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# Effect of Protein Conformation on Rate of Deamidation: Ribonuclease A

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***ABSTRACT*** **The effect of the folded conformation of a protein on the rate of deamidation of a specific asparaginyl residue has been determined. Native and unfolded ribonuclease A (RNase A) could be compared under identical conditions, because stable unfolded protein was generated by breaking irreversibly the protein disulfide bonds.**

**Deamidation of the labile Asn-67 residue of RNase A was followed electrophoretically and chromatographically. At 80°C, similar rates of deamidation were observed for the disulfide-bonded form, which is thermally unfolded, and the reduced form. At 37°C and pH 8, however, the rate of deamidation of native RNase A was negligible, and was more than 30-fold slower than that of reduced, unfolded RNase A. This demonstrates that the Asn-67 residue is located in a local conformation in the native protein that greatly inhibits deamidation. This conformation is the β-turn of residues 66–68.**

**Key words: ribonuclease A, protein deamidation, protein conformation, disulfide bonds**

## INTRODUCTION

Protein engineering has become a powerful experimental tool in the study of recombinant DNA-derived proteins, permitting the modification of polypeptide molecules at discrete residues by site-directed mutagenesis. One use of this technology is to improve the stabilities of enzymes, which would increase their medical and industrial usefulness. In the case of nonenzymic protein deamidation[1,2] enzyme stabilization is straightforward: thermolabile asparaginyl and glutaminyl residues may be substituted with thermostable amino acids.[3,4]

For rational design of improved proteins, the parameters determining the rate of deamidation of individual glutaminyl and asparaginyl residues must be considered, so that only the most labile are removed. Previous studies of protein deamidation have been unable to quantify the relative contributions of primary sequence and higher orders of structure.[5–8] The effect of primary structure has been demonstrated using small synthetic peptides,[9,10] where small or potentially reactive amino acids adjacent to labile residues were found to lead to increased rates of deamidation. The most labile Asn residues are those followed by a Gly residue.[11]

No such correlation was found in a recent study[12] in which neutron diffraction was used to identify which of the 13 asparaginyl residues in trypsin were deamidated in the folded protein at pH 7. The residues that deamidated were characterized by distinct local conformations and hydrogen-bonding structures, and the folded conformation was concluded to be predominant in determining the site of modification.

Experimental determination of the effect of tertiary structure on the rate of protein deamidation could be made by comparing the rates in native, folded protein and in unfolded protein. Such a comparison, however, requires that the native and unfolded species be studied under the same conditions, which normally is impossible. This precondition may be satisfied where a protein requires disulfide bonds for retention of its native structure, for unfolding will occur upon disulfide bond reduction and irreversible blockage of the thiol groups.[13]

During a study of disulfide formation and refolding of reduced RNase A, a more acidic species was found to be generated from the unfolded protein under mild conditions.[14] The rate of this modification seemed too rapid to be due to a spontaneous event, but it was subsequently demonstrated by Thannhauser and Scheraga[15] to be deamidation of Asn-67. At alkaline pH values, Asn-67 is deamidated much more rapidly than the remaining nine asparaginyl residues, due to the adjacent Gly residue.[11]

The high rate of deamidation of Asn-67 in reduced, unfolded RNase A contrasts with an apparently much lower rate in native RNase A, which is an exceptionally stable protein.[16] By electrophoresis, we could detect no deamidated form in native RNase A that had been stored for nearly 9 years in 0.1 M phosphate buffer (pH 6.5) in the cold room. Therefore, it is apparent that both the primary se-

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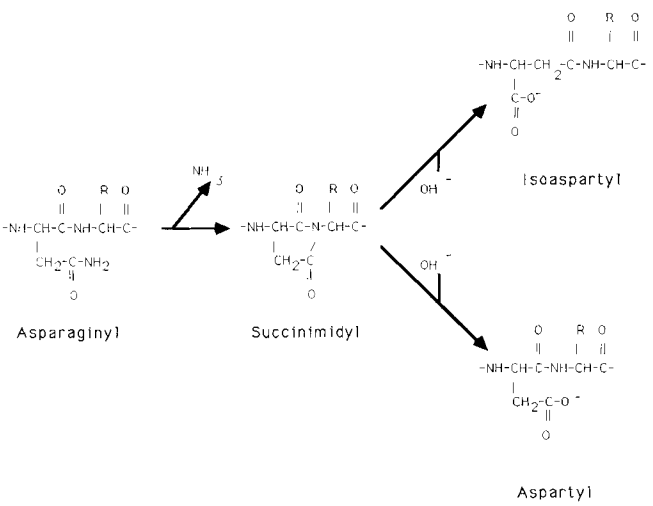


Fig. 1. Mechanism of deamidation of RNase A by spontaneous formation of the succinimide ring and subsequent hydrolysis.

