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Acid catalysis of the formation of the slow-folding species of RNase A: Evidence that the reaction is proline isomerization

(protein folding)

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ABSTRACT Unfolded RNase A is known to contain an equilibrium mixture of two forms, a slow-folding form (U_1) and a fast-folding form (U_2). If U_1 is produced after unfolding by the slow *cis-trans* isomerization of proline residues about X-Pro imide bonds, then the formation of U_1 should be catalyzed by strong acids. Therefore, the rate of formation of U_1 has been measured at different $HClO_4$ concentrations. After rapid unfolding of the native protein in concentrated $HClO_4$ at 0°, the slow formation of U_1 was measured by use of refolding assays. Catalysis of its formation was found at $HClO_4$ concentrations above 5 M. The uncatalyzed reaction follows apparent first-order kinetics but, in the acid-catalyzed range, two reactions are found. The faster reaction produces two-thirds of the slow-folding species and shows acid catalysis above 5 M $HClO_4$. Catalysis of the slower reaction begins at 8 M $HClO_4$. The faster reaction shows a 100-fold increase in rate at 10.6 M $HClO_4$ over the rate of the uncatalyzed reaction at 5 M. The activation enthalpy of the uncatalyzed reaction has been measured in two sets of unfolding conditions: $\Delta H^{\ddagger}$ is 21.5 kcal/mol (1 kcal = 4.2 $\times 10^3$ J) in 3.3 M $HClO_4$ and 21.0 kcal/mol in 5 M guanidine HCl, pH 2.5.

Both acid catalysis of the formation of U_1 and its high activation enthalpy are consistent with the rate-limiting step being *cis-trans* isomerization either of X-Pro imide bonds or of peptide bonds. The rate of the uncatalyzed reaction is in the range expected for proline isomerization and is 0.1% of that of peptide bond isomerization; thus, the simplest explanation for the formation of U_1 is proline isomerization. Earlier data, showing that the kinetic properties of the $U_1 \rightleftarrows U_2$ reaction in refolding conditions differ from those of proline isomerization, can be explained if there is kinetic coupling between early steps in the folding of U_1 and its conversion to U_2.

The existence of two acid-catalyzed reactions that are distinguished by the $HClO_4$ concentration at which catalysis begins suggests that at least two essential proline residues produce slow-folding species of RNase A by isomerization after unfolding. Because protonation of imide bonds is responsible for acid catalysis of proline isomerization, the slower reaction probably involves an imide bond with a low pK. It may be the bond connecting Lys-41 and Pro-42, because the positive charge on Lys-41 could make this bond more difficult to protonate.

It is now well established that RNase A, unfolded either by low pH (1) or by guanidinium chloride (GdmCl) contains a mixture of fast-folding and slow-folding species and that both the fast- and slow-folding reactions produce native RNase (1–6). The equilibrium ratio of these species is not changed by 6 M GdmCl or 8.5 M urea (2), and both the slow-folding species (U_1) and the fast-folding species (U_2) appear to be completely unfolded. Two explanations have been suggested: (*i*) the slow *cis-trans* isomerization of proline residues after unfolding could produce slow-folding species (5); and (*ii*) loop-threading reactions, involving the disulfide-bonded loops of RNase A, could be responsible (7).

If the isomerization of proline residues produces U_1, the reaction should be acid catalyzed (8). Imide bonds, like peptide bonds, are planar and have partial double-bond character. This is responsible for the slow rate of *cis-trans* isomerization and the high activation enthalpy. Although protonation of substituted amides occurs primarily on the carbonyl oxygen, a tautomeric form is also produced in which the nitrogen is protonated. This is responsible for the acid catalysis of *cis-trans* isomerization and of proton exchange (9). Acid catalysis of the isomerization of proline residues has been demonstrated (8) for the interconversion of poly(L-proline) form I (all *cis*) to form II (all *trans*).

In refolding conditions, the kinetic properties of the $U_1 \rightleftarrows U_2$ reaction are complex (7). The rate depends on temperature, pH, and GdmCl concentration in ways unlike those of proline isomerization. However, proline isomerization could still be the rate-limiting step in the slow-folding reaction if the $U_1 \rightleftarrows U_2$ reaction is coupled to the first steps in the folding of U_1 so that the measured rate reflects both processes (7). To avoid this problem, the rate of formation of U_1 has been studied in strongly unfolding conditions. The procedure is to begin with a concentrated solution of native RNase A at pH 7, to dilute it into a concentrated $HClO_4$ solution at 0°, causing unfolding, and then to measure the appearance of slow-folding species in refolding assays.

