Selective Deamidation of Ribonuclease A

Selective deamidation of proteins and peptides is a reaction of great interest, whether it has physiological significance as in protein aging, or occurs as a disturbing event in the preparation of natural or recombinant proteins. Deamidation of bovine pancreatic ribonuclease A, RNase A, a classical model protein, has been reported to occur only after denaturation of the protein, or under harsh conditions. In this paper convenient procedures are described for selective deamidation of Asn\(^{\text{67}}\) in native RNase A under mild conditions. Furthermore, for the first time, both products of deamidation were isolated: the aspartyl and the isoaspartyl containing protein derivatives. Replacement of Asn\(^{\text{67}}\) with either residue lowers the catalytic activity of the enzyme, on RNA and on model substrates, except when a dinucleotide with a purine on the 5' side is the substrate. In the latter case an intriguing increase in the specificity constant is observed. The Asp\(^{\text{67}}\) derivative was found to refold, after full denaturation and reduction, at the same rate as the fully amidated protein, whereas the iso-Asp\(^{\text{67}}\) derivative refolded at half that rate. It is hypothesized that this effect is due to a delayed formation of disulfide 65-72 for the presence of the abnormal isopeptide bond between residues 67 and 68.

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knowledge, the two protein derivatives produced by the deamidation reaction were isolated: one containing an isopeptide, the other a normal peptide bond, linking to Gly the aspartyl produced through deamidation. The main catalytic properties and the refolding rates of these derivatives were investigated and compared with those of the native fully amidated protein.

**EXPERIMENTAL PROCEDURES**

**Materials**—Substrates (yeast RNA, cCMP, CpC, UpA, and UpC) and RNase A, type XII-A, were from Sigma. RNase A was purified to homogeneity by chromatography on carboxymethylcellulose as described previously (Goren and Barnard, 1970). Trypsin was sequencing grade from Boehringer. Fully reduced and carboxymethylated RNase A was prepared as described for seminal RNase A (Di Donato et al., 1986).

**Chromatographic Separations**—Peptides were separated by RP-HPLC on Ultrasphere ODS columns (Beckman). Elution of peptides was achieved with gradients of acetonitrile in 0.1% trifluoroacetic acid. The column was equilibrated and loaded in 5% acetonitrile, 0.1% trifluoroacetic acid; the concentration of acetonitrile was then raised to 12.6% in 23 min, kept constant for 5 min, raised again to 15% in 7 min, kept constant for 10 min, and then raised to 38% in 70 min.

Ion-exchange separations of proteins were carried out with a fast protein liquid chromatography apparatus (Pharmacia), monitoring the effluent with a Uvicord monitor (Pharmacia) at 206 or 280 nm. Chromatographic separations were carried out on Mono S column as follows. The column was equilibrated in a mixture of 10% buffer B (0.05 M Tris-Cl, pH 7.2, containing 0.3 M NaCl) and 90% buffer A (0.05 M Tris-Cl, pH 7.2). Elution was started 3 min after loading by raising the concentration of buffer B to 70% in 20 min. The flow rate was 1 ml/min.

Hydrophobic chromatography was carried out on a Spherigel HIC-CAА column (Beckman) equilibrated in 70% buffer A (3 M ammonium sulfate, 0.5 M ammonium acetate, pH 6.0) and 30% buffer B (0.5 M ammonium acetate, pH 6.0). Elution was carried out by increasing the concentration of buffer B from 30 to 70% in 10 min, at a flow rate of 1 ml/min.

**Sequence and Amino Acid Analyses**—Sequence determinations were performed on an Applied Biosystems Sequencer model 473A, connected on-line with an HPLC for identification of phenylthiohydantoin derivatives. Amino acid analyses were performed after hydrolysis of peptides in HCl (vapor phase, 20 h at 110 °C), on a Beckman System Gold equipped with a Spherogel cation-exchange column (Beckman) and a ninhydrin detection system with a coil temperature of 130 °C.