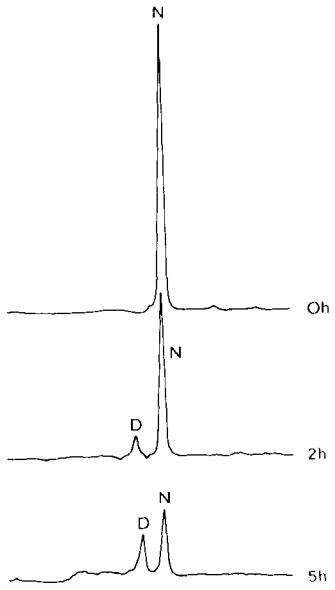


Fig. 2. Time course of deamidation of native RNase A, analysed by cation exchange chromatography[15] of fractions taken after the indicated periods of incubation at 60°C, pH 7.5. N indicates nondeamidated RNase A, D indicates deamidated RNase A. A small fraction of the eluted protein (less than 15% of the total) did not bind to the column; this fraction increased to 25% of the total on prolonged incubation.

quence and the tertiary structure determine the rate of deamidation of RNase A, but their relative contributions are not known.

The availability of both folded and unfolded, reduced (Rcam) forms of the protein, and the presence of a single Asn residue that deamidates rapidly, made RNase A an attractive model protein with which to quantify the effect of the folded conformation on the rate of deamidation. The rates of deamidation of native and of reduced, unfolded RNase A are compared under identical conditions of pH and temperature.

## MATERIALS AND METHODS

### Materials

The RNase A used in this study was of chromatographically pure grade from Miles Research Laboratories. The native protein was used without further purification. For the production of Rcam-RNase A, the native protein was reduced as described previously.[13] Iodoacetamide [in 0.5 M Tris–HCl (pH 6.8)] was added to the reduced protein to a final concentration of 0.5 M. After 2 minutes at room temperature, Rcam-RNase A was isolated by gel filtration on a Sephadex G-25 column (20 × 2 cm) at 4°C; the column was equilibrated and eluted with 0.01 M HCl.

### Methods

To initiate deamidation, freshly prepared solutions of RNase A (either native or Rcam) were diluted to a final concentration of 30 μM in 0.1 M Tris–HCl at pH 8, 9, and 10 (the actual pH was measured at the temperature of incubation). After various periods of incubation at 37°, 60°, and 80°C, aliquots were removed for analysis by either cation exchange chromatography or gel electrophoresis.

Cation exchange chromatography was performed on a Mono-S 5/5 column as described by Thannhauser and Scheraga[15]; 250 μl aliquots taken from the deamidation reactions were added to 750 μl of 25 mM sodium phosphate buffer (pH 6.5), then applied to the column. The elution profile was followed by monitoring the absorbance at 280 nm. In addition, eluted fractions of initially native samples were assayed for RNase A activity.[17]

Low-pH discontinuous polyacrylamide gel electrophoresis[18] was performed on native and Rcam-RNase A as described previously.[13,14] For measurements of the extent of deamidation of initially native RNase A, aliquots were reduced prior to electrophoresis by addition of dithiothreitol to a final concentration of 0.1 M, and urea to saturation; after incubation for 15 minutes at 37°C, an equal volume of 1.0 M iodoacetamide [in 0.5 M Tris–HCl (pH 6.8)] was added and allowed to react at room temperature for 2 minutes. The Coomassie Blue-stained gels were subjected to densitometry.

The relative amounts of original and deamidated species were determined by integration of the absorbance traces of the cation exchange column eluate, and of the densitometer scans.

