Fast- and Slow-Refolding Forms of Unfolded Ribonuclease A Differ in Tyrosine Fluorescence†

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ABSTRACT: Unfolded ribonuclease A (RNase A) consists of a 20:80 mixture of fast-folding (U_F) and slow-folding (U_S) molecules. Both species were assumed to be maximally unfolded by a number of criteria, the only difference being in the cis/trans configuration of one or more X–Pro peptide bonds. Here we show that U_F and U_S differ in their tyrosine fluorescence properties. The fluorescence of U_S is about 20% higher than the fluorescence of U_F. This finding is based on the analysis of fluorescence-detected unfolding kinetics of RNase A. The slowest kinetic phase is compared with the known properties of the $U_F \rightleftharpoons U_S$ equilibration reaction, which was measured by the assay for slow-folding molecules. The identity of both kinetics is shown, using the distinctive character of sequential reactions as a kinetic test. Because of the difference in fluorescence between U_F and U_S, the relative amplitude of the fast-refolding reaction ($U_F \rightarrow N$) decreases from 20% to 15% when monitored by fluorescence. U_F and U_S are both unfolded molecules which lack specific long-range interactions; therefore, the difference in fluorescence must depend on local changes in the environment of one or more tyrosine residues, correlated with proline peptide bond isomerization during the $U_F \rightleftharpoons U_S$ reaction. We conclude that the fluorescence difference is probably caused by tyrosine-92, which is next to proline-93 in sequence. When the Tyr-92–Pro-93 peptide bond is in the native cis configuration (as in U_F), the fluorescence of Tyr-92 is quenched by local interactions with the 40–95 disulfide bond and/or by hydrogen bonding to the carbonyl group of Lys-37. Upon isomerization of the Tyr-92–Pro-93 bond to the nonnative trans configuration, the spatial orientation of Tyr-92 is changed so that these interactions are abolished. Fluorescence of tyrosine-92 can be used as a local probe for the isomerization of the Tyr-92–Pro-93 bond in the unfolded protein as well as in partially folded intermediates.

In 1975, Brandts et al. proposed that the presence of more than one unfolded form of a protein [such as ribonuclease A (RNase A)[1]] results from the slow cis/trans isomerization about peptide bonds preceding proline (X–Pro bonds). Only those unfolded molecules having all X–Pro bonds in the native configuration (U_F) are able to refold fast; U_S molecules have at least one nonnative X–Pro bond. Brandts et al. (1975) proposed further that U_S cannot fold until the slow isomerization of the nonnative proline peptide bond(s) to the native configuration has occurred and that the complex refolding kinetics of small monomeric proteins result entirely from the presence of multiple unfolded forms.

This model has been tested for RNase A, and the first part of the model appears to be correct. Earlier it had been found that unfolded RNase A consists of 20% fast-folding species U_F and 80% slow-folding species U_S (Garel & Baldwin, 1973; Garel et al., 1976; Hagerman & Baldwin, 1976). After native RNase A is rapidly unfolded ($N \rightarrow U_F$), U_S is formed slowly from U_F until a U_F:U_S 20:80 equilibrium is reached. This equilibration reaction shows all the properties of proline peptide bond isomerization as measured in model compounds (Schmid & Baldwin, 1978). Hence, U_F has all its proline peptide bonds that are essential for folding in the native configuration, whereas U_S has at least one essential proline in the nonnative state. U_F is a single species with respect to the state of its essential proline residues; U_S may be composed of subspecies containing different sets of proline peptide bonds.

Both U_F and U_S are considered to be completely unfolded species, based on the following observations. (i) The equilibrium between U_F and U_S cannot be shifted toward U_S by structure–destabilizing agents, such as increasing concentrations of Gdn·HCl (Garel et al., 1976; Schmid & Baldwin, 1978). (ii) The kinetics of interconversion between U_F and U_S are independent of Gdn·HCl concentration (Schmid & Baldwin, 1979a). (iii) U_F and U_S do not differ detectably in their tyrosine absorption properties, which depend on the exposure of tyrosine residues to the aqueous solvent (Garel & Baldwin, 1973). The only physical difference between U_F and U_S known to date is a small difference of 0.05 in the pK values of NO_2-tyrosine residues in partially nitrated RNase A (Garel & Baldwin, 1975; Garel, 1980). Recently it was shown that under strongly native conditions tyrosine fluorescence is sensitive to proline isomerization during refolding of RNase A (Schmid, 1980, 1981).

In this paper, we ask whether this is also true for unfolding of the protein; i.e., we ask whether the fast-folding species U_F and the slow-folding species U_S differ in their tyrosine fluorescence. To answer this question, we investigate the fluorescence changes which occur upon unfolding of native RNase A. The observed kinetics are compared to unfolding monitored by tyrosine absorbance and to "double-jump" experiments, which detect the $U_F \rightleftharpoons U_S$ reaction. The results demonstrate that the $U_F \rightleftharpoons U_S$ reaction is accompanied by an increase in tyrosine fluorescence and that U_S shows a substantially higher fluorescence emission than U_F. If U_F and U_S are not equivalent in their fluorescence, then the relative amplitudes of the fast- and slow-refolding reactions, $U_F \rightarrow N$ and $U_S \rightarrow N$, are expected to differ from the 0.2:0.8 ratio, which was derived from stopped-flow refolding kinetics measured by tyrosine absorbance and by 2′-CMP binding. To test this prediction, we determined the relative amplitudes of the

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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 a chemical reaction (reciprocal of the apparent rate constant, k^{-1}); f, relative amplitude of a kinetic phase (fraction of the total amplitude); I_X and I_N, folding intermediates.

