Proc. Natl. Acad. Sci. USA
Vol. 73, No. 6, pp. 1853–1857, June 1976
Biochemistry

Guanidine-unfolded state of ribonuclease A contains both fast- and slow-refolding species

(protein folding/proline isomerism)

JEAN-RENAUD GAREL*, BARRY T. NALL, AND ROBERT L. BALDWIN

Department of Biochemistry, Stanford University Medical School, Stanford, California, 94305

Communicated by Walter Kauzmann, March 11, 1976

ABSTRACT **The kinetics of the refolding reaction of ribonuclease A from high concentrations of guanidine hydrochloride or urea are biphasic, and show two refolding reactions whose rates differ 450-fold at pH 5.8 and 25°. Measurements of cytidine 2′-phosphate binding during refolding, after stopped-flow dilution of guanidine hydrochloride (Gdn·HCl) or urea, show that functional bovine pancreatic ribonuclease A (RNase A; ribonucleate 3′-pyrimidino-oligonucleotidohydrolase, EC 3.1.4.22) is formed in both the fast and slow phases of the refolding process. We conclude that the guanidine-unfolded state of RNase A is an equilibrium mixture of fast- and slow-refolding species, as was found previously for the heat-unfolded state at low pH. The fraction of the fast-refolding species in guanidine or urea-unfolded RNase A is the same as that in the heat-unfolded protein at pH 2.**

Previous work has shown that the fast-refolding species disappears as the pH is raised from 3 to 5 for heat-unfolded RNase A. This pH effect is not present in refolding from concentrated Gdn·HCl solutions: the same proportion of the fast-refolding species is found from pH 2 to pH 6, and also from 2 M to 6 M Gdn·HCl at pH 5.8. We conclude that the same proportion of the fast-refolding species is present at equilibrium whenever the residual structure in unfolded RNase A is reduced to a low level, and that the structural difference between the fast-refolding and slow-refolding species of RNase A lies in the configuration of the random coil polypeptide chain.

The observed rates of protein folding reactions are many orders of magnitude faster than predicted from a purely random search of all possible configurations for a random coil polypeptide chain devoid of structure†. A possible explanation for this difference is that an unfolded protein is not a random coil. The elements of residual structure play a crucial role in directing the course of protein folding, and thus considerably restrict the possible pathways (1) to the native state. Indeed, long polypeptide fragments of staphylococcal nuclease, which appear unfolded by several criteria, still react specifically with antibodies directed against the native conformation of the intact enzyme (2). Guanidine hydrochloride (Gdn·HCl) is often used to disrupt the structure of proteins, since detailed studies have suggested that, at high Gdn·HCl concentrations, many proteins behave as random polypeptide chains (3–5), i.e., do not show any detectable elements of organized structure. This has led to the belief that a protein unfolded by Gdn·HCl can be considered as a single species in refolding experiments, since rotation about single bonds occurs in a much faster time range ($\tau_2 = 0.06$ sec at pH 5.8 and 25°; Table 2) than the fastest rate-limiting step observed in the refolding of bovine pancreatic ribonuclease A (RNase A); ribonucleate 3′-pyrimidino-oligonucleotidohydrolase, EC 3.1.4.22).

There seems to be a contradiction between the two proposals: (*a*) that a guanidine-unfolded protein behaves as a homogeneous random chain and (*b*) that this same unfolded state still possesses some elements of residual structure which increase the rate and determine the pathway(s) of folding. However, these proposals are compatible if the residual structure, important for the folding process, is not present as such in the unfolded state, but is rapidly formed after refolding is initiated. For instance, formation of α-helical segments could take place within the dead time of stopped-flow measurements (6) and limit the possible pathways for refolding, if the α-helical segments are stable under the conditions in which refolding is initiated, without a requirement of prior slow steps in refolding to provide a stabilizing environment.