## RESULTS AND DISCUSSION

Deamidation of Asn residues that precede Gly residues in the primary structure are believed to occur via a cyclic imide intermediate that is favored by the

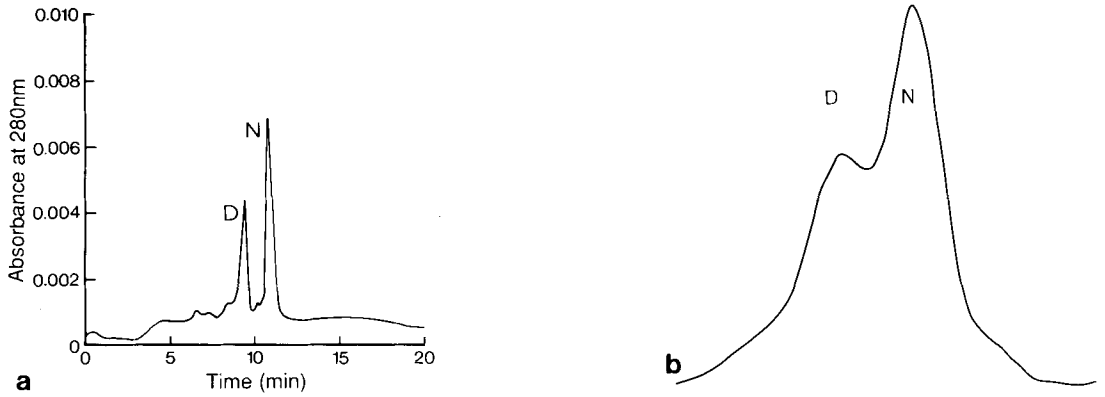


Fig. 3. Deamidation of native RNase A determined chromatographically (**a**) and electrophoretically (**b**). Native RNase A incubated at 80°C, pH 7.2 for 1.5 hours; N indicates nondeamidated RNase A, D indicates deamidated RNase A. The enzymic activity of the deamidated species was comparable to that of the native species, concurring with previous reports of monodeamidated RNase A having greater than 90% the enzymic activity of the native protein.[15,28]

absence of a side chain on the Gly residue (Fig. 1).[11,19] The imide ring is hydrolyzed on either side of the nitrogen atom, to yield either the Asp or iso-Asp residues.

Deamidation of Asn-67 of RNase A was followed in this study by either electrophoresis or ion-exchange chromatography, on the basis of the additional negative charge of the final products. Therefore, the Asp and iso-Asp forms of RNase A should be separated from the original Asn and the imide intermediate, but without distinguishing between either of the pairs with the same net charge. Representative results monitored by cation exchange chromatography are illustrated in Figure 2. Very similar results were obtained with both techniques whenever they were compared (Fig. 3).

No deamidation of native RNase A was detected after 96 hours at 37°C and pH 7.9, whereas the Rcam form deamidated with a rate of 0.030/hour (Fig. 4a). The methods used were found to detect readily as little as 5% deamidation; therefore, the rate of deamidation of native RNase A under these conditions must be less than $10^{-4}$/hour (Table I). This is consistent with the known stability of RNase A.[16] At higher pH values, some deamidation of native RNase A was apparent, but only after more than 48 hours at 37°C (Fig. 4a). The Rcam form also deamidated more rapidly at higher pH.

At higher temperatures, the rates of deamidation of both the native and Rcam forms increased (Fig. 4b,c). The temperature dependence of the Rcam form, at constant pH 7.2, indicated an activation energy of 15.2 kcal/mol, somewhat less than the value of 21.2 kcal/mol estimated in model peptides.[20] The temperature dependence of the native protein was even greater, undoubtedly due to the additional contribution of thermal unfolding of the protein. The midpoint of thermal unfolding at these pH values is in the region of 60°C.[21,22] At 60°C in these experiments, the rate of deamidation of the disulfide-bonded protein was approximately 10% that of the Rcam form (Table I), suggesting that the protein was unfolded about 10% of the time. The protein should be fully unfolded at 80°C, and the rate of deamidation was found to be 80% that of the Rcam form. The slightly lower rate in the disulfide bonded form may result from sterochemical constraints on Asn-67 imposed by the Cys-65–Cys-72 disulfide bond.