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fast ($U_F \rightarrow N$) and the slow ($U_S \rightarrow N$) refolding reactions of RNase A by stopped-flow experiments by using tyrosine fluorescence to monitor the folding reaction. The results are compared to absorbance-detected refolding kinetics (Garel et al., 1976). The observed difference in fluorescence between U_F and U_S is discussed in terms of local structure changes involving Pro-93, Tyr-92, and the 40–95 disulfide bond.

Materials and Methods

Materials. Ribonuclease A (type XII A, lots 17C–8098 and 49C–8047) was purchased from Sigma, St. Louis, MO; Gdn·HCl (ultrapure) was from Schwarz/Mann, Orangeburg, NY; sodium cacodylate was from Serva (Heidelberg). All other substances (p.a. grade) were purchased from Merck (Darmstadt).

Methods. A Cary 118 C spectrophotometer and a Hitachi Perkin-Elmer MPF 44A fluorescence spectrophotometer, both with jacketed cell holders, were used. RNase A concentrations were determined spectrophotometrically by using a molar absorption at 278 nm of 9800 M^{-1} cm^{-1} (Sela & Anfinsen, 1957). Temperatures were measured by a thermistor dipped into the cell after each kinetic experiment.

Slow-unfolding kinetics were initiated by a 20-fold dilution of native RNase A (in H_2O) into the unfolding buffer in the cuvette. Dead times of mixing were in the range of 5–15 s. Tyrosine absorption changes were measured at 287 nm and tyrosine fluorescence changes at 305 nm (with 10-nm slit width); excitation was at 268 nm. The assay for slow-folding molecules was carried out as described by Schmid & Baldwin (1978).

Stopped-flow fluorescence measurements were performed with a modified Gibson-Durrum stopped-flow spectrophotometer. Tyrosine fluorescence was monitored at $\lambda \geq 300$ nm with excitation at 277 nm. All experiments were carried out at 35 °C. The absence of thermal and Gdn·HCl dilution artifacts was verified by a 1:1 dilution of a solution of tyrosine in 1 M Gdn·HCl, pH 2, with 0.2 M sodium cacodylate. All solutions were degassed and passed through filters (0.45 μm) before use. The fast- and slow-refolding reactions were assumed to be single exponentials (Garel et al., 1976). They were resolved as the sum of two exponentials by using standard methods ("peeling back"). The results given in Table IV represent averages of fifteen single-kinetic experiments.

Results

Fluorescence-Detected Unfolding of RNase A Does Not Always Coincide with Absorbance-Detected Unfolding. The tyrosine absorbance of RNase A at 287 nm decreases upon unfolding of the protein. This property has been used extensively to monitor folding and unfolding of RNase A (Tsong et al., 1972; Hagerman & Baldwin, 1976; Nall et al., 1978). Tyrosine fluorescence of RNase A, which is low in the native state, increases approximately 1.5-fold upon unfolding (Gally & Edelman, 1962; Cowgill, 1967; Schmid, 1981).

Here we compare unfolding monitored by the increase in tyrosine fluorescence to absorbance-detected unfolding. The measurements are restricted to strongly unfolding conditions, where only unfolded species are present at equilibrium. Table I presents a comparison of fluorescence-detected and absorbance-detected unfolding kinetics of RNase A at 10 °C and under varying unfolding conditions.

At neutral pH, unfolding of RNase A is represented by a $N \rightarrow U_F \rightleftharpoons U_S$ mechanism. The rate of the $N \rightarrow U_F$ step increases with the concentration of Gdn·HCl, whereas the rate of the $U_F \rightleftharpoons U_S$ equilibration remains constant, as it is governed by proline isomerization. The two steps are about equal in rate at 5 M Gdn·HCl, pH 6, at 10 °C. Under these conditions, both probes (absorbance and fluorescence) yield identical kinetics. Semilogarithmic plots of the data give straight lines, which yield a time constant of about 200 s. The kinetic amplitudes account for the entire change in absorbance as well as in fluorescence, as obtained from equilibrium unfolding transitions (that is, $\Delta\epsilon_{287} = 2700$ M^{-1} cm^{-1} and $\Delta F_{305} = 0.6F^{\infty}_{305}$).

Table I: Unfolding of RNase A. Comparison of Fluorescence-Detected to Absorbance-Detected Kinetics[a]

unfolding conditions	method[b]	τ_1[c] (s)	τ_2[c] (s)	f_1[d]	f_2[d]
pH 6, 5 M Gdn·HCl	abs	180		1.0	
pH 6, 5 M Gdn·HCl	fluor	220		1.0	
pH 6, 7 M Gdn·HCl	abs		50		1.0
pH 6, 7 M Gdn·HCl	fluor	160	47	0.33	0.67
pH 2, 5 M Gdn·HCl	abs	180	*e*	0.03	*e*
pH 2, 5 M Gdn·HCl	fluor	160	*e*	0.30	*e*

[a] Initial conditions were 600 μM RNase A in H_2O; final conditions were 30 μM RNase A in 0.2 M glycine at pH 2 or 0.2 M sodium cacodylate at pH 6; 10 °C. [b] Abs, unfolding monitored by the absorbance at 287 nm; fluor, unfolding monitored by the fluorescence at 305 nm. [c] Time constants of the kinetic phases that can be resolved by manual mixing (dead time, 5–10 s). [d] Relative amplitudes expressed as the fraction of the total change in absorbance or fluorescence as derived from equilibrium unfolding transitions. [e] At pH 2, 5 M Gdn·HCl, the initial unfolding reactions were too fast and could not be resolved by manual mixing.