MATERIALS AND METHODS

Materials. The RNase A was Sigma type XIIA, lot 55C-8250; cytidine 2′,3′ > p was Sigma, lot 64C-7460; GdmCl was Schwarz/Mann ultrapure; cacodylic acid was from Fisher; perchloric acid was Baker reagent grade. The concentration of the perchloric acid was determined by titration with 1 M NaOH (Harleco). The concentration of RNase A was determined by using its absorbance at 278 nm and a molar absorbance of 9.8×10^3 cm^{-1} M^{-1} (10).

Methods. The enzymatic activity of RNase A was assayed on cytidine 2′,3′ > p (11). The kinetics of formation of the slow-folding species of RNase A was measured by a double-jump technique (5, 7) using refolding assays to determine U_1. Unfolding was started by adding 10 µl of a 60 mg/ml solution of RNase A to 90 µl of concentrated $HClO_4$ in a 10-ml glass tube in an ice bath, followed by thorough mixing with the pipette tip. The small volume-to-surface ratio ensured rapid dissipation of the heat evolved by dilution of the $HClO_4$. The amount of U_1 present after different times was assayed by adding 20-µl aliquots to 0.98 ml of 0.4 M Na cacodylate/1.5 M GdmCl at 25° in a spectrophotometer cell. The final pH was 6.3. A Cary 118

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Abbreviations: RNase A, bovine pancreatic ribonuclease A with disulfide bonds intact; GdmCl, guanidinium chloride; U_1 and U_2, slow-folding and fast-folding species, respectively, of RNase A; τ, time constant of a chemical reaction; ϵ, molar absorbance.

spectrophotometer was used, and formation of native RNase A was measured by the change in tyrosine absorbance at 287 nm.

RESULTS

Rate of Formation of U_1 after Unfolding. The rate of formation of U_1 after unfolding was determined by taking aliquots after different time intervals and measuring the amount of U_1 already formed by refolding assays (Fig. 1). Under these conditions (25°, 1.5 M GdmCl), folding of U_1 follows apparent first-order kinetics and the reaction goes to completion in a convenient time range. At temperatures below 25° and in the absence of GdmCl, the folding of U_1 shows biphasic kinetics. The faster reaction has the major amplitude and a low apparent activation enthalpy, as reported (7). The slower reaction has a small amplitude and an apparent activation enthalpy close to 20 kcal/mol. Similar results have been observed independently by J.-R. Garel (personal communication).

$HClO_4$ Catalysis of the Formation of U_1. To search for acid catalysis, $HClO_4$ was chosen for its high acidity in concentrated solutions (12). Figs. 2 and 3 show that the formation of U_1 is not catalyzed at $HClO_4$ concentrations up to 5 M but that strong catalysis is observed between 5 and 10.6 M. There are two acid-catalyzed reactions, for which catalysis begins at 5 M and at 8 M respectively (Fig. 3). The two reactions, which can be seen by inspection of Fig. 2, have been analyzed by "peeling back exponentials." The faster reaction produced two-thirds of the total U_1 formed; the total amount of U_1 is the same, within experimental error, in the range of acid catalysis as in the uncatalyzed reaction. Above 8 M $HClO_4$, the rate of each reaction increased with $HClO_4$ concentration in approximately the same manner. The data give linear plots for the logarithm of the rate constant against the Hammett acidity function. However, a different acidity function is needed, one developed specifically for the reaction of $HClO_4$ with proline imide bonds, before such a plot can be compared with a theoretical one for acid catalysis (12, 13).