**Enzymatic Assays**—Activity on RNA was assayed by the method of Kunitz (1946). Kinetic measurements on cCMP and on CpC were performed by spectrophotometric methods as described previously (Goren and Barnard, 1970). Trypsin was sequencing grade from Boehringer. Fully reduced and carboxymethylated RNase A was prepared as described for seminal RNase A (Di Donato et al., 1986).

**RESULTS**

**Preparation of Selectively Deamidated RNase A**—RNase A (1–5 mg/ml) was incubated in 1% ammonium bicarbonate, pH 8.2, at 37 °C. At appropriate time intervals, aliquots of protein were withdrawn, taken to dryness, resuspended into 50 µl of column equilibrium buffer, and subjected to cation-exchange chromatography on a Mono S column as described under "Experimental Procedures." A chromatogram of the reaction products after 360 h of incubation is shown in Fig. 1. Fraction C, eluted with the same retention time as native RNase A, and fraction B, more anionic than native RNase A, were tentatively identified respectively as unreacted protein and deamidated RNase A (d-RNase A). The material in peak A, which did not bind to the column, was found to be a complex mixture of protein species, resolved by anion-exchange chromatography on a Mono Q column (data not shown), presumably multiply deamidated forms of RNase A.

Under the conditions described above, more than 60% of native protein was transformed into d-RNase A in 360 h, with about 15% of the protein eluting with peak A. The kinetic rate of d-RNase production was calculated assuming first-order kinetics (see inset of Fig. 1), and assuming that the production of multiply deamidated forms (fraction A) did not affect the kinetics. The value of \( k \) was \( 5.4 \times 10^{-3} \) h⁻¹, with a \( t_{1/2} \) of 178 h.

**Identification of the Deamidation Site in RNase A**—The nature of d-RNase A was investigated by comparing tryptic digests of the native and of unreacted RNase A. Trypsin digestion was carried out on denatured, fully reduced, and carboxymethylated proteins at a 1/100 (w/w) ratio of trypsin (Supelchem). Internal standards were included in each run and consisted of \([^{1}H]\text{Ala} \) and \([^{14}C]\text{Leu} \).

**Computational Procedures**—Kinetic parameters for the enzymatic measurements and for the refolding experiments, as well as standard deviations were calculated using the computer program "Regression" (Blackwell Scientific Software, Oxford, United Kingdom) based on a nonlinear regression method (Marquardt, 1963).

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**Fig. 1.** Ion-exchange separation on a Mono S column of RNase A. Ashed 1% ammonium bicarbonate, pH 8.2. The arrow indicates the elution volume of untreated RNase A. In the inset is a first-order plot of the formation of fraction B.
to protein in 1% ammonium bicarbonate, pH 8.2. After 1 h at 37 °C, an equal amount of trypsin was added, and the incubation continued for 1 h. The two peptide maps obtained by RP-HPLC are illustrated in Fig. 2. The peaks in the two patterns are numbered according to their elution order and given the prefix “A” or “d” according to the source protein, RNase A and d-RNase A, respectively. The two chromatograms are very similar and show well resolved peaks except for the material not retained by the column. This material was resolved into discrete peptide peaks by re-chromatography with a more shallow gradient (data not shown).

After amino acid analyses, all the peptide fragments separated from the tryptic digest of native RNase A were satisfactorily accommodated in the known sequence of the protein (Spackman et al., 1960).

As for the peptide map obtained from d-RNase A, each peptide fragment was found to coelute with a corresponding tryptic fragment of RNase A, with a single exception, that of peptide d12*, absent in the peptide map from RNase A. By amino acid analyses, coeluting peptides in the maps from RNase A and d-RNase A were found to have identical amino acid compositions. This also allowed us to calculate peptide yields after normalization of the content of each peptide to two reference peptides: A8 and d8, for RNase A and d-RNase A, respectively. This revealed that the peptides from d-RNase A were produced in the same proportions as the corresponding peptides from RNase A, with two very noticeable exceptions: those of peptides d6 and d12, found to be present in lower amounts than peptides A6 and A12 from untreated RNase A (see Fig. 3).