The rates of deamidation of Rcam-RNase A at different temperatures had similar pH dependence, as did the native protein at 60°C (Fig. 5), consistent with deamidation occurring by the same process in each case. The pH dependence and the rates of deamidation were consistent with those reported by others in octa-*S*-sulforibonuclease[15] and in model peptides.[20] For example, Geiger and Clarke reported a half-time of 1.4 days at 37°C, pH 7.4 for the peptide Val-Tyr-Pro-Asn-Gly-Ala, whereas the value for Rcam RNase A, with the local sequence -Ala-Rcam-Cys-Lys-Asn-Gly-Gln- was found to be 1.2 days under the same conditions (Fig. 5).

Under all conditions, a single major deamidated species was observed, even when the deamidated species predominated. In these cases, it is evident that deamidation was limited to the labile Asn-67. Only in the case of the native protein at 37°C, where deamidation was very slow or negligible, was it possible that other Asn or Gln residues were also deamidated. Disulfide rearrangements[23,24] were apparent in native RNase A only after more than 8 hours incubation at 80°C; electrophoresis of the protein demonstrated more slowly migrating species that disappeared when the disulfide bonds were reduced and electrophoresis was of the Rcam form.

The kinetics of deamidation were normally of apparent first order (Fig. 4). Studies of deamidation of small model peptides have observed the initial formation of the imide intermediate (Fig. 1) to be rate

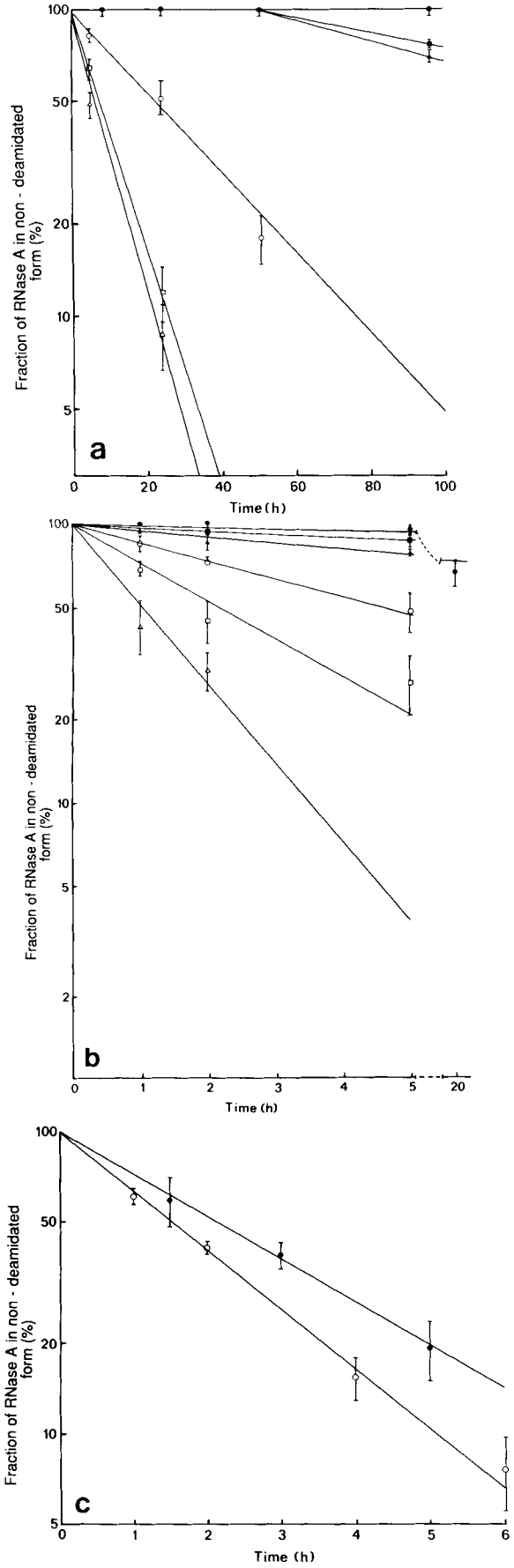


**TABLE I. A Comparison of the Rates of Deamidation (*k*) of Native RNase A (N) and Rcam-RNase A (R) Under a Range of Conditions of Temperature and pH**

| Temperature (°C) | pH* | $k$ ($h^{-1}$) Initial RNase A species N | R | Ratio† $k$ (R):$k$ (N) |
|---|---|---|---|---|
| 37 | 7.9 | $<1\times10^{-4}$ | 0.030 | >30 |
| | 8.9 | Biphasic | 0.087 | — |
| | 9.6 | Biphasic | 0.11 | — |
| 60 | 7.5 | 0.016 | 0.15 | 9.3 |
| | 8.4 | 0.029 | 0.33 | 11 |
| | 9.0 | 0.053 | 0.69 | 13 |
| 80 | 7.2 | 0.35 | 0.46 | 1.3 |