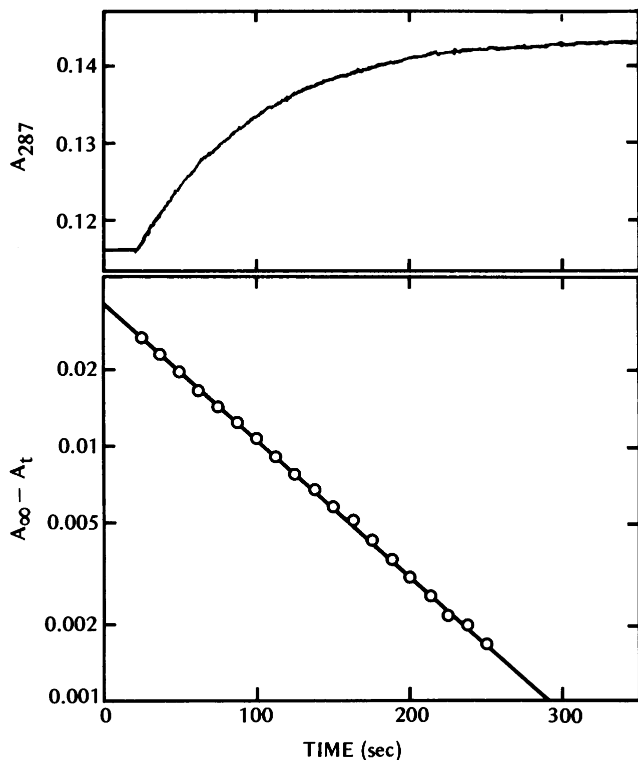

FIG. 1. Representative refolding assay: determination of the amount of slow-refolding species present after 480-sec unfolding at 0° in 6.4 M $HClO_4$; refolding conditions, 0.4 M cacodylate, pH 6.3, 1.5 M GdmCl, 25°, 22 μM RNase A. (*Upper*) Cary 118 chart recording of the refolding reaction. (*Lower*) Semilogarithmic plot of these data, giving $\tau^{-1} = 1.24 \times 10^{-2}\ s^{-1}$ and $\Delta\epsilon = 1630$ (the amplitude of the refolding reaction, expressed as the change in extinction coefficient at 287 nm).

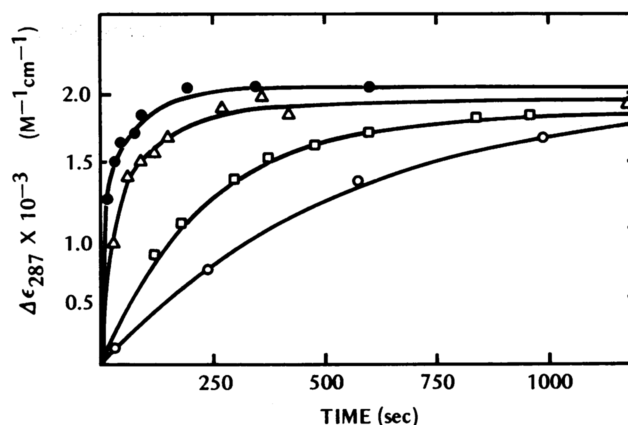

FIG. 2. Catalysis of $HClO_4$ of the formation of the slow-folding species. Each data point was obtained by a refolding assay of the type shown in Fig. 1. At zero time, a solution of native RNase A was diluted into concentrated $HClO_4$ at 0° to give: 3.3 M $HClO_4$ (○), 6.4 M $HClO_4$ (□), 9.0 M $HClO_4$ (△), or 10.6 M $HClO_4$ (●). The presence of two kinetic phases can be seen at 9.0 and 10.6 M.

There is a significant heat of dilution for concentrated $HClO_4$ solutions, and the temperature rise was measured with a thermistor in control experiments. The instantaneous rise was 3.5° at 10.6 M $HClO_4$ and 1.5° at 9 M; it decreased to 0.5° within 20 sec. To find out if the increase in rate shown in Figs. 2 and 3 could be an effect of ClO_4^- ion rather than of acid catalysis, the rate of formation of U_1 in 8.5 M $NaClO_4$ at 0° was measured. No acceleration was found.

Chemical Stability of RNase A in 9.6 M $HClO_4$. Some hydrolysis of chemical bonds may occur in these strongly acidic

