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Kinetics and Mechanism of the Refolding of Denatured Ribonuclease A†

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ABSTRACT: On the basis of two experimental observations, it is established that the refolding mechanism of ribonuclease A (RNase A) is independent of the nature of the denaturant used [urea or guanidine hydrochloride (Gdn·HCl)]. First, by use of a double-jump technique, it is demonstrated that a similar nativelike intermediate exists on the major slow-folding pathway of both urea- and Gdn·HCl-denatured RNase A. Second, from the temperature dependence of the slow-refolding kinetics, it is shown that the activation parameters (both enthalpy and entropy) of the rate-limiting steps, as monitored by tyrosine absorbance and fluorescence, are identical for the refolding of urea- and Gdn·HCl-denatured RNase A. A refolding scheme involving one intermediate on each of the two slow-folding pathways is proposed by adopting the notion that RNase A refolds through a sequential mechanism. However, these two intermediates are formed from their respective unfolded forms (U_S^{II} and U_S^{I}) through two different processes of distinct physical origin. The intermediate I_N, which is formed from the major slow-folding species U_S^{II} through a conformational folding step, already possesses many properties of the native protein. In contrast, the intermediate (designated as I′) on the minor slow-folding pathway is formed from U_S^{I} by the isomerization of a proline residue (possibly Pro^{93}) and is still conformationally unfolded. It is shown that such a refolding scheme can account for the known kinetic features of *both* major and minor slow-refolding pathways of RNase A. Specifically, it can account for the following experimental observations: (i) the merging of the major and minor slow-folding phases into a single one at high concentrations of denaturant; (ii) the retention of the biphasic behavior of the slow-folding kinetics at high concentrations of denaturant when $(NH_4)_2SO_4$ is added; and (iii) the reversal of the relative amplitudes of the two phases when detected by tyrosine fluorescence as compared to UV absorbance. As an alternative to proline isomerization, it is also hypothesized that the stereochemical inversion of the chirality of a disulfide bond in the protein molecule might be the underlying molecular basis for the $I_N \rightarrow N$ step.

The existence of multiple unfolded forms of bovine pancreatic ribonuclease A (RNase A)[1] in its denatured state is now well established (Garel & Baldwin, 1973; Garel et al., 1976; Hagerman & Baldwin, 1976). From its biphasic refolding kinetics, it has been concluded that the different unfolded forms interconvert slowly and refold to the native enzyme on vastly different time scales, ranging from milliseconds to seconds. These unfolded forms are designated as fast-folding (U_F) and slow-folding (U_S) species and exist at an equilibrium ratio of 20:80 in the denatured state. To account for the slow interconversion between the different unfolded conformations and the difference in refolding rates, Brandts et al. (1975) proposed that, while all of the prolyl residues of U_F are in their native conformations, U_S contains one or more nonnative proline conformations and the cis–trans isomerization(s) of these residues is (are) responsible for the slow-refolding kinetics. Since then, considerable experimental evidence has been accumulated in support of the involvement of proline isomerization in the folding/unfolding of RNase A (Nall et al., 1978; Schmid & Baldwin, 1978, 1979a,b) and of other proteins (Ko et al., 1977; Crisanti & Matthews, 1981; Desmadril & Yon, 1981; McPhie, 1982; Goto & Hamaguchi, 1982; Nall, 1983).

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[1] Abbreviations: RNase A, bovine pancreatic ribonuclease A with intact disulfide bonds; U_S and U_F, slow- and fast-folding species of denatured RNase A, respectively; BPTI, bovine pancreatic trypsin inhibitor; Gdn·HCl, guanidine hydrochloride; NaOAc, sodium acetate; 2′-CMP, cytidine 2′-phosphate; CD, circular dichroism; NMR, nuclear magnetic resonance; Gly·HCl, glycine hydrochloride.

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It is also known that, under strongly native conditions, the slow-folding kinetics itself resolve further into a faster, major (80%) and a slower, minor (20%) phase (Schmid & Baldwin, 1978; Cook et al., 1979; Schmid & Blaschek, 1981; Schmid, 1981; Lin & Brandts, 1983b). This biphasic behavior of the slow phase of the refolding kinetics indicates that there are at least two different forms[2] of slow-folding species, which are designated as U_S^{II} (major) and U_S^{I} (minor).

There are currently two proposed models[3] for the folding mechanism of RNase A. In the sequential model (Nall et al., 1978; Cook et al., 1979), U_S refolds through recognizable partially folded intermediates, and the definite and unique sequence of the formation of such intermediates defines its folding pathway. Recently, Lin & Brandts (1983b,c) proposed an alternative two-state model to account for the folding kinetics of RNase A. In this model, it is assumed that there are several different forms of RNase A in its denatured state, and the complex kinetics observed experimentally during the refolding process arise from the interconversion between different denatured species.

The fundamental difference between these two models centers upon the question as to whether intermediates with folded structures can exist on the folding pathway. In addition, it is also obligatory for U_S to refold through U_F in the model of Lin & Brandts (1983b,c). It has been demonstrated that, under strongly native conditions following denaturation with Gdn·HCl, intermediates with both ordered (I_1) and nativelike (I_N) structures are populated on the major slow-folding pathway of RNase A (Blum et al., 1978; Nall et al., 1978; Schmid & Baldwin, 1979b; Kim & Baldwin, 1980; Cook et al., 1979; Schmid & Blaschek, 1981, 1984; Schmid, 1983). However, Lin & Brandts (1983b,c) observed no evidence to support the existence of the nativelike intermediate on the major slow-folding pathway of RNase A. It must be pointed out that, in the study of Lin & Brandts (1983b), the refolding kinetics were measured under conditions where urea, rather than Gdn·HCl, was used as the denaturant.[4,5] It is, therefore, important to establish the identity of the refolding mechanism under two different sets of conditions (Gdn·HCl vs. urea).

In this paper, we present refolding experiments performed on urea-denatured RNase A simply to remove any possible doubt that urea and Gdn·HCl denaturation might lead to different refolding kinetics. Specifically, we shall establish that the refolding mechanism of RNase A is independent of the nature of the denaturant used (urea or Gdn·HCl) by demonstrating (i) the existence of a similar nativelike intermediate on the major refolding pathway and (ii) the coincidence of the refolding kinetics under the two different sets of conditions.

After this possible doubt is disposed of, we are then concerned not with accounting for the limited amount of experimental results presented in this paper but primarily with the general literature concerning the mechanism of refolding of RNase A. The latter is the primary goal of this paper.

The sequential folding model proposed by Baldwin and co-workers (Nall et al., 1978; Cook et al., 1979) is applicable only under strongly native conditions. However, there still remain certain questions about the slow-refolding kinetics that have not been addressed. For example, the amplitudes of the two slow-folding processes are not the same when the refolding kinetics are monitored by tyrosine fluorescence as compared to tyrosine absorbance (Lin & Brandts, 1983b). Furthermore, is there an alternative folding pathway whereby the two slow-folding species refold through U_F? More importantly, it is experimentally observed that the slow-folding kinetics deviate from its biphasic behavior and merge into a single phase as the denaturant concentration is increased, even before the transition region (Henkens et al., 1980; Lin & Brandts, 1983b). Such peculiar features cannot, as yet, be accounted for by any refolding scheme available in the literature. In this paper, we shall attempt to propose a scheme that will account for such features of the refolding of RNase A.