At higher concentrations of Gdn·HCl, such as 7 M (Table I), the rate of the $N \rightarrow U_F$ reaction increases, and both spectral probes produce different unfolding kinetics. Absorbance-detected unfolding is still represented by a single exponential with $\tau = 50$ s and an amplitude of $\Delta\epsilon_{287} = 2640$ M^{-1} cm^{-1}, which is about equal to the entire change in absorbance upon unfolding. On the other hand, fluorescence-detected unfolding is complex. The observed kinetics can be resolved into two phases: a faster one with 67% relative amplitude and $\tau = 47$ s (in accordance with the absorbance kinetics) and a slower phase with 33% relative amplitude and $\tau = 160$ s. The sum of both amplitudes is about equal to the change expected from equilibrium unfolding curves; i.e., there are no substantial fast reactions which have not been resolved by the manual mixing technique.

At low pH and high concentrations of Gdn·HCl, the major unfolding reaction of RNase A is known to become very rapid (Hagerman & Baldwin, 1976; K. H. Cook, R. L. Baldwin, and F. X. Schmid, unpublished experiments), whereas the $U_F \rightleftharpoons U_S$ reaction is independent of pH (Schmid & Baldwin, 1978). Therefore, unfolding of RNase A has also been measured at pH 2 and 5 M Gdn·HCl (Table I). Under these conditions, the major unfolding reactions which yield U_F have gone to completion within the time required for mixing; nevertheless, slow changes in absorbance as well as in fluorescence are detectable. In absorbance, only a very small phase with $\tau = 180$ s and $\Delta\epsilon \approx 80$ M^{-1} cm^{-1} (corresponding to about 3% of the entire change in absorbance) is visible. Fluorescence-detected kinetics yield approximately the same time constant of $\tau = 195$ s; however, the amplitude of this phase is much larger in fluorescence. It accounts for about 30% of the entire change in fluorescence upon unfolding (derived from equilibrium unfolding transitions). Comparison of data at pH 6 and 7 M Gdn·HCl and at pH 2 and 5 M Gdn·HCl (Table II) indicates that the time constant and the relative amplitude of the slowest phase of fluorescence-detected unfolding are in-

Table II: Effect of Final pH on the Slowest Step of Fluorescence-Detected Unfolding of RNase A[a]

pH	τ[b] (s)	F_{rel}[c] (%)
2.0	160	18
3.0	170	19
4.0	180	19
4.0[d]	190	17
4.5	190	20
5.0[d]	180	15
6.0[d]	170	15
7.0[d]	180	16

[a] Initial conditions were 600 μM RNase A in H_2O, and final conditions were 30 μM RNase A in 5 M Gdn·HCl and 0.2 M glycine (pH 2–3); 0.2 M sodium acetate (pH 4–5); 0.2 M sodium cacodylate (pH 6–7); 10 °C; pH as indicated. [b] Time constant of the slowest fluorescence-detected unfolding reaction. [c] Amplitude of the slowest phase, expressed as percent of the final value of fluorescence. [d] Results derived from pH-jump experiments: RNase A is preincubated at pH 2, 5 M Gdn·HCl, and 10 °C for 20 s to bring all earlier unfolding reactions to completion; then the protein is adjusted to the indicated pH, and the slowest step is monitored.

dependent of pH between pH 2 and pH 6.

Slowest Phase in Fluorescence-Detected Unfolding Shows Properties of the $U_F \rightleftharpoons U_S$ Reaction. The results shown in Table I suggest that the slowest phase in fluorescence-detected unfolding may be caused by the $U_F \rightleftharpoons U_S$ isomerization reaction of unfolded RNase A. The properties of the $U_F \rightleftharpoons U_S$ equilibration have been characterized extensively in order to show that this reaction is correlated to isomerization about X–Pro peptide bonds in the unfolded polypeptide chain (Brandts et al., 1975; Nall et al., 1978; Schmid & Baldwin, 1978, 1979a). In the following, we compare the properties of the slowest unfolding reaction (detected by fluorescence) to the $U_F \rightleftharpoons U_S$ reaction (as measured by use of the assay for slow-folding molecules).

(i) At 10 °C, both kinetics are about equal in rate. The time constant of the $U_F \rightleftharpoons U_S$ reaction is 200–220 s (Schmid & Baldwin, 1979a,b); the slowest fluorescence step yields τ = 160–200 s (cf. Table I).

(ii) In contrast to refolding and unfolding reactions of RNase A that are governed by conformational events, the $U_F \rightleftharpoons U_S$ reaction is independent of pH in both its rate and its amplitude (i.e., the equilibrium constant $K_{eq} = [U_F]/[U_S]$ is independent of pH). Therefore, the corresponding effect of the final pH on the slowest phase of fluorescence-detected unfolding was determined, the results being presented in Table II.

The separation of the slowest phase from the preceding kinetic steps is difficult above pH 4.5, because the initial unfolding reaction of RNase A ($N \rightarrow U_F$) becomes slow at neutral pH values and is shifted into the time range of the $U_F \rightleftharpoons U_S$ reaction. In order to avoid kinetic coupling and to facilitate quantitative determination of the slowest step, results at pH 4–7 (Table II) are derived from pH-jump experiments: The enzyme is preincubated at pH 2 in 5 M Gdn·HCl, 10 °C, for 20 s. Under these conditions, all unfolding reactions on the pathway from N to U_F are fast and have gone to completion within a few seconds. After 20 s, the protein is adjusted to the final pH, and the slowest step is monitored without interference from the preceding unfolding reactions. At pH 4, kinetics were determined directly as well by use of the pH-jump technique. Both methods yield identical kinetics. The results given in Table II prove that, in contrast to the early unfolding reactions, the slowest step of fluorescence-detected unfolding shows rates and amplitudes independent of pH like the $U_F \rightleftharpoons U_S$ reaction.