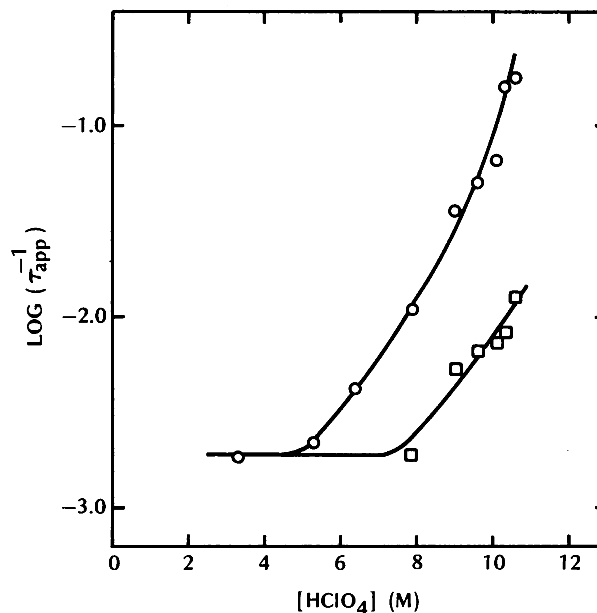

FIG. 3. Dependence on $HClO_4$ concentration, at 0°, of the apparent rate constant (τ^{-1}) for formation of the slow-folding species. In the region where acid catalysis is observed, two reactions are seen and the apparent rate constant (○,□) for each one is given. The kinetic curves of Fig. 2 have been analyzed by "peeling back exponentials"; the faster reaction (○) accounts for two-thirds of the total amplitude.

Table 1. Apparent activation enthalpy of the $U_1 \rightleftarrows U_2$ reaction in RNase A

Conditions	τ^{-1} (apparent rate constant), s^{-1} At 0°	At 18°	$\Delta H^{\ddagger}$, kcal/mol
From unfolding experiments:			
3.3 M $HClO_4$	1.70×10^{-3}	2.10×10^{-2}	21.5
5.0 M GdmCl, pH 2.5	1.15×10^{-3}	1.25×10^{-2}	21.0
From refolding experiments:[a]			
0.2 M GdmCl, pH 6.0	Measured in the temperature		4.8
2.0 M GdmCl, pH 6.0	range 10°–45°		17.4

[a] Data from ref. 7.

solutions; deamidation of glutamine and asparagine residues (14, 15) is the most likely source of damage. The results of exposure of RNase A to 9.6 M $HClO_4$ for 5 min at 0° has been characterized as follows [note that formation of U_1 is complete after 5 min at 9.0 M (Fig. 2)].

Refolding behavior. Data in Fig. 2 show that the amount of U_1 able to refold does not decrease with increasing time of exposure to $HClO_4$ under any of the conditions in Fig. 2. Furthermore, the refolding assays used to determine U_1 show that the folding rate does not change with increasing time of exposure.

Stability to unfolding by GdmCl. Fig. 4 compares the GdmCl-induced unfolding transition of native RNase A with that of a sample exposed to 9.6 M $HClO_4$ for 5 min and then allowed to refold. There are no significant differences between the two curves; the transition midpoints are the same, and the change in tyrosine absorbance across the transition curve is the same.

Recovery of enzymatic activity. With cytidine 2′,3′ > p as the substrate (11), a significant loss of enzymatic activity was observed for the treated sample. In two experiments, the recovery of activity was 80 ± 5%. Probably some deamidation occurred, causing a loss in enzymatic activity without affecting either the ability to refold or the stability of the final folded product.

Activation Enthalpy of the Formation of U_1. A second distinctive property of proline isomerization is its high activation enthalpy: 20 ± 3 kcal/mol in a range of model compounds (5, 16). The $\Delta H^{\ddagger}$ values measured for the formation of U_1 fall within this range. Table 1 shows results for two different sets of unfolding conditions: $\Delta H^{\ddagger}$ = 21.5 kcal/mol in 3.3 M $HClO_4$, which is below the range of acid catalysis, and $\Delta H^{\ddagger}$ = 21.0 kcal/mol in 5 M GdmCl at pH 2.5. The actual rates measured in 3.3 M $HClO_4$ and in 5 M GdmCl were significantly different, suggesting that specific ion effects on the rate of formation of U_1 can be observed. This salt effect is comparable in magnitude to that of 12 M NaSCN on the rate of proline isomerization in acetyl-L-proline (16). When the rate of the $U_1 \rightleftarrows U_2$ reaction was studied in refolding conditions, a complex dependence of rate on pH, temperature, and GdmCl concentration was found (7) (see *Discussion*).