Experimental Procedures

Materials. Bovine pancreatic ribonuclease A (type IIA) was purchased from Sigma Chemical Co. and was purified on a (carboxymethyl)cellulose (CM-52, Whatman Ltd.) column by the method of Taborsky (1959). Ultrapure urea and guanidine hydrochloride were from Schwarz/Mann and were used without further purification. Ammonium sulfate and sodium acetate, both of analytical grade, were purchased from Mallinckrodt, Inc.

The concentrations of urea and Gdn·HCl solutions were checked by refractive index measurements at 20 °C (Warren & Gordon, 1966; Nozaki, 1972) using an Abbé refractometer (Bausch & Lomb Optical Co.). All solutions were filtered before use with filters (0.45-μm pore size) purchased from Millipore Corp. pH measurements were made on a Radiometer pH meter (Model PHM 84) equipped with a glass combination electrode.

Absorbance Measurements. All UV absorption measurements were made on a modified Cary Model 14 spectrophotometer (Denton et al., 1982). The data were automatically

[2] A third very slow phase, accounting for about 5% of the total amplitude, has recently been resolved spectroscopically by Lin & Brandts (1983b) when denatured RNase A is refolded at high concentrations of urea. This might indeed suggest the existence of a third slow-folding species of RNase A.

[3] Most recently, on the basis of their own experimental observations, Biringer & Fink (1984) proposed a convergent sequential model for the refolding of RNase A. However, in their experimental study of the refolding of RNase A, three slow kinetic phases of *equal* amplitude (24% each) were observed. Since their refolding experiments were carried out by using aqueous methanol cryosolvents and at subzero temperatures, it is difficult to compare their results directly with those obtained in aqueous solution and at ambient temperatures, discussed in this paper.

[4] Recently, Schmid et al. (1984) investigated the folding kinetics in urea. They showed that, for *urea-denatured* RNase A, the rate of conversion of U_S^{II} to the native form is much slower when monitored in a double-jump experiment (which, by analogy with folding of Gdn·HCl-denatured RNase A, essentially observes the $I_N \rightarrow N$ reaction) than when monitored by UV absorbance (which, by a similar analogy, observes the faster folding step, $U_S^{II} \rightarrow I_N$). Moreover, they also showed that the rate of the $U_F \rightleftarrows U_S^{II}$ interconversion, as it occurs in the denatured state, is independent of urea concentration, implying that the observed dependence of the refolding kinetics of U_S^{II} on urea concentration (Lin & Brandts, 1983b) cannot be due to the $U_S^{II} \rightarrow U_F$ step. These results are in contradiction to the model of Lin & Brandts (1983b,c), even though these authors (Lin & Brandts, 1984), solely on the basis of the earlier work of Schmid (1983) on the refolding of *Gdn·HCl-denatured* RNase A, subsequently included I_N on an *additional* pathway for refolding in urea. However, in the present context, the study of Schmid et al. (1984) did not provide an answer to the key question (except by *analogy* to the behavior in Gdn·HCl) as to whether folded intermediates (such as I_N) also exist on the major refolding pathway of urea-denatured RNase A.

[5] The transient species I_1, detected by Baldwin and co-workers (Blum et al., 1978; Schmid & Baldwin, 1979b; Kim & Baldwin, 1980), was shown to form very rapidly. Presumably, it lies on the $U_S^{II} \rightarrow I_N$ pathway and is not considered in our model. Furthermore, as mentioned in footnote 2, it is possible that a third slow-folding species, amounting to about 5% of the total of the unfolded forms, exists in denatured RNase A. However, because of its small refolding amplitude and hence its relative insignificant contribution to the overall refolding kinetics, the refolding pathway of this very minor slow-folding species (U_S^{III}) will not be discussed in this paper.

digitized with an analog to digital converter connected to the spectrophotometer. The temperature was controlled with a Forma-Temp Jr. water bath and was measured with a calibrated thermistor dipped directly into the optical cell. The accuracy of the temperature measurements is estimated to be ±0.1 °C.

Native RNase A was denatured in 4.0 M Gdn·HCl or 5.2 M urea in 50 mM Gly·HCl (15 mg/mL, pH 2.0). For refolding, 0.10 mL of the unfolded protein solution was pipetted into a cuvette containing 1.50 mL of 0.050 M NaOAc buffer (pH 5.4) that had been thermostated at the desired temperature for at least 15 min. The solution was then mixed manually for 15 s. The final pHs for urea and Gdn·HCl-denatured refolding were 4.8 and 5.0, respectively. The absorbance change at 287 nm was monitored to follow the refolding kinetics. Digitized data were recorded every second and were input directly into a Prime 550 computer. The time course of the refolding process was monitored for a time period equivalent to at least 6 times its half-life, or until there was no observable change in absorbance. The temperature dependence of the refolding kinetics was measured in the range of 1–15 °C.

Fluorescence Measurements. The experimental procedure for the fluorescence measurements was essentially the same as that for the absorbance measurements. All measurements were carried out on a Perkin-Elmer Model MPF-44B fluorescence spectrophotometer equipped with jacketed cell holders. The excitation wavelength was 268 nm, and the tyrosine fluorescence emission at 305 nm was monitored as a function of time to follow the refolding kinetics. Both excitation and emission slit widths were set at 5 nm. The temperature was controlled and thermostated to ±0.1 °C by a circulating water bath and was measured directly with a calibrated, dipping thermister.

Experiments for Detection of the Existence of I_N. To achieve maximal accumulation of I_N, the denatured protein was allowed to refold under very strongly native conditions for a short period of time. The refolding process was then interrupted, and the prefolding solution was jumped to different final conditions to establish the stability of I_N (Schmid, 1983). For the prefolding step, 0.10 mL of the denatured protein solution (3.3 mM RNase A in 5.2 M urea and 50 mM Gly·HCl, pH 2.0) was pipetted into 1.50 mL of 0.05 M NaOAc buffer (pH 5.4) and 0.6 M $(NH_4)_2SO_4$ and 0.05 M NaOAc, pH 5.0 at 0 °C. After being mixed for 20 s, 0.125 mL of the prefolding solution was pipetted into a cuvette that had been thermostated at 10.0 °C and contained 1.125 mL of 0.053 M NaOAc and varying amounts of urea. The solution was then mixed manually for about 15 s, and the absorbance change at 287 nm was followed. The final conditions for the refolding kinetics were 20 μM RNase A in 0.06 M $(NH_4)_2SO_4$, 0.05 M NaOAc, and 6.0–7.0 M urea, pH 5.0 at 10.0 °C.

For comparison, the analogous experiments of Schmid (1983), in which denaturation was brought about by Gdn·HCl, were also repeated here.