Peptides A6 and d6 had the composition of segment 62–66 of RNase A, and peptides A12 and d12 that of segment 67–85, following in the protein sequence (see Fig. 3). As for peptide d12*, its amino acid composition was found to coincide with that of the sequence segment 62–85 of RNase A, i.e. the segment spanning the sequence of both peptides d6 and d12. Sequence analyses by automatic Edman degradation confirmed the assignments but revealed that (i) while at position 67 of RNase A (position 1 of peptide A12) Asn was present as expected at the corresponding position of d12 (from d-RNase A) asp substituted for Asn; (ii) Edman degradation could not be carried out in d12* beyond Lys69 (see Fig. 3).

It should be underlined that all the tryptic peptides obtained for the sequence segments 1–61 and 86–124 of RNase A were indistinguishable on the basis of their amino acid compositions and elution volumes on the RP-HPLC column, from the corresponding peptides from untreated RNase A. Thus they could be unequivocally accommodated in the known sequence of RNase A. This clearly indicated that the only region affected by the deamidation event was that comprised between residue 62 and 85.

These results led to the following main conclusions. (i) d-RNase was the product of a single deamidation event, occurring at position 67 of RNase A; (ii) for a fraction of d-RNase A, at position 67 (position 6 of peptide d12*) an isoaspartyl residue was present. The latter conclusion was strongly suggested by the evidence that the peptide bond linking Lys69 to the residue following in sequence (an aspartyl residue in the amino acid composition) was resistant under the conditions employed to tryptic hydrolysis as well as completely resistant to Edman degradation.

**Isolation of Isoaspartyl 67 and Aspartyl 67 Derivatives of RNase A** — The results reported above suggested that d-RNase A was not a homogeneous protein derivative, rather, that it was a mixture of two derivatives produced by deamidation at position 67 of RNase A: one derivative with Asp and the other with iso-Asp replacing the original Asn.

The separation of the two isomers could be accomplished...
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by hydrophobic chromatography on a Spherogel HIC-CAA column (see "Experimental Procedures"). As shown in Fig. 4, d-RNase A was separated into two components, denoted as d1-RNase A and d2-RNase A, found in a ratio of 3:2.

For the identification of these components, the experimental approach described above for identifying d-RNase A was followed. Fig. 5 shows that the chromatographic profiles of the tryptic digests of d1- and d2-RNase A are largely superimposable, and superimposable on those of untreated RNase A and of d-RNase A (cf. Fig. 2), with a few significant exceptions. Namely, d1-RNase A contains almost exclusively peptide d12*, with low amounts of peptides d6 and d12 (17 and 19%, respectively, relative to the content of peptide d8). On the other hand, d2-RNase A contains peptides d6 and d12 (97 and 78%, respectively), whereas peptide d12* is less than 10%.

Amino acid analyses of the peptides separated in the peptide maps of d1-RNase A and d2-RNase A revealed that coeluting peptides in the chromatographic patterns of the tryptic digests of RNase A, d-RNase A, d1-RNase A, and d2-RNase A had in fact identical amino acid compositions.

Sequence analyses of peptides d6, d12, and d12* from d1- and d2-RNase A confirmed the sequence identifications reported above for these peptides as isolated from the map of d-RNase A (see Fig. 3). They also confirmed that peptide d12 contained an aspartic acid residue at position 67 and that peptide d12* was resistant beyond Lys67 to Edman degradation.

Conclusive evidence for the presence of an isopeptide linkage in peptide d12* was secured by the procedure employed previously by Di Donato et al. (1986) for the identification of iso-Asp in deamidated BS-RNase, based on the method proposed by Matsuo and Narita (1975) for the determination of COOH-terminal groups in proteins and peptides. Iso-Asp residues are readily detectable by this method, as they possess an a-COOH group just like COOH-terminal residues. Acid hydrolysates (see "Experimental Procedures") of peptide d12* and peptide d12, previously reacted with acetic anhydride in the presence of [3H]H2O, and [3H]Ala and [14C]Leu added as internal standards, were analyzed for their contents of radioactive amino acids. The results (data not shown) indicated that the only radioactive, i.e. a-COOH-containing, amino acid found for peptide d12 was Arg, the peptide COOH-terminal residue. For peptide d12*, however, two amino acids with a-COOH groups were detected: the COOH-terminal Arg and Asp, indicating that the latter residue possessed a free a-COOH function and hence was linked through an isopeptide bond to the residue following in sequence.