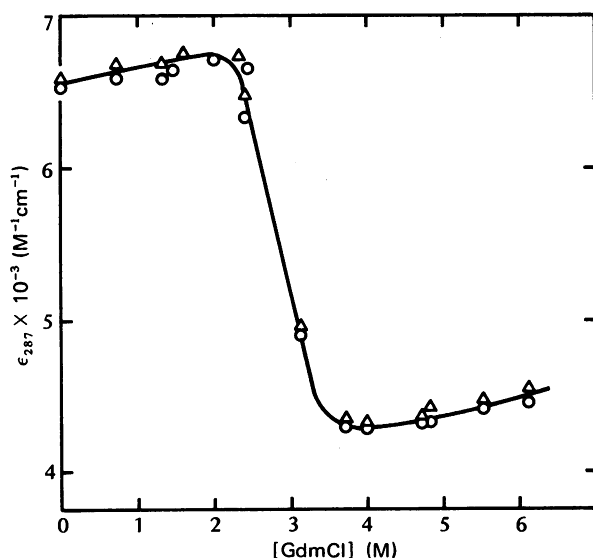

FIG. 4. Comparison of native RNase A (△) with a sample exposed to 9.6 M $HClO_4$ at 0° for 5 min and then allowed to refold (○). The GdmCl-induced unfolding transition curves are shown for 25° and pH 6.3. The buffer was 0.4 M Na cacodylate-cacodylic acid, and the solution also contained 0.2 M $NaClO_4$. RNase A concentration, 18.2 μM. Unfolding was monitored by tyrosine absorbance at 287 nm.

DISCUSSION

Comparison of Properties of Proline Isomerization and of $U_2 \rightleftarrows U_1$ Reaction of RNase A. Table 2 compares the kinetic and thermodynamic properties of proline *cis-trans* isomerization with those of the $U_2 \rightleftarrows U_1$ reaction of RNase A measured in unfolding conditions. In making this comparison, it must be remembered that RNase A has four proline residues and at least two of them appear to be "essential" prolines (see

Table 2. Properties of proline isomerization and of $U_1 \rightleftarrows U_2$ reaction of RNase A[a]

	Proline *trans* ⇄ *cis*	RNase A $U_1 \rightleftarrows U_2$
	Equilibrium properties	
K_{eq}	0.1–1 (5, 16–18)	0.25 (1–3, 6)
K_{eq} = f(pH)?	No[b]	No (2)[c]
K_{eq} = f(GdmCl)?	?	No (2)
ΔH	0–1 kcal/mol (8, 16)	0 kcal/mol (3)
	Kinetic properties	
τ^{d} at 25°	10–100 sec (5, 16)	40 sec (7)
τ = f(pH)?	No[b]	No[e]
τ = f(GdmCl)?	No (7)	No[e]
$\Delta H^{\ddagger}$	20 ± 3 kcal/mol	21 kcal/mol
Acid catalysis	In strong acids (8)	In $HClO_4$ ≥ 5 M

[a] Only those properties of the $U_1 \rightleftarrows U_2$ reaction that have been measured under strongly unfolding conditions (see text) are given. References are given in parentheses.

[b] Special cases in which the proline residue interacts with a neighboring ionized group are excluded.

[c] An apparent pH dependence of the U_1/U_2 ratio (resulting in the disappearance of U_2 at and above pH 5) has been reported (3) for conditions inside the pH-induced unfolding transition at 50° (pH 3–5). Data measured inside the unfolding transition zone are excluded from this table (see text). Later measurements under strongly unfolding conditions (2) (in either 6 M GdmCl or 8.5 M urea) show a normal 4:1 ratio at pH 6.

[d] The apparent rate constant, τ^{-1}, is the sum of both the forward and reverse rate constants for $U_1 \rightleftarrows U_2$ reaction when measured under unfolding conditions (6).

[e] Unpublished data.

following section), whereas the data for proline isomerization refer to the *cis-trans* isomerization of a single residue. The results of earlier investigations of the $U_1 \rightleftarrows U_2$ reaction of RNase A are summarized in Table 2, but the entries are restricted to measurements made in unfolding conditions. Rates of the $U_1 \rightleftarrows U_2$ reaction that have been measured in refolding conditions are excluded because, as suggested earlier (7), they are affected by a kinetic coupling between the $U_1 \rightleftarrows U_2$ reaction and early steps in the folding of U_1. A possible mechanism has been given to show how this can explain the low apparent activation enthalpy for the $U_1 \rightleftarrows U_2$ reaction observed in refolding conditions (7). Equilibrium data for the ratio U_1/U_2 are included in Table 2 only for conditions of complete unfolding. Data taken inside the unfolding transition zone are excluded because partially structured intermediate species may participate in the overall equilibrium.