Table III: Effect of Increasing Concentrations of Gdn·HCl on the Slowest Step of Fluorescence-Detected Unfolding of RNase A[a]

[Gdn·HCl] (M)	τ[b] (s)	ΔF_{rel}[c] (%)
3.0	195	16
3.2	160	16
3.5	150	17
4.0	160	16
5.0	160	18
6.0	155	16

[a] Initial conditions were 600 μM RNase A in H_2O; final conditions were 30 μM RNase A in 0.2 M glycine, pH 2.0; 10 °C. Unfolding was monitored at 305 nm (excitation at 268 nm). [b] Time constant of the slowest phase of fluorescence-detected unfolding. [c] Amplitude of the slowest phase, expressed as percent of the final value of fluorescence.

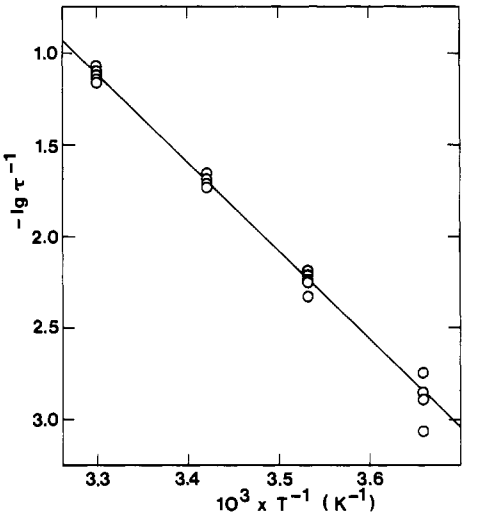

FIGURE 1: Temperature dependence of the apparent rate constant (τ^{-1}) of the slowest step of fluorescence-detected unfolding of RNase A. Unfolding was carried out in 0.1 M HCl, pH 1, and variable concentrations of Gdn·HCl ranging from 3 to 6 M at 0–19 °C and from 2 to 3 M at 30 °C. Fluorescence changes were monitored at 305 nm (excitation at 268 nm). The slope of the line yields an activation enthalpy of 22.0 kcal/mol.

(iii) Protein unfolding reactions typically become faster with increasing concentrations of Gdn·HCl in the unfolding conditions (Tanford et al., 1973; Nall et al., 1978; Lin & Brandts, 1978), whereas the $U_F \rightleftharpoons U_S$ reaction of RNase A is independent of Gdn·HCl concentration (Schmid & Baldwin, 1979a,b). The influence of increasing concentrations of Gdn·HCl on the slowest step of fluorescence-detected unfolding has been determined at 10 °C and pH 2. The results (Table III) show that the rate as well as the relative amplitude of this reaction is essentially independent of the concentration of Gdn·HCl.

(iv) A peculiar feature of the $U_F \rightleftharpoons U_S$ reaction is its high activation enthalpy of 21 kcal/mol (Schmid & Baldwin, 1979b), which is characteristic of the cis/trans isomerization of proline peptide bonds. The dependence on temperature of the slowest step of fluorescence-detected unfolding of RNase A was determined between 0 and 30 °C at pH 1 and varying concentrations of Gdn·HCl. The resulting Arrhenius plot of the observed rate constants (Figure 1) is linear and yields an activation enthalpy of 22 kcal/mol. The relative amplitudes are practically independent of temperature and show a constant value of about 25–30% of the entire change in fluorescence upon unfolding.

Slowest Fluorescence-Detected Phase and the $U_F \rightleftharpoons U_S$ Reaction Monitored by the Double-Jump Technique Are Identical. Evidence gained so far is quite strong that the

slowest step of fluorescence-detected unfolding and the reaction monitored by the assay for slow-folding molecules, U_S (double jumps), are identical; i.e., both follow the $U_F \rightleftharpoons U_S$ isomerization reaction in unfolded RNase A. However, an alternative model is conceivable, in which both probes do not monitor the same reaction but different steps in a sequential unfolding scheme. In this scheme, the fluorescence-detected reaction precedes the final $U_F \rightleftharpoons U_S$ equilibration, which is measured by the assay for slow-folding molecules, U_S. These two alternative mechanisms are represented by eq 1 and 2. The

$$N \xrightarrow{\text{fast}} U_F \underset{k_{SF}}{\overset{k_{FS}}{\rightleftharpoons}} U_S \qquad (1)$$

assay for slow-folding molecules and slow fluorescence changes

$$N \xrightarrow{\text{fast}} I_x \xrightarrow{k_{IF}} U_F \underset{k_{SF}}{\overset{k_{FS}}{\rightleftharpoons}} U_S \qquad (2)$$

slow fluorescence changes; assay for slow-folding molecules

kinetics of formation of U_S provide a sensitive test to decide which of the two alternatives is the correct one. The scheme in eq 1 results in simple first-order kinetics of the formation of U_S:

$$[U_S] = [U_S]_{eq}e^{-k't} \qquad (3)$$

Sequential reactions involving two steps that are comparable in rate (as in the scheme in eq 2) show a characteristic lag phase in the formation of U_S:

$$[U_S] = [U_S]_{eq}\left[1 + \frac{1}{k_{IF} - k'}(k'e^{-k_{IF}t} - k_{IF}e^{-k't})\right] \qquad (4)$$