Studies of the refolding of heat-unfolded RNase A (7–11) have shown that the heat-unfolded state does not behave as a single species in refolding. Instead, distinct fast-refolding and slow-refolding species of the heat-unfolded protein have been found to coexist in a slow, pH-dependent equilibrium (9). If the fast-refolding species owes its existence to elements of residual structure that provide nuclei for rapid refolding, then it should disappear at concentrations of Gdn·HCl sufficient to break up such residual structure. If, on the other hand, the fast- and slow-refolding species are different random coil forms that slowly equilibrate, then a division into fast- and slow-refolding forms should be maintained even at a high concentration of Gdn·HCl. For example, the interconversion of *cis* and *trans* forms of prolyl residues is a slow reaction that may have a profound effect on the refolding kinetics of a random coil polypeptide chain (11), and could account for the existence of fast- and slow-refolding forms of all proteins with prolyl residues.

To find out if more than one unfolded species of RNase A is present in concentrated guanidine solutions, we have made a study of the refolding kinetics. Complex refolding kinetics after guanidine-induced unfolding have already been reported for horse heart cytochrome *c* (12, 13), hen lysozyme (14), and bovine carbonic anhydrase (15). The kinetic complexity shows that at least one intermediate must be present during refolding but *does not necessarily show the presence of more than one unfolded form.* In earlier studies (12–15), it was presumed that the intermediates responsible for the kinetic complexity were formed *after* refolding was initiated.

Our results show that slow- and fast-refolding forms are present *before* refolding is initiated when RNase A is unfolded in concentrated Gdn·HCl solutions. The approach is the same as in an earlier study of the refolding of heat-unfolded RNase A (8). Well separated, fast- and slow-refolding reactions are found, and so the stopped-flow apparatus can be used as a

Abbreviations: RNase A, bovine pancreatic ribonuclease A with disulfide bonds intact; 2′-CMP, cytidine 2′-phosphate; τ, time constant of a chemical reaction; Gdn·HCl, guanidine hydrochloride.

* Present address: Service de Biochimie Cellulaire, Institut Pasteur, 25 Rue du Dr Roux, 75015, Paris, France.

† The term random coil can refer only in a limited sense to the unfolded polypeptide chain of RNase A, since it is a copolymer of 20 amino acid residues, and the chain is constrained by four disulfide cross-links. We use the term here as it has been previously defined (21).

separation method. The complete fast-refolding reaction can be characterized by rapid-response methods before the slow-refolding reaction proceeds to a significant extent. Formation of functional RNase A is monitored by the rapid binding of a specific ligand, 2′-CMP. Since there is one strong binding site for 2′-CMP per RNase A molecule, the stoichiometry of binding can be used to obtain the concentration (8) of native RNase A present at any time. A different measurement of the refolding process is provided by the change in absorbance at 287 nm, which accompanies the burial of three tyrosine groups in native RNase A and monitors the exclusion of water from the interior of the protein. Refolding is studied in jumps across the entire transition zone, so that only unfolded species are present in the initial conditions and, since the reaction is completely reversible (16), only native RNase A is present in the final conditions.

MATERIALS AND METHODS

Materials. RNase A (Sigma type XII A, lot no. 35G-8190), 2′-CMP (P. L. Biochemicals lot no. 273-10), Gdn·HCl (Heico lot no. 217,005), urea (Mann ultrapure), and cacodylic acid (Fisher) were used. Some of the experiments were done with Sigma RNase A that was purified further by chromatography on Sephadex CM-50; the basic properties of the refolding kinetics are the same for both preparations.