Analysis of Refolding Kinetic Data. Under strongly native conditions, the slow-refolding kinetics of RNase A are biphasic in character (Cook et al., 1979; Schmid, 1981; Lin & Brandts, 1983b). The experimentally observed refolding kinetic data for the absorbance and fluorescence changes were fitted to the following empirical equations involving two exponential terms:

$$A_\infty - A_t = A_1 \exp(-t/\tau_1) + A_2 \exp(-t/\tau_2) \quad (1)$$

$$f_1 = A_1/(A_1 + A_2) \quad (2)$$

Here A_∞ and A_t are, respectively, the relative absorbances (or fluorescence) at infinite time and at time t after the solvent jump. A_1 and A_2 represent the two amplitudes associated with the kinetic phases, and f_1 is the fraction of the total amplitude associated with the shorter kinetic time constant, τ_1. In the computer-fitting procedure, the error of fit was treated as a quadratic function. The five adjustable parameters, A_1, A_2, A_∞, τ_1, and τ_2, were systematically varied to minimize the error between the observed and calculated values of A_t.

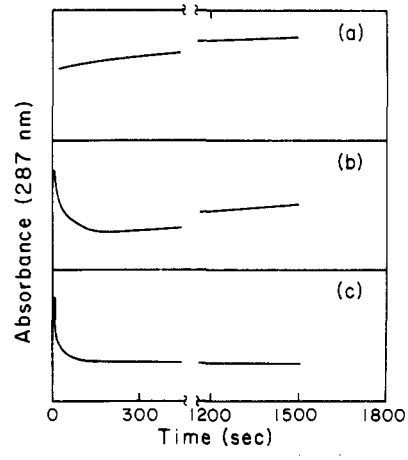

FIGURE 1: Tests for the existence of I_N and its relative stability as compared to that of the native protein toward urea denaturation. Denatured RNase A (3.3 mM RNase A in 5.2 M urea and 50 mM Gly·HCl, pH 2.0) was subjected to a prefolding step for approximately 20 s. The final condition for the prefolding step was 0.2 mM RNase A in 0.35 M urea, 0.6 M $(NH_4)_2SO_4$, and 0.05 M NaOAc, pH 5.0 at 0 °C. Refolding or unfolding was induced by a 10-fold dilution of the prefolding solution with a solution containing varying amounts of urea at 10 °C. The absorbance change at 287 nm as a function of time is shown for the final assay conditions of (a) 6.0 M urea, (b) 6.5 M urea, and (c) 7.0 M urea.

RESULTS

Detection of a Nativelike Intermediate in Urea-Denatured Refolding. It has been demonstrated that, after denaturation with Gdn·HCl, refolding under strongly native conditions involves a nativelike intermediate, I_N, on the major $U_S^{II} \rightarrow N$ folding pathway (Cook et al., 1979; Schmid & Blaschek, 1981; Schmid, 1983). I_N already possesses many properties of the native protein, as indicated by its similar tyrosine absorbance, binding of the specific inhibitor 2′-CMP (Cook et al., 1979), enzymatic activity (Schmid & Blaschek, 1981), and stability toward Gdn·HCl denaturation (Schmid, 1983). Here, we examine the possibility of the existence of a similar intermediate during refolding after denaturation with urea.

The denatured protein was first prefolded under strongly native conditions for a brief period of time. Under these conditions, all the fast-folding species (U_F) have been refolded to the native protein after the prefolding step, whereas essentially all the minor slow-folding species (U_S^I) remain unfolded. Moreover, most of the major slow-folding species (U_S^{II}) have been converted into a folded intermediate (I_N) (Cook et al., 1979; Schmid & Blaschek, 1981; Schmid, 1983), if indeed such an intermediate exists on the major slow-folding pathway of RNase A after denaturation with urea. It is conceivable that the native protein and any folded intermediate(s) accumulated after the prefolding step might have different stabilities toward urea denaturation. If so, jumping the prefolded solution to different final folding conditions will give an indication as to the relative stability of any such accumulated intermediate(s).

Figure 1a shows the time course of the absorbance change for refolding in 6.0 M urea (transition midpoint at 6.8 M urea),

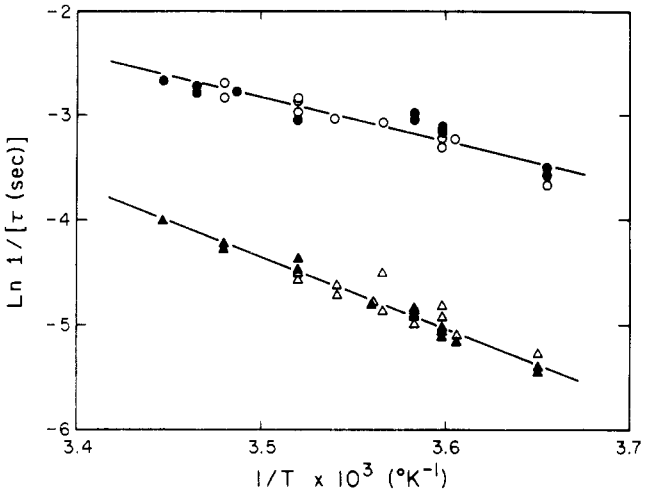

FIGURE 2: Temperature dependence of the apparent rate constants (τ^{-1}) of urea-denatured [major phase (○); minor phase (△)] and Gdn·HCl-denatured [major phase (●); minor phase (▲)] refolding kinetics of RNase A as monitored by absorbance. Final conditions were as follows: (i) for urea-denatured refolding, 1.0 mM RNase A and 0.33 M urea, pH 4.8; (ii) for Gdn·HCl-denatured refolding, 1.0 mM RNase A and 0.25 M Gdn·HCl, pH 5.0.

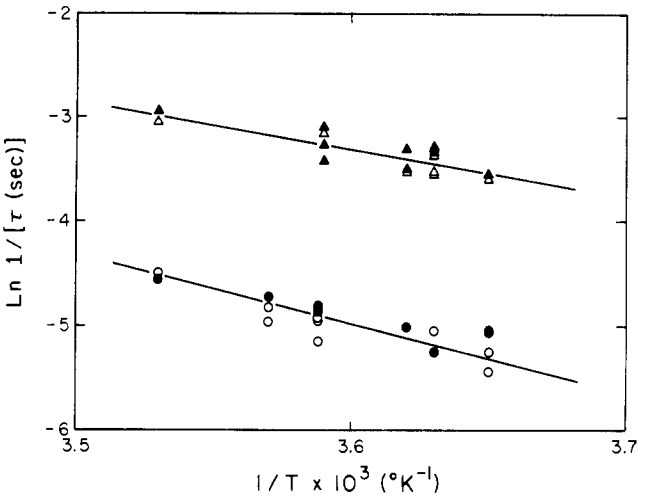

FIGURE 3: Same as in Figure 2 except that the refolding kinetics were monitored by fluorescence. It should be noted that, for fluorescence-detected kinetics, the major phase is the *slower* phase.

pH 5.0 at 10.0 °C, after the denatured protein had been subjected to a brief prefolding period under very strongly native conditions [0.35 M urea and 0.6 M $(NH_4)_2SO_4$, pH 5.0 at 0 °C]. It can be seen that there is a continuous increase in absorbance at 287 nm, indicating the refolding of any accumulated intermediates, along with the remaining unfolded molecules (U_S^I), to the native state.

