} e^{\Lambda t} \mathbf{S}^{-1} U_0 \tag{A-3}$$

where $e^{\Lambda t}$ is a diagonal matrix, with diagonal elements $e^{\lambda_i t}$, and λ_i is the ith eigenvalue of matrix $\mathbf{A}$. Details of this approach can be found in Strang (1980). For the particular 4 × 4 matrix equation describing the box mechanism, we have solved for $\mathbf{S}$, $\mathbf{S}^{-1}$, and Λ by using standard algebraic methods. These matrices are

$$\mathbf{S} = \begin{bmatrix} k_{YX}k_{TC} & k_{YX} & k_{TC} & 1 \\ k_{YX}k_{CT} & -k_{YX} & k_{CT} & -1 \\ k_{XY}k_{TC} & k_{XY} & -k_{TC} & -1 \\ k_{XY}k_{CT} & -k_{XY} & -k_{CT} & 1 \end{bmatrix} \quad \text{(A-4a)}$$

$$\mathbf{S}^{-1} = \frac{1}{(k_{YX} + k_{XY})(k_{CT} + k_{TC})} \times \begin{bmatrix} 1 & 1 & 1 & 1 \\ k_{CT} & -k_{TC} & k_{CT} & -k_{TC} \\ k_{XY} & k_{XY} & -k_{YX} & -k_{YX} \\ k_{XY}k_{CT} & -k_{XY}k_{TC} & -k_{YX}k_{CT} & k_{YX}k_{TC} \end{bmatrix} \quad \text{(A-4b)}$$

and

$$\Lambda = \begin{bmatrix} 0 & 0 & 0 & 0 \\ 0 & -(k_{CT} + k_{TC}) & 0 & 0 \\ 0 & 0 & -(k_{XY} + k_{YX}) & 0 \\ 0 & 0 & 0 & -(k_{CT} + k_{TC} + k_{XY} + k_{YX}) \end{bmatrix} \quad \text{(A-4c)}$$

The matrices used in eq A-3 provide the exact closed-form solution to the chemical kinetics of the box mechanism; they give the time dependencies of the concentrations of the four unfolded protein species. The solution can be applied to arbitrary initial conditions specified in the initial vector U_0. We give the time dependence of U_{CX}, starting from the initial condition specified below:

$$U_{CX}(0) > 0 \qquad U_{TX}(0) = 0$$
$$U_{CY}(0) = 0 \qquad U_{TY}(0) = 0$$

This is written for eq A-3 as

$$U_0 = [U_{CX}(0) \quad 0 \quad 0 \quad 0]'$$

The matrices S, S^{-1}, and Λ are put into eq A-3, along with U_0 above, and the sequence of matrix vector products carried out to arrive at the solution

$$U_{CX}(t) = \frac{U_{CX}(0)}{(k_{XY} + k_{YX})(k_{CT} + k_{TC})}(k_{YX}k_{TC} + k_{YX}k_{CT} \exp[-(k_{CT} + k_{TC})t] + k_{TC}k_{XY} \exp[-(k_{XY} + k_{YX})t] + k_{XY}k_{CT} \exp[-(k_{CT} + k_{TC} + k_{XY} + k_{YX})t]) \quad \text{(A-5a)}$$

Straightforward manipulation yields

$$U_{CX}(t) = U_{CX}(0)\left[\frac{1}{k_{TC} + k_{CT}}(k_{TC} + k_{CT} \exp[-(k_{CT} + k_{TC})t])\right]\left[\frac{1}{k_{XY} + k_{YX}}(k_{YX} + k_{XY} \exp[-(k_{XY} + k_{YX})t])\right] \quad \text{(A-5b)}$$

Further algebraic analysis shows that eq A-5b, which is derived from consideration of the complete kinetic description involving all four unfolded protein species, is equal to the expression for $U_{CX}(t)$ obtained using the simpler probability arguments outlined above.

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