Methods. Most of the methods have been described before (8, 9). Concentrations of stock Gdn·HCl solutions were determined by density measurements (17) with a pycnometer and checked by refractometry. Stopped-flow mixing controls were made by mixing water either with tryptophan solutions or with RNase A in 0.6 M Gdn·HCl at pH 5.8. The absence of any thermal artifact was checked (8). Variable-ratio mixing syringes were obtained from Durrum Instruments, and used in combination with syringes from Metrohm A. G. (Herisau, Switzerland). The mixing ratio was checked with a known solution of tryptophan; for the syringe combination giving a 10-fold dilution, the actual dilution was found to be 9.8 ± 0.2. The mixing time was found to be slightly longer (10 msec) when diluting 6 M Gdn·HCl with the 9:1 ratio syringes than when mixing solutions without Gdn·HCl (3 msec). Both the fast- and slow-refolding reactions were assumed to be single exponentials except as described in the text, and the standard method of resolving two exponentials ("peeling back exponentials") has been used. The time constants of the reactions given in Tables 1 and 2 are average values from three to six photographs of duplicate mixing experiments.

RESULTS

Measurement of 2′-CMP binding in 0.6 M Gdn·HCl

Two properties of the reaction between 2′-CMP and native RNase A make it particularly useful for monitoring the kinetics of refolding. (*a*) The binding of 2′-CMP to native RNase A is a fast reaction, complete within 3 msec; it provides a nearly instantaneous probe of the extent of refolding. (*b*) At 250 nm there is a substantial change in absorbance caused by 2′-CMP binding but only a small absorbance change caused by protein refolding, so that binding can be measured separately from refolding. To use 2′-CMP binding to study refolding in guanidine dilution experiments, it is necessary to find out first if 2′-CMP binding can be measured in 0.6 M Gdn·HCl. Fig. 1 shows equilibrium measurements of ΔA_{250} made by the tandem cell technique for the binding of 2′-CMP to native RNase A in 0.6 M Gdn·HCl, and in 0.1 M $NaClO_4$ at pH 5.8 and 25°. The results show that 0.6 M Gdn·HCl does reduce the affinity of RNase A for 2′-CMP but that binding can be observed satisfactorily via ΔA_{250}. When stopped-flow measurements of refolding are made in 0.6 M Gdn·HCl at pH 5.8 and 25°, ΔA_{250} has opposite signs in the presence and absence of 2′-CMP, such that an increase in transmittance gives a positive test for binding.

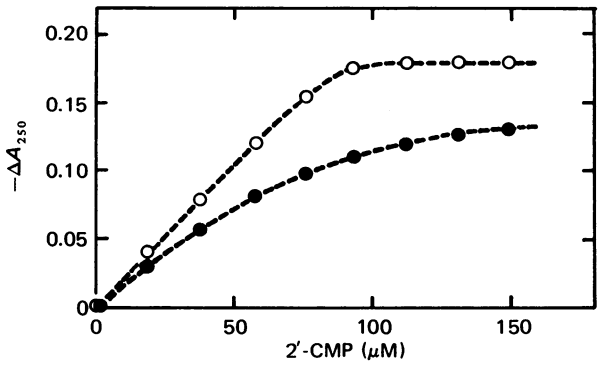

FIG. 1. Equilibrium measurements of the absorbance difference at 250 nm resulting from the binding of 2′-CMP to native RNase A measured in tandem cell difference experiments. Conditions: 0.05 M Na cacodylate at pH 5.8, 85 μM RNase A and 25°. ○, 0.1 M $NaClO_4$; ●, 0.6 M Gdn·HCl.

Characterization of the fast- and slow-refolding reactions observed in guanidine dilution experiments