The situation is quite different, however, when the final refolding step is carried out at a urea concentration of 6.5 M (Figure 1b). Under these conditions, the transient intermediates accumulated in the prefolding step are no longer stable and are rapidly unfolded, thus giving rise to the initial decrease in absorbance immediately after the solvent jump. However, since these are folding conditions, the unfolded molecules can still refold to the native conformation, as indicated by the slow, continual increase in absorbance after the initial decrease.

To provide further support for the above analysis, the prefolding solution is jumped to an unfolding condition (7.0 M urea, pH 5.0 at 10.0 °C.). The spectrophotometric trace at 287 nm (Figure 1c) shows a rapid decrease as before (Figure 1b), but there is no observable subsequent recovery phase, indicating that *all* folded molecules are being denatured under these conditions.

It can be inferred from the results of this study that a folded intermediate with buried tyrosine groups exists on the urea-denatured major slow-folding pathway of RNase A. Furthermore, its stability toward urea denaturation is rather close to that of the native protein (it starts to unfold at 6.5 M urea at pH 5.0 as compared to 7 M urea for native RNase A), implying that this nativelike intermediate can exist under a wide range of folding conditions. Similar results have been obtained by Schmid (1983) using Gdn·HCl as the denaturing agent. (The data from our repeat of Schmid's experiments with Gdn·HCl are in essential agreement with those that he reported.) It can, therefore, be concluded that both urea- and Gdn·HCl-denatured RNases A refold through a nativelike intermediate on its major slow-folding pathway.

Comparison of Refolding Kinetics of Urea- and Gdn·HCl-Denatured RNase A. To address the question of whether the refolding pathway is identical for urea- and Gdn·HCl-denatured RNase A, we examined the energetics of their refolding kinetics. Figure 2 shows the temperature dependences of the relaxation times of the major and minor slow phases as monitored by UV absorbance. The kinetic time constants were derived from manual-mixing refolding experiments in which the fast phase is essentially complete within the mixing dead time and were obtained by fitting the time course of the absorbance change at 287 nm to eq 1. It can be seen that, within experimental error, the relaxation time constants of both major and minor phases of urea- and Gdn·HCl-denatured refolding kinetics lie on the same straight lines in the experimental temperature range of 1–15 °C. This indicates that, for either slow-folding species (U_S^{II} or U_S^I), the activation parameters (both enthalpy and entropy) of the refolding process as monitored by absorbance are the same regardless of the nature of the denaturant used.

It is known that the observed slow-refolding kinetics depend on the physical probes employed for their detection (Schmid, 1981; Lin & Brandts, 1983b). For example, it has been demonstrated that the slow-refolding kinetics monitored by fluorescence and absorbance are noncoincident (Schmid, 1981; Lin & Brandts, 1983b). This has been attributed to the fact that different rate-limiting steps are being monitored by different probes (Schmid, 1981). Thus, we also examined the temperature dependence of the slow-refolding kinetics using fluorescence as a monitoring probe.

The fluorescence-detected relaxation time constants of the two slow phases of urea- and Gdn·HCl-denatured refolding kinetics in the temperature range of 1–11 °C are shown in Figure 3. Within experimental error, the kinetic time constants derived from fluorescence monitoring also lie on two straight lines. This demonstrates, once again, that the activation energies of the rate-limiting steps as monitored by fluorescence are identical for urea- and Gdn·HCl-denatured refolding processes.

It must be emphasized that so far we have demonstrated only that the activation energies of the rate-limiting processes of the two slow-refolding phases are the same regardless of the nature of the denaturant used. This does not necessarily imply that the entire folding pathways for the regeneration of the native protein from its denatured state are identical under the two sets of conditions. However, it was also determined from the refolding kinetic experiments that the relative amplitudes of the two slow phases remain constant for both absorbance-detected ($f_1 = 0.75$) and fluorescence-detected ($f_1 = 0.35$) refolding of urea- and Gdn·HCl-denatured RNase A. This, coupled with the fact that a similar kinetic

intermediate is populated on both major slow-refolding pathways, does make it highly likely that urea- and Gdn·HCl-denatured RNase A refold through identical pathways. It is interesting to note that it has also been demonstrated that the refolding kinetics of hen egg white lysozyme are the same regardless of the nature of the denaturant used (Gdn·HCl or acetic acid), if the final refolding conditions are the same with both acetic acid and Gdn·HCl diluted to a low concentration (Kato et al., 1981).

Discussion

Kinetic Equations for the Refolding of a Single Denatured Species. Hereinafter, we are interested in discussing only the general kinetic features of the refolding of RNase A currently known in the literature. However, before we proceed further to discuss the refolding mechanism of RNase A, we think it is appropriate to consider here first some general theoretical aspects of its refolding kinetics. As mentioned in the previous section, our results indicate that U_S^{II} refolds to the native conformation through I_N on a sequential pathway that is independent of the nature of the denaturant used. In general, for a linear, sequential three-state model involving a single intermediate I, i.e.

$$U \xrightarrow{k_1} I \xrightarrow{k_2} N \tag{3}$$

the time dependences of the concentrations of the three individual species are given by the following expressions (Szabo, 1969):

$$[U] = [U]_0 e^{-k_1 t} \tag{4}$$

$$[I] = \frac{k_1}{k_2 - k_1}[U]_0(e^{-k_1 t} - e^{-k_2 t}) \tag{5}$$

$$[N] = [U]_0\left[1 - e^{-k_1 t} - \frac{k_1}{k_2 - k_1}(e^{-k_1 t} - e^{-k_2 t})\right] \tag{6}$$

Here, it is assumed that only the denatured species U, at a concentration of $[U]_0$, exists at $t = 0$. Any experimentally monitored property, such as absorbance or fluorescence intensity, will simply be the sum of the contributions from all the individual species present weighted by their corresponding concentrations. For example, by use of eq 4–6, the total molar absorption extinction coefficient, ϵ_I, can be shown to be given by

$$\epsilon_T = (\epsilon_U[U] + \epsilon_I[I] + \epsilon_N[N])/[U]_0 = \epsilon_N + \left[(\epsilon_U - \epsilon_N) + (\epsilon_I - \epsilon_N)\frac{k_1}{k_2 - k_1}\right]e^{-k_1 t} - (\epsilon_I - \epsilon_N)\frac{k_1}{k_2 - k_1}e^{-k_2 t} \tag{7}$$

It can be seen from the above expression that, for the refolding of a *single* denatured species according to the mechanism depicted in eq 3 involving two different rate constants k_1 and k_2, the refolding kinetics will become monophasic only under the limiting conditions of (i) $\epsilon_I = \epsilon_N$ and/or (ii) $k_2 >> k_1$. The significance of these two conditions will become apparent later.