$[U_S]_{eq}$ is the final concentration of U_S present at equilibrium; k' is the apparent rate of the $U_F \rightleftharpoons U_S$ reaction, which is equal to the sum of the individual rate constants, $k' = k_{FS} + k_{SF}$. The formation of U_S after unfolding is determined by refolding assays, the concentration of U_S being proportional to the amplitude of the slow-refolding assay, $\Delta\epsilon_{287}$. Substituting $\Delta\epsilon_{287}$ for $[U_S]$ in eq 3 and 4 yields eq 5 and 6, respectively:

$$\Delta\epsilon_{287} = (\Delta\epsilon_{287})_{eq}e^{-k't} \qquad (5)$$

$$\Delta\epsilon_{287} = (\Delta\epsilon_{287})_{eq}\left[1 + \frac{1}{k_{IF} - k'}(k'e^{-k_{IF}t} - k_{IF}e^{-k't})\right] \qquad (6)$$

The formation of U_S has been determined by using the assay for slow-folding molecules (Schmid & Baldwin, 1978) at 10 °C in 4 M Gdn·HCl, pH 1. Under these conditions, fluorescence-detected unfolding shows a final slow phase with $\tau = 160$ s. All the preceding steps in unfolding are very fast. The $U_F \rightleftharpoons U_S$ reaction shows a time constant $\tau = (k')^{-1} = 200$ s at 10 °C, independent of pH and of Gdn·HCl concentration (Schmid & Baldwin, 1979a,b). The results of double jumps at 4 M Gdn·HCl, pH 1, are shown in Figure 2. The observed refolding amplitudes are compared to two kinetic progress curves, calculated by using eq 5 (based on the scheme in eq 1) and eq 6 for the alternative sequential scheme (eq 2). The experimental points coincide with the single exponential expected for model 1. Hence, a mechanism where the slowest phase of fluorescence-detected unfolding precedes the $U_F \rightleftharpoons U_S$ reaction (eq 2) can be ruled out.

The distinct properties of sequential reactions can be further exploited to locate the relative position of the experimentally obtained kinetic phases on the unfolding pathway. At low pH, unfolding kinetics are described in terms of a $N \rightarrow I \rightarrow U_F \rightleftharpoons U_S$ pathway (Hagerman & Baldwin, 1976; Hagerman et al., 1979; K. H. Cook, R. L. Baldwin, and F. X. Schmid, unpublished experiments). Fluorescence kinetics at pH 2, 2.8

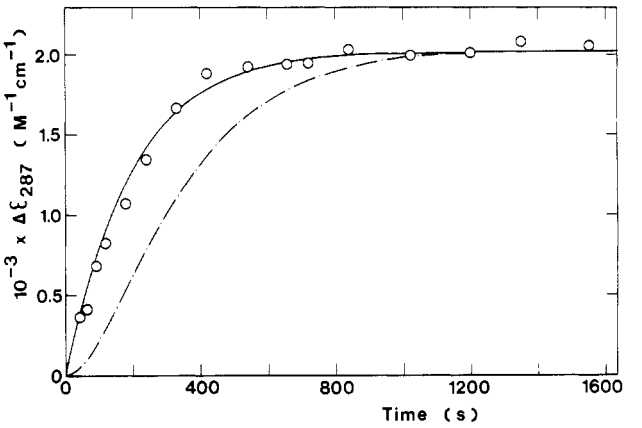

FIGURE 2: Kinetics of formation of the slow-folding species U_S after unfolding at pH 1, 4 M Gdn·HCl, 10 °C. The amount of U_S present after varying times of unfolding was determined by use of refolding assays carried out at 1.3 M Gdn·HCl, pH 6.4, at 25 °C. The amplitudes of the refolding assays (expressed as the change in extinction coefficient at 287 nm) are shown as a function of the time of sample withdrawal. Experimental data (O) are compared to two theoretical curves: (—) computed by using eq 5 with $\Delta\epsilon_{eq} = 2030$ M^{-1} cm^{-1} and $\tau = 200$ s; (-----) computed by using eq 6 for a sequential mechanism with $\Delta\epsilon_{eq} = 2030$ M^{-1} cm^{-1}, $\tau_1 = 160$ s, and $\tau_2 = 200$ s.

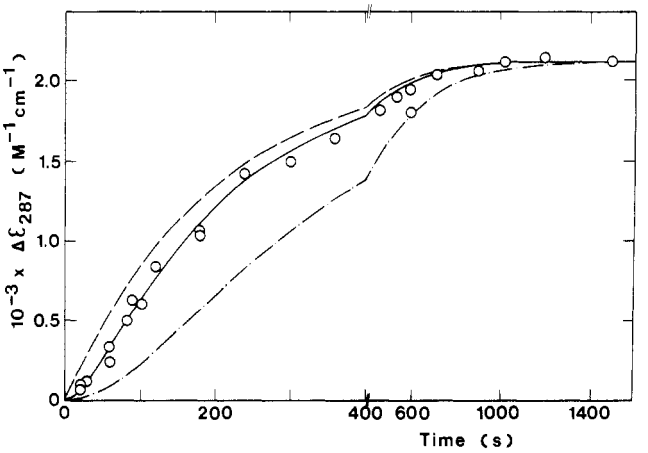

FIGURE 3: Kinetics of formation of the slow-folding species U_S after unfolding at 2.8 M Gdn·HCl and 0.05 M glycine, pH 2.0, at 10 °C. Refolding assays were carried out as described in the legend to Figure 2. Experimental data (O) are compared to three calculated kinetic curves: (—) a sequential mechanism (eq 6) with $\Delta\epsilon_{eq} = 2120$ M^{-1} cm^{-1}, $\tau_1 = 30$ s, and $\tau_2 = 200$ s; (-----) a sequential mechanism (eq 6) with $\Delta\epsilon_{eq} = 2120$ M^{-1} cm^{-1}, $\tau_1 = 160$ s, and $\tau_2 = 200$ s; (- - -) a simple first-order reaction (eq 5) with $\Delta\epsilon_{eq} = 2120$ M^{-1} cm^{-1} and $\tau = 200$ s.