Two refolding reactions are seen in guanidine dilution experiments: Fig. 2a shows refolding measured by tyrosine absorbance at 287 nm for 6 M to 0.6 M Gdn·HCl at pH 5.8 and 25°. These two reactions closely resemble the fast and slow reactions observed in pH-jump refolding experiments, and in certain conditions they appear to be identical. By comparison with the more extensive studies of pH-jump refolding (8–11), the following observations are sufficient to show that the two refolding reactions arise from two conformationally different forms of unfolded RNase A present in concentrated guanidine solutions. (*a*) The guanidine-induced unfolding transition is complete near 4 M (Fig. 3a) and all species present in 6 M Gdn·HCl are unfolded species by this criterion. Therefore, the fast-refolding reaction does not arise from a partly unfolded intermediate that is present only inside the transition zone. (*b*) In guanidine dilution experiments, both the fast- and slow-refolding reactions yield native RNase A able to bind 2′-CMP (Fig. 2b, Table 1). Therefore, complete refolding occurs in the fast reaction as well as in the slow reaction, since only unfolded species are present initially. (*c*) Comparison of the refolding kinetics measured at 287 nm, which monitors buried tyrosine groups, and at 250 nm, which follows 2′-CMP binding, shows identical results (i) Only two refolding reactions are seen by each probe; (ii) the same values of τ_1 and τ_2 are found; and (iii) the ratio of the absorbance changes in the fast and slow reactions is the same for both probes. These results are expected if the fast- and slow-refolding reactions are produced by two different unfolded species of RNase A, both of which have the same molar extinction coefficient at 287 nm. (*d*) The fraction of the fast-refolding species is the same in pH-jump refolding experiments that start below pH 3 (9) as in guanidine dilution experiments at pH 5.8. Therefore, conditions which minimize residual structure in unfolded RNase A show the same proportion of the fast-refolding species in both types of refolding experiments. (*e*) In specified conditions (Table 2), the number of fast- and slow-refolding reactions observed in guanidine dilution experiments is the same as in pH-jump experiments and both types of experiments show the same values of τ_1 and τ_2. This indicates that