Refolding Kinetics of RNase A. (1) Fast-Refolding Phase. From stopped-flow kinetic studies, it has been demonstrated that the fast-refolding phase (τ = 50 ms) shows simple, first-order kinetics and accounts for 20% of the total amplitude change (Garel & Baldwin, 1973; Garel et al., 1976; Hagerman & Baldwin, 1976; Lin & Brandts, 1983b). Furthermore, there is also strong experimental evidence that the $N \rightarrow U_F$ reaction is the first step during unfolding (Rehage & Schmid, 1982; Lin & Brandts, 1983b). Thus, these authors concluded that the transition between U_F and N is reversible in both directions of the folding/unfolding of RNase A and does not involve proline isomerization.

(2) Slow-Refolding Phases. In contrast to the fast-refolding phase, the slow-refolding kinetics are known to be complex. Moreover, it has also been demonstrated that the observed slow-folding kinetics depend strongly on the physical probe employed for its detection (e.g., fluorescence vs. absorbance), as well as on the final refolding conditions (Schmid, 1981; Lin & Brandts, 1983b). We shall attempt to provide here a consistent physical picture as to the origin of such complexities.

As mentioned earlier, the major slow-folding species U_S^{II} refolds by way of a three-state mechanism involving a nativelike, folded intermediate, I_N. It has been proposed that the $I_N \rightarrow N$ step involves the isomerization of a prolyl residue (Cook et al., 1979; Schmid, 1981; Schmid & Blaschek, 1981). However, not much is known about the minor $U_S^I \rightarrow N$ pathway, although there is experimental evidence indicating the involvement of the isomerization of Pro^{93} from a trans to a cis conformation (Lin & Brandts, 1983a,b). Here we propose, as will become apparent later from an analysis of the slow-folding kinetics, that U_S^I also refolds through an intermediate (designated as I′) which is formed by the isomerization of Pro^{93} as a *first* step in its refolding pathway. Our proposed refolding pathways for the two slow-folding species are depicted schematically as follows:[5]

$$U_S^{II} \xrightarrow{k_1} I_N \xrightarrow{k_2} N \tag{8}$$

$$U_S^{I} \xrightarrow{k_1'} I' \xrightarrow{k_2'} N \tag{9}$$

Here k_1 and k_2' are rate constants that represent conformational chain folding processes [i.e., with low activation enthalpy and strong dependence on denaturant and $(NH_4)_2SO_4$ concentrations etc.]. While the $I_N \rightarrow N$ and $U_S^I \rightarrow I'$ reactions may or may not represent genuine proline isomerization steps (see later discussions), their associated rate constants k_2 and k_1' do show such characteristics [i.e., high activation enthalpy, little or no dependence on denaturant or $(NH_4)_2SO_4$ concentrations, catalyzed in strong acidic medium, etc.]. Adopting the above refolding scheme, we shall now proceed to show that its resulting kinetics are consistent with the observed kinetic features of the slow-refolding of RNase A (at concentrations of denaturant below the transition zone).

(A) Absorbance-Detected Kinetics. When UV absorbance is used to follow the refolding kinetics, it is usually the change in the degree of exposure of tyrosine residues to the solvent that is being monitored. We have demonstrated in this study that the stability of I_N is very close to that of native RNase A, in agreement with other earlier experimental results (Cook et al., 1979; Schmid, 1981, 1983; Schmid & Blaschek, 1981). It is also known that I_N already possesses a folded, nativelike structure (Cook et al., 1979). Indeed, it has been shown that the optical properties of I_N, as measured by tyrosine absorbance, are very close to those of the native protein (Cook et al., 1979). Adopting the same line of reasoning, since proline isomerization is supposed to be a local effect, the optical properties of U_S^I and I′ will also be equivalent. This is supported by the fact that all the unfolded forms (U_F, U_S^{II}, and U_S^I) of RNase A, presumably differing only in the conformation of a proline residue(s), possess identical tyrosine absorption properties (Garel & Baldwin, 1973; Lin & Brandts, 1983b). Thus, for absorbance-detected refolding kinetics

$$\Delta\epsilon = \epsilon_{U_S^{II}} - \epsilon_N = \epsilon_{U_S^{II}} - \epsilon_{I_N} = \epsilon_{U_S^{I}} - \epsilon_N = \epsilon_{I'} - \epsilon_N \tag{10}$$

(*i*) *Refolding at Low Denaturant Concentration* (*e.g., 0.5 M Urea*) ($k_1 >> k_2$; $k_2' >> k_1'$). Under strongly native conditions, i.e., low denaturant concentration, it has been established that the rate of the conformational folding step is much faster than that of proline isomerization (Cook et al., 1979). This implies that, under strongly native conditions, $k_1 >> k_2$; from a similar argument, we may also infer that $k_2' >> k_1'$. Using the aforementioned condition, eq 10, and applying eq 7 to the refolding pathways of both U_S^{II} and U_S^{I} (eq 8 and 9), we can obtain the following expression for the total molar extinction coefficient, ϵ_{total}, for the slow refolding of RNase A:

$$\epsilon_{total} = \epsilon_N + f_{II}\Delta\epsilon e^{-k_1 t} + f_I\Delta\epsilon\frac{k_2'}{k_2' - k_1'}e^{-k_1' t} - f_I\Delta\epsilon\frac{k_1'}{k_2' - k_1'}e^{-k_2' t} \quad (11)$$

where f_{II} and f_I represent the relative amounts of U_S^{II} and U_S^{I}, respectively, in the denatured state.

Because $k_2' >> k_1'$, and also because f_I is relatively small compared to f_{II}, the amplitude associated with the last term in eq 11 will be negligible, and consequently, only two refolding phases will be observed. The kinetic time constants of the two phases are given by k_1^{-1} and $k_1'^{-1}$, which are characteristic of conformational folding and proline isomerization processes, respectively. Moreover, the ratio of the amplitudes of the two kinetic phases will be approximately equal to the ratio of f_{II}:f_I. All of the above kinetic features are consistent with experimental observations, viz., the existence of two slow phases, one characteristic of conformational folding and the other of proline isomerization, with relative amplitudes of f_{II}:f_I = 75:25.

It is important to point out that, if we were to assume that proline isomerization is the *second* step in the refolding pathway of U_S^{I} (viz., I′ → N and $\epsilon_{I'} = \epsilon_N$), then eq 11 would become

$$\epsilon_{total} = \epsilon_N + f_{II}\Delta\epsilon e^{-k_1 t} + f_I\Delta\epsilon e^{-k_1' t} \quad (12)$$

i.e., two phases with kinetic constants k_1 and k_1', both characteristic of conformational folding, would be obtained for the slow-refolding of RNase A. This, of course, is contradictory to the experimental observation that only *one* of the two phases showed characteristics of conformational folding [i.e., low activation enthalpy, strong dependence on denaturant and $(NH_4)_2SO_4$ concentrations, etc.]. Thus, the I′ → N step cannot involve proline isomerization.