Some well-known properties of proline isomerization, included in Table 2, are: an equilibrium constant (*trans* → *cis*) whose order of magnitude is unity and which is nearly independent of temperature, and a reaction rate whose time constant at 25° is in the range of 10–100 sec and which shows both a high activation enthalpy and catalysis by strong acids. All of these properties have now been found for the $U_2 \rightleftarrows U_1$ reaction of RNase A. Special properties of proline isomerization that are dependent on the neighboring amino acid residues are not given in Table 2. These include a dependence of the isomerization rate on the bulkiness of neighboring residues (5) and a dependence of the *cis-trans* ratio not only on interactions with neighboring residues (17) but also on conformational equilibria involving an entire oligopeptide (18). It will be important to learn how the kinetic and equilibrium properties of proline isomerization change in the presence of denaturants such as GdmCl and NaSCN because these agents can be used to investigate the coupling between early steps in folding and the $U_1 \rightleftarrows U_2$ reaction. A few results indicate that GdmCl (7) and NaSCN (16) have only small effects on the rate of proline isomerization, whereas denaturing salts have striking effects on the rate of the $U_1 \rightleftarrows U_2$ reaction in refolding conditions (7, 19).

Revision of One of the Postulates of the Proline Isomerism Model. A specific model has been given to show how proline isomerization might produce the slow-folding form of RNase A (5). It has three basic postulates. (*i*) In a native protein, each proline residue has a unique *cis* or *trans* conformation. (*ii*) After the unfolding, isomerization occurs to give a mixture of the *cis* and *trans* forms. (*iii*) Before any folding can occur, each proline residue must have the same *cis* or *trans* conformation as in the native protein.

Because the model is both simple and explicit, it makes predictions that are easily tested experimentally. For example, it predicts that the kinetic properties of the $U_1 \rightleftarrows U_2$ reaction are the same in refolding as in unfolding conditions. This prediction has been tested (see Table 1) and found not to be valid for RNase A (7). By changing postulate *iii*, the model can be modified to fit these data for RNase A.

Revised postulate *iii* would be: Before folding can *go to completion to give the native structure*, each *essential* proline residue must have the unique *cis* or *trans* conformation characteristic of the native structure. In this form, postulate *iii* allows some steps in folding to occur before the isomerization of an essential proline residue takes place. The distinction between essential and nonessential proline residues is made because, as H. W. Wyckoff pointed out (personal communication), the x-ray structure of RNase S suggests that certain "permissive" proline residues may be accommodated in either the *cis* or *trans* conformation in the native structure.

Other Tests for Proline Isomerization as a Rate-Limiting Step in Folding. (*i*) A fruitful approach has been to compare the folding kinetics of two homologous proteins, one with and one without a proline residue (20, 21). The carp parvalbumins provide such a system; one without a proline residue fails to show any slow phase in folding (20) whereas a related parvalbumin with one proline residue does show a slow-folding reaction (21), indicating that isomerization of the proline residue is responsible for the slow reaction.

(*ii*) Direct measurement of the isomerization of proline residues in a protein during folding and unfolding should be possible by use of reporter groups attached to the side chains of neighboring residues.

(*iii*) If the original postulates of the proline isomerism model (5) were valid, it would be possible to determine whether a slow reaction in folding is caused by proline isomerization simply by comparing the kinetic properties of proline isomerization with those of the slow-folding reaction, when folding takes place outside the folding transition zone. As discussed above, this approach has been found not to be valid for RNase A, and postulate *iii* of the proline model has been revised.

(*iv*) The conditions used here to test for acid catalysis of the formation of slow-folding species of RNase A should be generally applicable to other monomeric proteins if they are sufficiently stable and soluble in $HClO_4$ solutions, because the rates of proline isomerization should be roughly comparable in different proteins.

Nature of the Two Acid-Catalyzed Reactions. The observation of two well-separated reactions in the acid-catalyzed range (Fig. 3) indicates that there are at least two essential proline residues in RNase A and that they have different pKs for protonation. The slower reaction, with the lower pK, may be isomerization of the imide bond connecting Lys-41 to Pro-42. The positive charge on Lys-41 may make this bond more difficult to protonate. Such an effect has been observed in a study of the acid catalysis of proton exchange in model peptides (22).

Dr. J.-R. Garel sent us his results of a study probing the nature of the $U_1 \rightleftarrows U_2$ reaction of RNase A by a different approach, and we are grateful for his discussion. F.X.S. acknowledges a fellowship from the Deutsche Forschungsgemeinschaft. This research has been supported by grants from the National Science Foundation (PCM 77-16834) and the National Institutes of Health (2 R01 GM 19988-18).

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