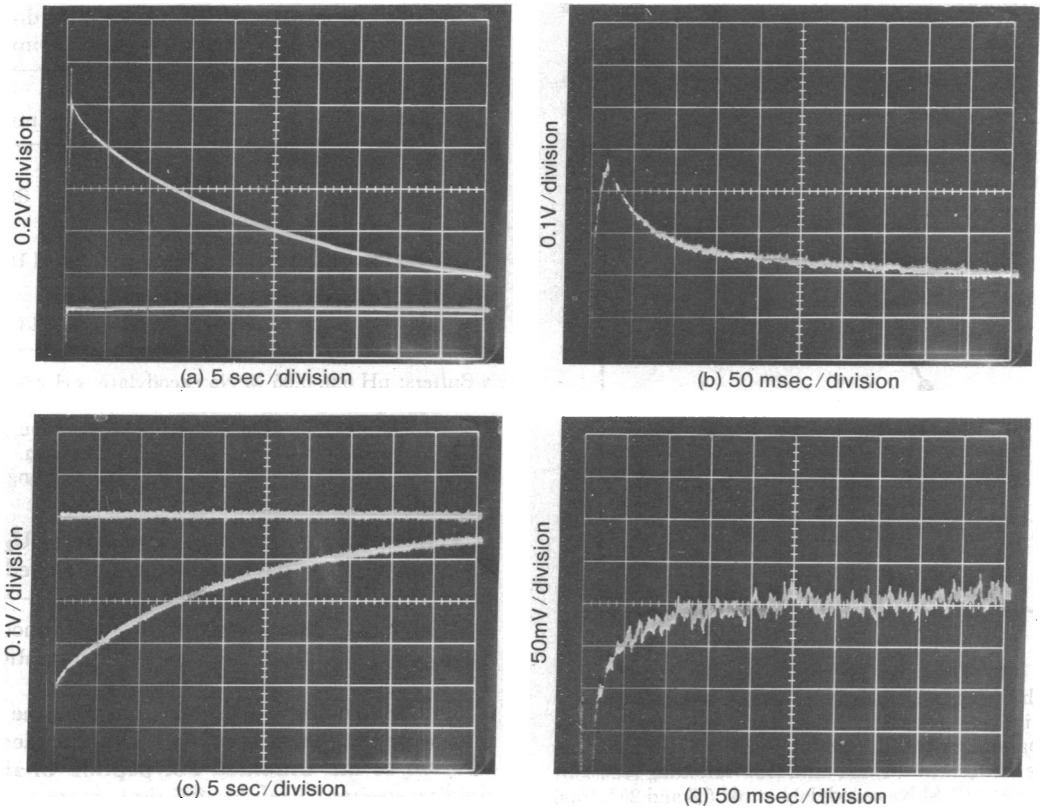

FIG. 2. Oscillograph records (change in transmittance versus time) of refolding from 6 M Gdn·HCl (6 M to 0.6 M Gdn·HCl, at pH 5.8 and 25°). (a,b) Measurements at 287 nm of the slow- and fast-refolding reactions observed by tyrosine absorbance, total signal 3.8 V. (c,d) Measurements at 250 nm of the absorbance change caused by 2′-CMP binding, total signal 3.2 V. Final RNase A concentration, 0.11 mM; final 2′-CMP concentration (c,d only), 0.13 mM.

the same rate-limiting steps are being observed in both types of refolding experiment. Table 2 first compares refolding at 35° initiated by guanidine dilution (5 M to 0.5 M at pH 6) with refolding initiated by a pH-jump (pH 2 to pH 6): the values of τ_1 and especially of τ_2 are somewhat different. However, when 0.5 M Gdn·HCl is included in the pH-jump experiment to make the final conditions of refolding the same, then identical values of τ_1 and τ_2 are found in both experiments. At temperatures below 35°, the slow-refolding reaction becomes kinetically complex in pH-jump refolding but not in guanidine dilution experiments. This subject will be discussed elsewhere (B. T. Nall, J.-R. Garel, and R. L. Baldwin, in preparation).

Refolding from 8.5 M urea

Refolding of RNase A from 8.5 M urea (8.5 M to 0.85 M urea at pH 5.8 and 25°) shows all of the properties (Table 1) just described for guanidine dilution experiments. Two refolding reactions are seen; τ_1 and τ_2 have values close to those found in 0.6 M Gdn·HCl; the refolded protein produced in both the fast and slow reactions binds 2′-CMP; and the fraction of RNase A refolded in the fast reaction is $f_2 = 0.21 \pm 0.02$ measured either at 287 nm by tyrosine absorbance or at 250 nm by 2′-CMP binding.

DISCUSSION

(*a*) Fast-Refolding and Slow-Refolding Species of Unfolded RNase A in Guanidine Hydrochloride. The refolding of guanidine-unfolded RNase A shows two widely separated reactions. Both yield native enzyme, as judged from studies of 2′-CMP binding. In this work two conditions are strictly fulfilled, (*a*) refolding is complete in the final conditions, and (*b*) the two reactions are always well separated, so that the relative amplitude of the fast phase gives the actual concentration of the fast-refolding species in the initial conditions (10). The guanidine-unfolded state of RNase A thus appears to be a mixture composed of about 20% and 80% of fast- and slow-refolding materials, respectively. The same fractional composition is found for the urea-unfolded state of RNase A (Table 1) and was also obtained previously for the heat-unfolded state at low pH (9, 10). There is no proof that the fast- and slow-refolding species are the same in RNase A unfolded by Gdn·HCl, urea, or heat at low pH. However, the division of the unfolded state into the same proportion of fast- and slow-refolding species in these three cases strongly suggests that this partition into fast-refolding and slow-refolding species is an intrinsic property of the unfolded state (or states) of RNase A. This conclusion is supported by the finding that the same rate-limiting steps are observed in guanidine dilution as in pH-jump refolding experiments (Table 2).