M Gdn·HCl, and 10 °C yield three phases. The first one is too fast to measure with manual mixing and shows a large amplitude (about 0.5); the second one shows a time constant of 30 s (relative amplitude 0.2); the slowest step is governed by a time constant of 160 s (relative amplitude 0.3). Under these final conditions, the formation of U_S has been measured by use of the assay for slow-folding molecules. The results are shown in Figure 3. Again the observed kinetics are compared with calculated progress curves: (i) for a single exponential, by using eq 5; (ii) assuming a sequential reaction as in the scheme in eq 2 where formation of U_S from U_F is preceded by the 30-s phase of the fluorescence-detected unfolding kinetics; and (iii) another sequential reaction, where formation of U_S is preceded by the slowest phase of the fluorescence kinetics (the 160-s phase). The experimental data show that unfolding is indeed a sequential process (because of the sigmoidal kinetics of formation of U_S) and that formation of the slow-folding species U_S is preceded by the

Table IV: Stopped-Flow Refolding Experiments with RNase A. Comparison of Fluorescence-Detected Kinetics with Absorbance-Detected Refolding[a]

parameter	τ_1[c] (s)	τ_2[d] (ms)	f_2[e]
absorbance[b]	19.2	110	0.19
fluorescence	19.8	97	0.15

[a] Initial conditions were 35 °C, pH 2.0, 1.0 M Gdn·HCl, 70 μM RNase A; final conditions were 35 °C, pH 6.0, 0.5 M Gdn·HCl, 35 μM RNase A. [b] From Table II of Garel et al. (1976). [c] Time constant of the slow-refolding reaction. [d] Time constant of the fast-refolding reaction. [e] Relative amplitude of the fast-refolding reaction ($f_1 + f_2 = 1$).

second-slowest phase of the fluorescence-detected unfolding kinetics. Hence, the slowest phase of these kinetics is identical with the $U_F \rightleftharpoons U_S$ reaction.

Figures 2 and 3 demonstrate that unfolding of RNase A at pH 2 and 10 °C is adequately described by a sequential $N \rightarrow I \rightarrow U_F \rightleftharpoons U_S$ mechanism (Hagerman & Baldwin, 1976). The slowest phase of unfolding, i.e., the $U_F \rightleftharpoons U_S$ equilibration reaction, is accompanied by a substantial change in fluorescence, while tyrosine abosrbance remains unchanged.

Fast-Refolding Reaction of RNase A Shows a Relative Amplitude of Only 0.15 in Fluorescence. It is well established that unfolded RNase A consists of 20% fast-folding species U_F and 80% slow-folding species U_S (Garel & Baldwin, 1973; Hagerman & Baldwin, 1976; Nall et al., 1978). If, as shown previously, U_S has a higher tyrosine fluorescence than U_F, then the specific decrease of fluorescence intensity upon refolding has to be larger (by about 30%) for the $U_S \rightarrow N$ folding reaction compared to the specific change during the $U_F \rightarrow N$ reaction.

Therefore, the relative amplitude of the fast-refolding reaction is expected to drop from $f_2 = 0.20$ (as detected by absorbance) to $f_2 = 0.14$, when monitored by fluorescence. In consequence, the relative amplitude of the slow-refolding reaction should rise to 0.86. Stopped-flow measurements of the decrease in tyrosine fluorescence during refolding were carried out to check this prediction. The protein was kept unfolded at 35 °C, 1 M Gdn·HCl, pH 2.0, and the refolding was initiated by a 1:1 dilution to 0.5 M Gdn·HCl, pH 6.0. These conditions were chosen (i) because the two refolding reactions are well separated ($\tau_1/\tau_2 = 200$), which facilitates accurate determination of the relative amplitudes, and (ii) because absorbance-detected refolding is well characterized under these final conditions (Garel et al., 1976). Table IV gives a comparison of the kinetic results obtained with fluorescence detection to the absorbance data of Garel et al. (1976). The time constants of the fast- and slow-refolding reactions derived from the fluorescence measurements agree well with the results of the absorbance kinetics. However, the relative amplitude of the $U_F \rightarrow N$ reaction, f_2, decreases as expected from 0.19 in the absorbance kinetics to 0.15 in the fluorescence-detected refolding kinetics.

Discussion

Fast- and Slow-Folding Species of RNase A, U_F and U_S, Differ in Their Fluorescence Properties. The fast-folding species U_F and the slow-folding species U_S are not equivalent in their tyrosine fluorescence emission although both species appear to be completely unfolded. The $N \rightarrow U_F$ reaction is accompanied by a 2-fold increase in fluorescence, whereas the fluorescence of U_S is about 2.5-fold higher than the emission of native RNase A. Refolding of a 20:80 mixture of U_F and U_S (as present in RNase A, unfolded at equilibrium; Garel et al., 1976) consequently yields a relative amplitude of $f_2 = 0.15$ only, in contrast to $f_2 = 0.20$ obtained in absorbance-detected refolding.