(*ii*) *Refolding at High Denaturant Concentration* (*e.g., >2 M but <6 M Urea*) ($k_2 >> k_1$; $k_1' >> k_2'$). The rate of a conformational folding process is strongly dependent on the concentration of the denaturant present. On the contrary, proline isomerization rates are relatively independent of denaturant concentration (Cook et al., 1979; Schmid, 1981; Lin & Brandts, 1983a,b). Hence, as the refolding condition is moved toward the transition region, the relative rates of the above two types of processes will be reversed; i.e., at high denaturant concentration, $k_2 >> k_1$ and $k_1' >> k_2'$. To obtain the limiting behavior under these conditions, we rewrite eq 11 as

$$\epsilon_{total} = \epsilon_N + f_{II}\Delta\epsilon e^{-k_1 t} + f_I\Delta\epsilon\frac{k_1'}{k_1' - k_2'}e^{-k_2' t} - f_I\Delta\epsilon\frac{k_2'}{k_1' - k_2'}e^{-k_1' t} \quad (13)$$

For the same reasons as mentioned before, the contribution of the last term in eq 13 to the overall refolding kinetics will be negligible. Since the U_S^{II} → I_N and I′ → N reactions are both supposed to be conformational folding steps, their associated rate constants, k_1 and k_2', are expected to be similar in magnitude. This, along with the relatively small concentration of U_S^{I} and the long relaxation time ($\tau = k^{-1}$) involved in both conformational folding steps, will make the distinction between the two kinetic phases difficult. The slow-refolding kinetics will then be observed as a single phase (Henkens et al., 1980; Lin & Brandts, 1983b). Addition of a stabilizing salt such as $(NH_4)_2SO_4$, however, would reverse the inequalities among the rate constants; i.e., now $k_2 << k_1$ and $k_1' << k_2'$, which is the same as the conditions at low denaturant concentrations. Thus, we would observe two kinetic phases, with an amplitude ratio given by f_{II}:f_I. This is, once again, in exact agreement with previously reported experimental results on the effect of the addition of $(NH_4)_2SO_4$ (Lin & Brandts, 1983b).

(*B*) *Fluorescence-Detected Kinetics.* In contrast to tyrosine absorbance, which is a measure of its degree of exposure to the solvent medium, tyrosine fluorescence is critically dependent on specific interactions within its local environment. To account for the fact that native RNase A has a lower fluorescence than its denatured conformation, it has been postulated that tyrosine fluorescence is internally quenched through hydrogen bonding to other residues in the native conformation (Cowgill, 1966, 1967). It has also been suggested that the presence of nonnative proline conformations could prevent the formation of such hydrogen-bonded pairs (Schmid, 1981). Hence, as a result of the change in proline conformation(s), U_S^{I} and I′, and I_N and N will have different tyrosine fluorescent properties, even though the absorbance is the same within each pair. In addition, the conformational folding steps (U_I^{II} → I_N and I′ → N) can also lead to changes in fluorescence because tyrosine fluorescence can be quenched by nearby disulfide bonds, as indicated by the fact that reduced RNase A has a higher fluorescence than the native protein (Cogwill, 1966, 1967; Morgan et al., 1978; Bodner et al., 1980; Némethy & Scheraga, 1981). As a consequence of these conformational changes, it seems reasonable that U_S^{II} and I_N, and I′ and N, will also have different tyrosine fluorescent properties. In view of all the above, as far as fluorescence-detected refolding kinetics are concerned, we shall assume that the intrinsic fluorescent properties (i.e., the quantum efficiency of fluorescence, ϕ) of all three species are different in either eq 8 or eq 9, i.e.

$$\phi_{U_S^{II}} \neq \phi_{I_N} \neq \phi_N \quad (14a)$$

$$\phi_{U_S^{I}} \neq \phi_{I'} \neq \phi_N \quad (14b)$$

(*i*) *Folding at Low Denaturant Concentration* ($k_1 >> k_2$; $k_2' >> k_1'$). Our results (Figures 2 and 3) indicate that the time constants of the two kinetic phases are identical for absorbance- and fluorescence-detected kinetics of the slow refolding of RNase A. As a matter of fact, the only difference between the two sets of refolding kinetics lies in the relative *amplitudes* of the two kinetic phases. In absorbance-detected kinetics, the major phase corresponds to the one with the shorter relaxation time and accounts for 75% of the total amplitude of the slow-folding phases. However, when fluorescence is used to monitor the refolding kinetics, the amplitude of this faster phase decreases to a value of 35% of the total amplitude, hence becoming, instead, the minor slow-refolding phase. Similar results have also been reported by Lin & Brandts (1983b).

Applying eq 7 once again to both slow-refolding pathways of RNase A, but allowing the fluorescent properties of all species on a given pathway to differ, we obtain

$$\phi_{total} = \phi_N + f_{II}\left[(\phi_{U_S^{II}} - \phi_N) - (\phi_{I_N} - \phi_N)\frac{k_1}{k_1 - k_2}\right]e^{-k_1 t} + f_{II}(\phi_{I_N} - \phi_N)\frac{k_1}{k_1 - k_2}e^{-k_2 t} + f_I\left[(\phi_{U_S^I} - \phi_N) + (\phi_{I'} - \phi_N)\frac{k_1'}{k_2' - k_1'}\right]e^{-k_1' t} - f_I(\phi_{I'} - \phi_N)\frac{k_1'}{k_2' - k_1'}e^{-k_2' t} \quad (15)$$

Use of the limiting condition of $k_1 >> k_2$ and $k_2' >> k_1'$ in eq 15 leads to the following expression for the quantum efficiency of fluorescence, ϕ_{total}, for the slow refolding of RNase A:

$$\phi_{total} = \phi_N + f_{II}[(\phi_{U_S^{II}} - \phi_N) - (\phi_{I_N} - \phi_N)]e^{-k_1 t} + f_{II}(\phi_{I_N} - \phi_N)e^{-k_2 t} + f_I(\phi_{U_S^I} - \phi_N)e^{-k_1' t} \quad (16)$$

From eq 16, we can see that, under these conditions, ϕ_{total} is independent of k_2' but not k_1 (both k_1 and k_2' correspond to fast steps in the two pathways). The underlying physical reason as to why this should be the case can be seen qualitatively as follows. Since it is assumed that only the denatured species U exists at $t = 0$, the amplitude of the kinetic phase corresponding to the *first* step in a three-state mechanism (eq 8 and 9) will vanish only under the limiting condition that the monitored physical property (e.g., ϵ or ϕ) is identical for the intermediate (I_N or I′) and the denatured species (U_S^{II} or U_S^I, respectively). The amplitude of the kinetic phase corresponding to the *second* step, however, is dependent on the ratio of the rate constants of the individual steps. In the case where the two rate constants are vastly different, the two kinetic steps will become decoupled. If the first step is *slow* compared to the second, as in the case of the $U_S^I \rightarrow I'$ reaction, I′ will be accumulated only to a small extent during the course of the $U_S^I \rightarrow N$ transition, thus making the *amplitude* of the second step (i.e., $I' \rightarrow N$) relatively small. Hence, ϕ_{total} is independent of k_2'. On the contrary, if the first step is the *faster* one (e.g., $U_S^{II} \rightarrow I_N$), there will be a significant accumulation of the intermediate (I_N) after a short lag time, and consequently, the amplitude associated with the second step will also be significant.