Proof that the two refolding reactions arise from different species of unfolded protein and not from transient intermediates in folding has been given earlier for heat-unfolded RNase A. There are three lines of evidence: (*a*) when the refolding conditions (pH and temperature) are fixed, the proportion of fast- to slow-refolding material depends on the *initial* pH (9). This shows that there is a pH-dependent equilibrium between the fast- and slow-refolding species in the initial conditions, and that the rate of readjustment of this equilibrium is slow compared to the stopped-flow mixing process (dead time, 3 msec). Because both the fast- and slow-refolding species are present at temperatures well above the transition zone for unfolding at pH 2 (8, 9), and their proportion does not change at lower temperatures, both forms must be unfolded species of RNase A. (*b*)

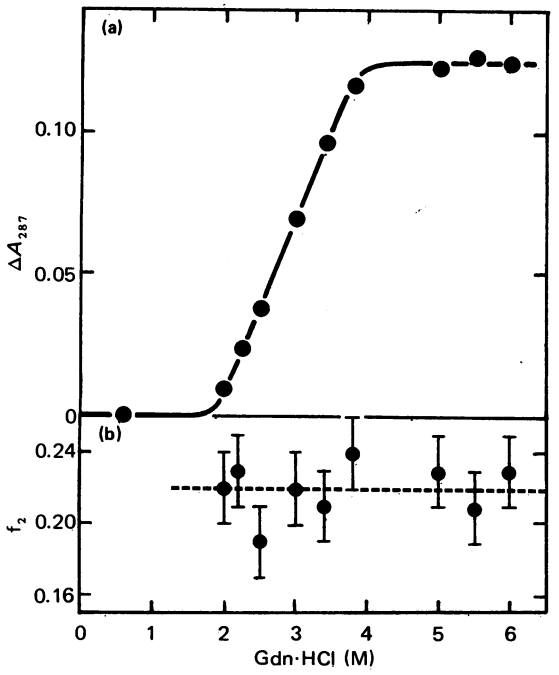

FIG. 3. (a) The extent of refolding versus the Gdn·HCl concentration used to induce unfolding; refolding is measured in the stopped-flow apparatus by tyrosine absorbance at 287 nm; the sum of the absorbance changes in the fast- and slow-refolding reactions is shown. Conditions: 0.05 M Na cacodylate at pH 5.8 and 25°; final Gdn·HCl concentration, 0.5–0.6 M; final RNase A concentration, approximately 0.1 mM. These data correspond to a kinetic measurement of the equilibrium transition curve for guanidine-induced unfolding. They agree satisfactorily with more accurate equilibrium measurements of this transition (16). (b) The fraction of the guanidine-unfolded protein present as U_2 in the initial conditions of these same experiments; f_2 is measured as the fraction of the total absorbance change upon refolding that occurs in the fast reaction.

In pH-jump experiments, the kinetics of the refolding reaction can be explained quantitatively (10) by a $U_1 \overset{\text{slow}}{\rightleftharpoons} U_2 \overset{\text{fast}}{\rightleftharpoons} N$ mechanism for refolding in which U_1 and U_2 are slow-refolding and fast-refolding forms of unfolded RNase A and N is the native enzyme. With refolding data only, it is possible to predict correctly both the proportion of the fast- and slow-unfolding reactions as a function of temperature at pH 3.0, and also the equilibrium transition curve. (*c*) A double-jump (first unfolding, then refolding) experiment (ref. 11. B. T. Nall, J.-R. Garel, and R. L. Baldwin, in preparation shows that the $U_2 \rightleftharpoons U_1$ reaction occurs slowly even though unfolding measured by absorbance appears to be complete in a fast reaction ($N \rightleftharpoons U_2$). When the total amount of refolding is measured as a function of the delay time after unfolding, the kinetics of formation of the slow-refolding species U_1 can be observed.

Table 2. Comparison of RNase A refolding kinetics in pH-jump and guanidine dilution experiments at 35°

Refolding experiment[a]	τ_1[b] (sec)	τ_2[c] (msec)	f_2[d]
pH 2.0 to pH 6.0, 0 M Gdn·HCl	14.3	38	0.21
pH 2.0 to pH 6.0, 0.5 M Gdn·HCl	19.2	110	0.19
5 M to 0.5 M Gdn·HCl, pH 6.0	17.9	111	0.21

[a] Buffers: pH 6.0, 0.05 M Na cacodylate; pH 2.0, sufficient $HClO_4$ to adjust pH.
[b] The time constant of the slow-refolding reaction.
[c] The time constant of the fast-refolding reaction.
[d] The fractional concentration of the fast-refolding species.