The small amplitude of $f_2 = 0.15$ does not agree with the results of Henkens et al. (1980), who monitored the refolding of RNase A at 1.9 M Gdn·HCl, pH 7.1, and 15.5 °C. After RNase was unfolded in 6 M Gdn·HCl at temperatures from 0 to 70 °C, they found the amplitude f_2 to be slightly dependent on the temperature of unfolding, ranging from $f_2 = 0.33$ at 0 °C to $f_2 = 0.20$ at 70 °C. However, in their approach, f_2 was not measured directly, but only the amplitude of the slow-refolding reaction was determined. f_2 was assumed to be the difference between the extrapolated fluorescence of the unfolded protein (which depends considerably on Gdn·HCl concentration) and the beginning of the slow phase. This indirect method may lead to an overestimate of f_2. Discussion of their data in terms of equilibrium constants for the $U_F \rightleftharpoons U_S$ reaction and the role of different proline residues has been based on the assumption that both unfolded species are equivalent in their tyrosine fluorescence properties. Our results show that this is not the case.

Parallel to the fluorescence changes, a difference in absorbance between U_F and U_S has been detected. It is very small (3–5% of the total change upon unfolding), and therefore it is only detectable under conditions where all preceding unfolding reactions are very fast, e.g., a combination of low pH and high concentrations of Gdn·HCl. A similar slow phase with an amplitude of 2–3% was observed earlier by Nall (1976) when RNase A was unfolded by high concentrations of Gdn·HCl at pH 2.1 and 45 °C.

Difference in Fluorescence Is Probably Caused by Isomerization of the Tyr-92–Pro-93 Peptide Bond. U_F and U_S are both completely unfolded species, which lack specific long-range interactions (Garel et al., 1976). They differ only in the configuration of proline peptide bonds. U_F has all its proline residues in the same configuration as in the native molecule, whereas U_S possesses at least one incorrect proline isomer (Schmid & Baldwin, 1978; Cook et al., 1979). Proline isomerization can influence tyrosine fluorescence in the unfolded disordered chain only at positions where proline and tyrosine are close in sequence.

To find out which of the four proline residues causes the observed change in tyrosine fluorescence upon isomerization, we have to search the RNase A structure for positions where tyrosine residues are next to such proline residues, which are in the cis configuration in the native molecule. In unfolded proteins and in short peptides in general, the trans configuration is predominant; often a ratio of 80% trans and 20% cis state is found in model systems (Brandts et al., 1975). For the same reason, *cis*-prolines are expected to isomerize after unfolding largely to the more favorable trans state. Out of the *trans*-prolines, only about 20% isomerize to the cis configuration after unfolding. They are not considered here, since they would cause only small fluorescence changes and the discussion is restricted to the *cis*-prolines.

There are two positions in the RNase A sequence where tyrosine residues are adjacent to *cis*-prolines: Pro-114–Tyr-115 and Tyr-92–Pro-93. We suggest that the difference in fluorescence between U_F and U_S is caused by Tyr-92 and Pro-93 for the following reasons.

(i) Pro-114 may be nonessential for folding [H. W. Wyckoff, as quoted by Schmid & Baldwin (1978)], and Tyr-115 is already exposed to solvent in the native state. Moreover, it is not hydrogen bonded, and the nearest disulfide bond (58–110) is five residues away (hydrogen bonding and nearby disulfides are assumed to quench tyrosine fluorescence; Cow-

gill, 1966, 1967; Richards & Wyckoff, 1971). Its spectroscopic properties, therefore, are not expected to change significantly during the $U_F \rightleftharpoons U_S$ reaction.

(ii) In the native molecule, Tyr-92 and Pro-93 are part of a β-bend structure; Pro-93 is cis, while Tyr-92 is close to the 40–95 disulfide bond, and its phenolic hydroxyl group is hydrogen bonded to the backbone oxygen of Lys-37. Both interactions quench the fluorescence of Tyr-92 (Cowgill, 1966, 1967). In U_F, Pro-93 is still in the native cis configuration, thus allowing Tyr-92 to maintain local interactions either with disulfide 40–95 or with Lys-37 or with both of them. Aromatic side chains are known to form specific noncovalent bonds of considerable strength with disulfides (Morgan et al., 1978; Némethy & Scheraga, 1981). These interactions could conceivably persist even in the unfolded chain, as Tyr-92 is close in sequence to disulfide 40–95, and also close even to its hydrogen-bond acceptor Lys-37 because of the covalent link provided by the 40–95 disulfide bridge.

(iii) Isomerization of the X–Pro peptide bond is known to strongly influence the steric orientation of residue X (Grathwohl & Wüthrich, 1976; Toma et al., 1980; Levitt, 1981). In corticotropin for instance, isomerization of the Tyr-23–Pro-24 bond strongly affects the NMR signals of the ring protons of Tyr-23 (Toma et al., 1978). Therefore, cis → trans isomerization of Pro-93 probably changes the steric orientation of Tyr-92 and prevents it from maintaining its interaction with the 40–95 disulfide bond and/or the hydrogen bond to Lys-37. As both interactions critically depend on distance, small changes in the spatial location of the aromatic ring of Tyr-92 may be sufficient to disrupt the interactions, thus leading to the observed increase in tyrosine fluorescence. The very small effect observed in tyrosine absorbance may be caused by minor changes in the solvent accessibility of Tyr-92 upon isomerization of Pro-93.

Experimental evidence taken together with the known structural properties of RNase A favors the assignment of the fluorescence changes to Tyr-92 and Pro-93. However, we cannot exclude the alternative explanation that both *cis*-prolines, 93 as well as 114, and their adjacent tyrosine residues contribute to the overall fluorescence change.