Since k_2 and k_1' are supposed to be of similar magnitude, the two phases corresponding to these two rate constants might become indistinguishable and hence merge into a single phase. As a consequence, as seen from eq 16, two instead of three kinetic phases with time constants k_1^{-1} (characteristic of conformational folding) and k_2^{-1} (or $k_1'^{-1}$, characteristic of proline isomerization) will be observed. Furthermore, the ratio of the amplitudes of the two kinetic phases (conformational folding step relative to that of proline isomerization), which is observed experimentally to be 35:65, is given by

$$R = \frac{f_{II}[(\phi_{U_S^{II}} - \phi_N) - (\phi_{I_N} - \phi_N)]}{f_{II}(\phi_{I_N} - \phi_N) + f_I(\phi_{U_S^I} - \phi_N)} = \frac{f_{II}(1 - r_2)}{f_{II}r_2 + f_I r_1} = \frac{35}{65} \quad (17)$$

Here r_1 denotes the *relative* intrinsic fluorescence properties of the two slow-folding species (U_S^{II} and U_S^I), and r_2 represents the *fractional* fluorescence change of the proline isomerization step ($I_N \rightarrow N$) in the $U_S^{II} \rightarrow N$ transition, i.e.

$$r_1 = \frac{\phi_{U_S^I} - \phi_N}{\phi_{U_S^{II}} - \phi_N} \quad (18)$$

$$r_2 \frac{\phi_{I_N} - \phi_N}{\phi_{U_S^{II}} - \phi_N} \quad (19)$$

In order to obtain rough estimates of r_1 and r_2, we shall consider the following. It is known that the fluorescence-detected *unfolding* kinetics are biphasic in character (Rehage & Schmid, 1982; Lin & Brandts, 1983b). Although the faster phase has been assigned to the $N \rightarrow U_F$ reaction, there remains the uncertainty as to the physical origin of the second, slower kinetic phase (Rehage & Schmid, 1982; Lin & Brandts, 1983b). Rehage & Schmid (1982) attributed the slow phase to the $U_F \rightleftarrows U_S$ reaction (without distinguishing U_S^I from U_S^{II}), thereby implying that the intrinsic fluorescent properties of U_S^{II} and U_S^I are identical, i.e., $r_1 = 1$. Alternatively, Lin & Brandts (1983b) assigned the slower phase to the $U_F \rightleftarrows U_S^I$ reaction, which led them to a value of 2.5 for r_1. Using the value of $r_1 = 1$ determined by Rehage and Schmid, and $f_{II} = 0.75$ in eq 17, we obtained a value of 0.5 for r_2. Even if one were to assign the slow-unfolding phase to the $U_F \rightleftarrows U_S^{II}$ reaction exclusively, a value of 1.3 would be obtained for r_1, which in turn yields the same value of 0.5 for r_2. The use of Lin and Brandts' value of $r_1 = 2.5$ leads to a value of $r_2 = 0.36$. Hence, quantitative agreement with the experimental observation of the reversal of the relative amplitudes of the two phases in fluorescence-detected (i.e., a ratio of 35:65) as compared to absorbance-detected refolding kinetics can be achieved with *either* value of r_2 in this range. In summary, the reversal of the relative amplitude arises from the fact that I_N and N (correspondingly U_S^I and I′) have different fluorescence but similar absorbance properties.

(*ii*) *Refolding at High Denaturant Concentration* ($k_2 >> k_1$; $k_1' >> k_2'$). As in absorbance-monitored refolding, the fluorescence-detected kinetics of RNase A also become monophasic when the refolding is carried out at higher denaturant concentrations (Lin & Brandts, 1983b). Applying the limiting conditions of $k_2 >> k_1$ and $k_1' >> k_2'$ to eq 15, we obtain the following expression for ϕ_{total}:

$$\phi_{total} = \phi_N + f_{II}(\phi_{U_S^{II}} - \phi_N)e^{-k_1 t} + f_I[(\phi_{U_S^I} - \phi_N) - (\phi_{I'} - \phi_N)]e^{-k_1' t} + f_I(\phi_{I'} - \phi_N)e^{-k_2' t} \quad (20)$$

As discussed previously, for a three-state kinetic mechanism consisting of a *faster* second step (i.e., $I_N \rightarrow N$), the amplitude associated with the second step will be relatively small. From eq 20, it can be seen that ϕ_{total} is indeed independent of k_2 under these conditions.

Using the same reasoning as before, the rate constants (k_1 and k_2') corresponding to conformational folding processes will likely be of similar magnitude and will merge into a single phase. Hence, under these conditions, the ratio of the amplitudes of the two kinetic phases (conformational folding step relative to that of proline isomerization) is given by

$$R = \frac{f_{II}(\phi_{U_S^{II}} - \phi_N) + f_I(\phi_{I'} - \phi_N)}{f_I[(\phi_{U_S^I} - \phi_N) - (\phi_{I'} - \phi_N)]} = \frac{f_{II}/r_1 + f_I r_3}{f_I(1 - r_3)} \quad (21)$$

where r_3 is the *fractional* fluorescence change of the conformational folding step ($I' \rightarrow N$) in the $U_S^I \rightarrow N$ pathway, i.e.

$$r_3 = \frac{\phi_{I'} - \phi_N}{\phi_{U_S^I} - \phi_N} \quad (22)$$

Using the assumption of Rehage & Schmid (1982) that the fluorescent properties of U_S^{II} and U_S^I are identical, i.e., $r_1 = 1$, we have already shown that the fluorescence change corresponding to the conformational folding step amounts to 50% of the total change of the $U_S^{II} \rightarrow N$ transition, i.e., $r_2 = 0.5$. We assume that conformational folding also accounts for 50% of the fluorescence change for the $U_S^I \rightarrow N$ transition, i.e., $r_3 = 0.5$. Substitution of $r_1 = 1$ and $r_3 = 0.5$ in eq 21 leads to a value of 7.0 for R, the ratio of the amplitudes of the two

kinetic phases. Alternatively, using the Lin & Brandts' (1983b) value of $r_1 = 2.5$, we find that the conformational folding step ($U_S^{II} \rightarrow I_N$) accounts for 64% of the total fluorescence change of the $U_S^{II} \rightarrow N$ pathway. Assuming the same fractional change for the corresponding conformational folding step ($I' \rightarrow N$) on the minor slow-folding pathway, viz., $r_3 = 0.64$, and using $r_1 = 2.5$ in eq 21, we obtain a value of 5.1 for R. Hence, using either large value for R, we see that this dominance in the amplitude of the conformational folding kinetic phase may make the experimental distinction of the second phase difficult, if not indeed impossible. On the other hand, under *low* denaturant conditions, the values of R are 75:25 and 35:65 for absorbance- and fluorescence-detected refolding, respectively, thereby enabling two phases to be observed.

Possibility of U_S^{II} or U_S^{I} Refolding through U_F to the Native Conformation. The question of whether the two slow-refolding species U_S^{II} and U_S^{I} can refold to the native conformation through U_F arises naturally. In the two-state model of Lin & Brandts (1983b,c), U_F is an obligatory intermediate on the refolding pathways of both U_S^{II} and U_S^{I}. However, in the literature adopting the sequential folding model, the above question has seldom been addressed.