(*b*) The Structural Difference Between the Fast-Refolding and Slow-Refolding Species of RNase A Lies in the Configuration of the Unfolded Polypeptide Chain. The fast-refolding species U_2 refolds 450-times more rapidly than U_1 in the present conditions (Table 1). Either U_2 might be nucleated for refolding, i.e., it might possess elements of secondary or tertiary structure that allow it to refold rapidly, or the difference between U_1 and U_2 might reside in the configuration of the unfolded polypeptide chain. On the one hand, certain properties of the refolding of heat-unfolded RNase A suggest that the second explanation is correct (8, 9). (*a*) U_2 is not melted out when the temperature is raised above the transition zone for thermal unfolding. (*b*) The equilibrium ratio of (U_2):(U_1) is 1:4. This is much too high for a nucleation reaction, if the equilibrium ratio is interpreted as a measure of the difficulty of nucleation. (*c*) U_2 disappears at neutral pH, whereas elements of secondary and tertiary structure that are needed for nucleation would be expected to form at neutral pH. On the other hand, the pH dependence of the (U_2):(U_1) ratio indicates that residual structure must also control the division into slow-refolding and fast-refolding forms. The proportion of U_2 begins to drop above

Table 1. Effect of initial pH or denaturant on RNase A refolding kinetics[a]

Initial		Final					
pH	Denaturant	pH	Denaturant	λ[b] (nm)	τ_1[c] (sec)	τ_2[d] (msec)	f_2[e]
2.0	6 M Gdn·HCl	5.8	0.6 M Gdn·HCl	287	28.5	62	0.21
5.8	6 M Gdn·HCl	5.8	0.6 M Gdn·HCl	287	26.5	55	0.23
5.8	6 M Gdn·HCl	5.8	0.6 M Gdn·HCl	250[f]	25.5	59	0.21
5.8	8.5 M urea	5.8	0.85 M urea	287	28	61	0.20
5.8	8.5 M urea	5.8	0.85 M urea	250[g]	26	64	0.22

[a] Temperature, 25°; pH 5.8 buffer, 0.05 M Na cacodylate.
[b] Wavelength of observation.
[c] Time constant of the slow-refolding reaction.
[d] Time constant of the fast-refolding reaction.
[e] The fractional concentration of the fast-refolding species, $(U_2)/[(U_1) + (U_2)]$.
[f] 2′-CMP present in the final conditions (0.13 mM).
[g] 2′-CMP present in the final conditions (0.11 mM).

pH 3, at 50°, and the decrease in (U_2) follows the titration curve of a carboxyl group (9). This cannot be explained by the proline isomerization hypothesis (11) without assuming the presence of residual structure since none of the four prolyl residues in RNase A has a neighboring aspartyl or glutamyl residue; the closest such pair is Glu 111-Pro 114. At this distance between residues, there is little influence of a neighboring ionizable group on the equilibrium between *cis* and *trans* isomers of a prolyl residue (18).

The pH dependence of the (U_2):(U_1) ratio disappears in concentrated Gdn·HCl solutions: the ratio is 1:4 at pH 5.8 over the range of Gdn·HCl concentration studied (2 M–6 M, Fig. 3a). The same (U_2):(U_1) ratio is found in refolding from 8 M urea at pH 5.8. If one accepts the idea that concentrated guanidine and urea solutions disrupt residual structure, then the persistence of the $U_2 \rightleftharpoons U_1$ equilibrium in these solutions shows that the difference between U_1 and U_2 lies in the configuration of the unfolded polypeptide chain. The proline isomerization hypothesis (11) provides a plausible explanation for a division into slow- and fast-refolding forms in the absence of residual structure. The pH dependence of the (U_2):(U_1) ratio, observed in the absence of guanidine, probably arises because residual structure is formed which creates new species with distinctive refolding properties and thus alters the proportion of fast- and slow-refolding forms. This interpretation is supported by equilibrium measurements (19) showing that Gdn·HCl induces further unfolding in heat-unfolded proteins.