*pH measured at indicated temperatures.
†As the rates of deamidation of N and R were measured under identical conditions, this ratio is a measure of the degree of inhibition of deamidation by any local secondary and tertiary structure which may exist. Unity indicates a rate of deamidation independent of any local conformational factors in the native structure.

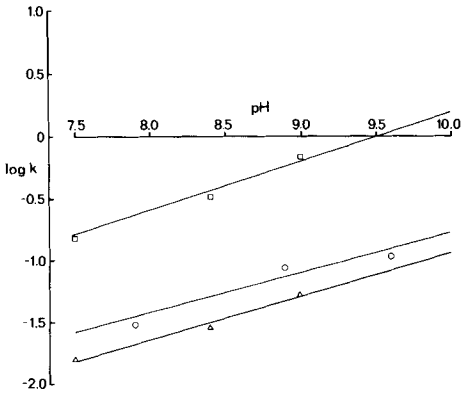


Fig. 5. Dependence of rate of deamidation (k) on pH. Rates of deamidation are calculated from Figure 4 for Rcam-RNase A at 37°C (○) and 60°C (□), and for native, nonreduced RNase A at 60°C (△).

limiting.[20] This is also likely to be the case with the RNase A deamidation studied here. Complex kinetics were observed with the native protein at 37°C, however, where molecules with an additional negative charge appeared only after a lag phase of more than 48 hours (Fig. 4a). One possibility is that the imide intermediate is accumulating during the lag

Fig. 4. The kinetics of deamidation of RNase A. The extent of deamidation was analyzed by chromatography and electrophoresis after various periods of incubation under conditions of temperature and pH given below. Open symbols represent initially native, nonreduced RNase A; closed symbols represent Rcam-RNase A. Error bars represent the standard deviation of two or three estimates of degree of deamidation by electrophoretic analysis and one estimate by chromatographic analysis. **a:** Incubation at 37°C; pH 7.9 (○,●), pH 8.9 (□,■), pH 9.6 (△,▲). **b:** Incubation at 60°C; pH 7.5 (○,●), pH 8.4 (□,■), pH 9.0 (△,▲). **c:** Incubation at 80°C; pH 7.2.

period; but this would imply that the native conformation has inhibited primarily the rate of the second step, hydrolysis of the imide, which seems unlikely. An alternative explanation is that some other covalent modification must precede deamidation of the folded protein.

The native conformation of RNase $A^{25-27}$ has clearly inhibited deamidation of Asn-67 at least 30-fold (Fig. 4a). This residue is on the surface of the protein and the side chain is mobile in the solvent. Therefore, the conformation has not acted simply by restricting access of water or hydroxide ion to the side chain. It is most likely that the backbone has been prevented from attaining the conformation necessary for the imide intermediate; this conformation is normally not populated significantly, due to steric constraints, but it obviously can be adopted transiently by a flexible peptide or an unfolded polypeptide chain.[2] In the case of Asn-67 of RNase A, attaining the imide structure would require that the backbone tortional angle psi be rotated by 125°.[2] Apparently, such rotations are substantially hindered by the local conformation of Asn-67, even though it is in a relatively flexible part of the molecule.[27] This local conformation is the β-turn of residues 66 to 68, rigidified by hydrogen bonding, and further restricted in flexibility by the Cys-65–Cys-72 disulfide bond.[25,26]

The local conformation has inhibited deamidation of this particular residue, but this need not always be the case. This demonstration of the importance of three-dimensional conformation for determining the rate of deamidation implies that the rate could vary widely with other residues, but would not be determined solely by the primary structure. This could be determined experimentally with other proteins by comparing the rates in native and irreversibly unfolded proteins.

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