From unfolding experiments, it has been determined that the $U_S \rightleftarrows U_F$ reaction (due to proline isomerization) is independent of denaturant concentration and has a relaxation time of 250 s at 10 °C (Cook et al., 1979; Lin & Brandts, 1983b). Since $[U_F]/[U_S^{II}]$ has an equilibrium ratio of 1:3 in the denatured state, the $U_S^{II} \rightarrow U_F$ step, as it occurs under such unfolding conditions, will have a kinetic time constant of about 1000 s at 10 °C. It is important to point out that the above estimation of rate constants is based on kinetic data obtained under *unfolding* conditions. However, since the $U_S^{II} \rightleftarrows U_F$ reaction is known to be independent of denaturant concentration (Cook et al., 1979), it can be inferred that the above estimate of 1000 s for the kinetic time constant of the $U_S^{II} \rightarrow U_F$ reaction at 10 °C is also applicable under folding conditions. As a comparison, it is known that, under folding conditions, the $U_S^{II} \rightarrow I_N$ step has a time constant of $\approx$20 s at 10 °C and is therefore faster than the $U_S^{II} \rightarrow U_F$ reaction by about a factor of 50. Furthermore, it has been established that I_N possesses high stability toward denaturation and presumably can exist under a wide range of refolding conditions. It seems, therefore, that U_S^{II} does not refold through U_F under refolding conditions below the transition region.

The situation concerning U_S^{I} is much less certain, however. Because of its lower equilibrium ratio to U_F (1:1), the $U_S^{I} \rightarrow U_F$ reaction, as it occurs in the denatured state, will have a relaxation time of about 500 s at 10 °C. Since the $U_S^{I} \rightleftarrows U_F$ reaction involves proline isomerization (Lin & Brandts, 1983a,b) and is, therefore, relatively independent of denaturant concentration, its kinetic time constant should be of similar magnitude under both folding and unfolding conditions. As a comparison, the kinetic time constant for the minor slow phase has a value of 90 s at 10 °C under strongly native conditions and is, therefore, about 5 times faster than the alternative $U_S^{I} \rightarrow U_F$ reaction. If the $U_S^{I} \rightarrow U_F$ reaction is indeed *completely* independent of denaturant concentration, then it is likely that, under strongly native conditions, U_F does not lie on the refolding pathway of U_S^{I}.

It must be pointed out, however, that even if it turns out that U_S^{I} does refold through U_F, i.e., if $U_F \equiv I'$ in eq 9, all the foregoing discussions in the previous sections remain unaffected. Moreover, it is also important to bear in mind that the refolding scheme proposed so far is applicable only for refolding conditions *below* the transition zone. Inside the transition zone, it is possible that the refolding mechanism may be different.

Molecular Bases for the $I_N \rightarrow N$ and $U_S^{I} \rightarrow I'$ Reactions. (A) Minor Slow-Folding Phase. It is known that the minor slow-folding phase of RNase A shows characteristics of proline isomerization (Cook et al., 1979; Schmid & Baldwin, 1978; Lin & Brandts, 1983b). Using isomer-specific proteolytic methods, Lin & Brandts (1983a) directly measured the kinetics of the isomerization of Pro^{93} during refolding. It has been shown that the isomerization kinetics of Pro^{93} match those of the minor slow-refolding phase of RNase A, both in relaxation time and in amplitude (Lin & Brandts, 1983b). It seems, therefore, that the $U_S^{I} \rightarrow I'$ reaction is in fact due to the isomerization of Pro^{93}.

(B) Major Slow-Folding Phase. It has been postulated that the $I_N \rightarrow N$ reaction is also due to the isomerization of a prolyl residue (Cook et al., 1979; Schmid, 1981, 1983). However, recent isomer-specific proteolytic experiments performed by Lin & Brandts (1984) indicate that Pro^{117} retains its native trans conformation in the denatured state and only 5% of Pro^{114} is changed to the nonnative trans conformation in denatured RNase A. This immediately raises the question as to the correct identity of I_N on a molecular level. We suggest that there are two possible candidates for I_N.

(i) A natural possibility would be that I_N still lacks the correct conformation in the Lys^{41}–Pro^{42} peptide bond. However, it has been firmly established that Lys^{41} is involved in the active site of RNase A (Hirs et al., 1961; Borkakoti et al., 1983; Palmer et al., 1984). Since I_N has been shown to be enzymatically active (Cook et al., 1979), it is difficult to imagine that the isomerization of the Lys^{41}–Pro^{42} peptide bond would not induce any significant conformational changes in Lys^{41}. Moreover, since Pro^{42} is trans in native RNase A, it seems rather unlikely that 60% of the Pro^{42} residues would be changed to the cis conformation upon denaturation. Hopefully, future isomer-specific proteolysis experiments will settle the feasibility of this possibility.

(ii) Another possibility is that I_N lacks the correct chirality of one or more disulfide bonds. It has been reported that, depending on the reoxidation conditions, the CD spectra of reoxidized RNase A may be different from that of the native protein, suggesting that the protein molecule may be trapped in a nonnative conformation during the reoxidation reaction (Beychok, 1966; Pflumm & Beychok, 1969). It is interesting to note that States et al. (1980) and Kosen et al. (1981) concluded from NMR and CD studies that, during regeneration from the reduced form, BPTI is also trapped in a nonnative conformation. We suggest here that one or more disulfide bonds of the wrong chirality may be formed (in I_N) during the refolding of RNase A and that the stereochemical changes of such disulfide bonds to their native conformations may represent the last rate-limiting step of the major slow-refolding phase. Concerning the energetics of such stereochemical processes, it has been determined that the activation enthalpy of the inversion of the chirality of the S–S bond in H_2S_2 amounts to 7–9 kcal/mol (Redington, 1962; Winnewisser et al., 1968; Veillard & Demuynck, 1970). It must be emphasized, however, that this value pertains to that of H_2S_2, where there is, presumably, little or no steric hindrance accompanying the inversion process. As a matter of fact, it has been demonstrated that, as the hydrogen atoms are replaced by more bulky groups, the values of the associated activation enthalpy are increased accordingly (Fraser et al., 1971). The highest value of the activation enthalpy obtained so far for

acyclic disulfides is 15.7 kcal/mol for bis(4-methyl-2,6-di-*tert*-butylphenyl) disulfide (Kessler & Rundel, 1968). For cyclic disulfide compounds, the values of the activation enthalpy are much higher, presumably because of the extra steric strain introduced in the ring. For example, the activation enthalpy of inversion for tetramethyldithiane has been determined to be 16.1 kcal/mol (Claeson et al., 1961). It is conceivable that, in a globular protein molecule, there will be some steric hindrance associated with the inversion process, although its magnitude cannot be predicted easily. As a comparison, the activation enthalpy for the $U_F \rightleftarrows U_S^{II}$ reaction has been determined to be about 20 kcal/mol (Schmid & Baldwin, 1978; Lin & Brandts, 1983b; Schmid et al., 1984). However, whether such disulfide inversion processes will show characteristics consistent with those of the major slow-refolding phase of RNase A remains to be tested experimentally.

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