It should be noted that high concentrations of Gdn·HCl may not be sufficient to break up residual structure for all proteins. For horse heart ferricytochrome *c*, which has a covalently linked heme group, conformational changes involving the heme group can be produced by changes in pH, after completion of the guanidine-induced unfolding transition (20). These changes in conformation have been observed by the fluorescence of the single tryptophan group and also by the absorption bands of the heme group (20).

This work has been supported by research grants from the U.S. National Institutes of Health (Grant, GM 19988-15) and National Science Foundation (Grant, BMS 720-2262). J.R.G. received an International Research Fellowship of the U.S. Public Health Service and wishes to acknowledge the financial support of the CNRS, France, and of the University of Paris VII for a travel grant.

1. Levinthal, C. (1968) *J. Chim. Phys.* **65**, 44–45.
2. Sachs, D. H., Schechter, A. N., Eastlake, A. & Anfinsen, C. B. (1972) *Proc. Natl. Acad. Sci. USA* **69**, 3790–3794.
3. Tanford, C., Kawahara, K. & Lapanje, S. (1966) *J. Biol. Chem.* **241**, 1921–1923.
4. Tanford, C., Kawahara, K. & Lapanje, S. (1967) *J. Am. Chem. Soc.* **89**, 729–736.
5. Tanford, C., Kawahara, K., Lapanje, S., Hooker, T. M., Zarlengo, M. H., Salahuddin, A., Aune, K. C. & Takagi, T. (1967) *J. Am. Chem. Soc.* **89**, 5023–5029.
6. Schwarz, G. (1965) *J. Mol. Biol.* **11**, 64–77.
7. Tsong, T. Y., Baldwin, R. L. & Elson, E. L. (1972) *Proc. Natl. Acad. Sci. USA* **69**, 1809–1812.
8. Garel, J.-R. & Baldwin, R. L. (1973) *Proc. Natl. Acad. Sci. USA* **70**, 3347–3351.
9. Garel, J.-R. & Baldwin, R. L. (1975) *J. Mol. Biol.* **94**, 611–620.
10. Hagerman, P. J. & Baldwin, R. L. (1976) *Biochemistry*, **15**, 1462–1473.
11. Brandts, J. F., Halvorson, H. R. & Brennan, M. (1975) *Biochemistry* **14**, 4953–4963.
12. Ikai, A., Fish, W. W. & Tanford, C. (1973) *J. Mol. Biol.* **73**, 165–184.
13. Henkens, R. W. & Turner, S. R. (1973) *Biochemistry* **12**, 1618–1621.
14. Tanford, C., Aune, K. C. & Ikai, A. (1973) *J. Mol. Biol.* **73**, 185–197.
15. Yazgan, A. & Henkens, R. W. (1972) *Biochemistry* **11**, 1314–1318.
16. Salahuddin, A. & Tanford, C. (1970) *Biochemistry* **9**, 1342–1347.
17. Sober, H. A., ed. (1970) in *Handbook of Biochemistry* (Chemical Rubber Co., Cleveland, Ohio), 2nd. ed., p. J278.
18. Fermandjian, S., Tran-Dinh, S., Savrda, J., Sala, E., Mermet-Bouvier, R., Bricas, E. & Fromageot, P. (1975) *Biochim. Biophys. Acta* **399**, 313–338.
19. Aune, K. C., Salahuddin, A., Zarlengo, M. H. & Tanford, C. (1967) *J. Biol. Chem.* **242**, 4486–4489.
20. Tsong, T. Y. (1975) *Biochemistry* **14**, 1542–1547.
21. Tanford, C. (1968) *Adv. Protein Chem.* **23**, 121–282: see pp. 127 and 150.