Tyrosine Fluorescence as a Local Probe for Proline Isomerization. A similar change in fluorescence has been detected in the refolding kinetics of RNase A. Under strongly native folding conditions, proline isomerization can occur in a nativelike intermediate I_N after the major folding events have taken place (Cook et al., 1979). This final isomerization step leads to a significant decrease in tyrosine fluorescence of the refolding molecule (Schmid, 1980, 1981). The dependence of the fluorescence changes on proline isomerization has been explained in similar terms. U_S species containing only Pro-93 in the incorrect trans state rapidly fold to a nativelike intermediate I_N, which possesses a compact structure, a substrate binding site, and catalytic activity (Schmid & Blaschek, 1981). In a subsequent slow reaction, Pro-92 isomerizes from the incorrect trans state to the native cis configuration, thereby allowing Tyr-92 to establish its native interaction with the 40–95 disulfide and its hydrogen bond to Lys-37; both lead to a quenching of fluorescence. Together with the above discussion concerning the fluorescence difference between U_F and U_S, this provides strong evidence that the molecular nature of the coupling between isomerization at Pro-93 and the fluorescence emission of Tyr-92 may be the same in the unfolded polypeptide and in a nativelike folded molecule.

So far, proline isomerization during the $U_F \rightleftharpoons U_S$ reaction of RNase A has been measured by the use of the assay for slow-folding molecules, which monitors the formation of U_S after unfolding (Brandts et al., 1975; Nall et al., 1978; Schmid & Baldwin, 1978, 1979a,b). This double-jump technique is not sensitive to a single proline, but monitors all prolines which are essential for folding. Comparison of the fluorescence-detected isomerization at Pro-93 and the isomerization of all essential prolines as monitored by the assay for slow-folding molecules gives important clues regarding the role of Pro-93 in the folding of RNase A.

(i) The similarity of both kinetics shows that Pro-93 is most important in determining the rate and the relative amplitude of the $U_F \rightleftharpoons U_S$ reaction. This is to be expected since Pro-93 is cis in the native state, but largely trans in the unfolded state. Pro-42 and -117 are both trans in the native molecule and remain predominantly in this state in the unfolded molecule. Therefore, they are not expected to strongly influence the properties of the $U_F \rightleftharpoons U_S$ reaction. (It is assumed that the other *cis*-proline, Pro-114, is nonessential for folding; this is still a hypothesis.)

(ii) The fluorescence-detected isomerization reaction shows a time constant in the range of 150–200 s (see Tables I–III) at 10 °C whereas the time constant derived from slow-refolding assays is 200–220 s (Schmid & Baldwin, 1979a,b). This small decrease in rate may be caused by the *trans*-prolines-42 and -117, as expected from model calculations (Brandts et al., 1975; Creighton, 1978). However, it cannot be ruled out that this small difference in rates is simply caused by experimental uncertainty. Therefore, this conclusion can only be regarded as tentative.

The pK value of one or more nitrotyrosine residues in nitrated RNase A has been shown to depend on proline isomerization and has been used to monitor the kinetics of the $U_F \rightleftharpoons U_S$ reaction (Garel & Baldwin, 1975; Garel, 1980). As only accessible tyrosines (tentatively, 73, 76, and 115) had been converted into nitrotyrosine, it was concluded that the pK shift occurred at Tyr-115, which is the only one next to a proline (Pro-114). Tyr-92 was assumed to remain unmodified by the nitration procedure; however, nitration may yield a mixture of molecules, which are modified to a varying extent (Van der Zee et al., 1977). The observed kinetics at pH 6.5 are similar to the fluorescence-detected kinetics of $U_F \rightleftharpoons U_S$ obtained with unmodified RNase A in this study.

Local Structures in Unfolded RNase A. The state of proteins which are unfolded by high concentrations of Gdn·HCl has been studied in detail by Tanford and co-workers [for a review, see Tanford (1968)]. On the basis of viscosity, circular dichroism, and titration data, they concluded that proteins in 6 M Gdn·HCl approach random-coil behavior. This is also true for unfolded proteins with intact disulfide bridges, if one takes into account the restrictions imposed on the chain by the covalent cross-links. Nevertheless, local nonrandom structures may exist even in high concentrations of Gdn·HCl, and the aromatic rings of tyrosine and phenylalanine may be part of such structures (Tanford, 1968). There is some evidence that the state of the tyrosine residues of RNase A in 6 M Gdn·HCl differs from the state of tyrosine in model compounds such as tyrosine amide or short oligopeptides containing tyrosine. (i) The six tyrosines are slightly heterogeneous in their pK values (they range from 9.7 to 10.3; the expected pK is 9.8). The slightly increased pK of three tyrosine residues was tentatively assigned to hydrophobic interactions with disulfide bonds or other adjacent hydrophobic residues in the unfolded chain (Nozaki & Tanford, 1967). (ii) Solvent perturbation measurements by Herskovits & Laskowski (1968) suggest that in 8 M urea the tyrosine residues are not com-

pletely accessible to solvent; accessibility can be increased by rupture of the disulfide bonds. (iii) Tyrosine fluorescence of unfolded RNase A increases upon reduction of the disulfide bridges (Cowgill, 1966). These results suggest that local hydrophobic interactions which are resistant to 6 M Gdn·HCl may be present in unfolded RNase A and that the tyrosine side chains may be involved in such structures. Fluorescence intensity is a potential probe for that kind of local interaction, as it is critically dependent on distance. Usually local structural fluctuations in unfolded proteins are extremely fast (Tsong, 1975); however, in the case of RNase A, the local structural changes around Tyr-92 are coupled to the cis/trans isomerization at Pro-93, and therefore, they are easily observable in the time range of minutes.

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