This conclusively identified d1-RNase and d2-RNase A as the expected products of deamidation of RNase A: the former with iso-Asp at position 67 (N67D-RNase A), the latter with Asp (N67D-RNase A).

Catalytic Properties of Native and Deamidated RNase A—Fig. 6 shows the saturation curves obtained for native RNase A, and the selectively deamidated derivatives N67D- and N67D-RNase A, with cCMP as a substrate for the hydrolytic step of the reaction. Surprisingly, the substitution of Asp for Asn provided RNase A with a significantly higher catalytic activity.
rate. In Table I are listed the values of $K_{on}$ and $K_{off}$ calculated for the substrate concentration range 0-10 mM for the native enzyme and for the deamidated derivatives. In fact, higher substrate concentrations were found to modulate the activity of both derivatives, as has been described for the native enzyme (Walker et al., 1975).

The first transphosphorylase step of the reaction was investigated with dinucleotide substrates. The results are summarized in Table I.

Refolding of Unfolded N67D- and N67D-RNase A—Asn$^67$ is in a sequence segment of special importance in the refolding of RNase A (Némethy and Scheraga, 1979; Montelione and Scheraga, 1989). This consideration led Thannhauser and Scheraga (1989) to suggest an experiment in which the refolding of fully amidated RNase A was compared with the refolding of the RNase derivatives containing Asp or iso-Asp, respectively, substituting for Asn$^67$.

The results of this experiment are illustrated in Fig. 7. Refolding of RNase A and N67D- and N67D-RNase A was followed by measuring with the Kunitz assay (Kunitz, 1946), the percent of renaturation taken as 100% the maximal activity of each enzyme protein. It should be noted that by this model for deamidation studies has been limited, apparently because of the difficulty in defining suitably mild conditions for selective deamidation of the native protein (Wright, 1991a, 1991b). In previous investigations, carried out under rather harsh conditions (Venkatesh and Vithayatil, 1984), mono- and polydeamidated derivatives were simultaneously obtained, which could only be partially characterized. On the other hand, denatured RNase A was readily and selectively deamidated (Thannhauser and Scheraga, 1986; Wearne and Creighton, 1989). These results led to speculation (Wright, 1991a, 1991b) that structural constraints, inherent in the native conformation, hindered selective deamidation of RNase A, so that this could occur only under harsh conditions or in the unfolded protein (Wearne and Creighton, 1989). Furthermore, there has been a general consensus, following the pioneering work by Bornstein and Balian (1970) on hydroxylamine cleavage of RNase A, for a mechanism of deamidation based on the formation of a cyclic succinimide (from Asn$^67$ attacked at its $\alpha$-carbonyl by the NH of Gly$^68$). When this imide hydrolyzes, two derivatives are produced; one with an Asp and the other with an iso-Asp replacing the Asn.

However, deamidated RNase A containing an iso-Asp residue has never been isolated. In this report, convenient methods are described for the selective deamidation of native RNase A and for the separation of the two products of deamidation: one containing Asp and the other iso-Asp, respectively, substituting for the deamidated Asn. This has been positively identified as Asn$^67$, and the presence of iso-Asp in one of the deamidated products has been determined by chemical methods. It should be noted that a ratio of 3:2 was found between the iso-Asp and the Asp-containing products of deamidation of RNase A, within the range of ratios previously found for peptides and proteins (Geiger and Clarke, 1987; Johnson et al., 1988; Artigues et al., 1990).

Ass$^67$ is part of an exposed loop of eight residues, cross-linked by the disulfide bond between Cys$^{56}$ and Cys$^{72}$, and adopts the same conformation in RNase S (Wyckoff et al., 1970) and in various refined models of RNase A (Wlodawer and Sjolin, 1983; Borkakoti et al., 1983). This loop experiences several interactions with the region containing active site residues such as His$^{110}$ and Asp$^{121}$. The residues surrounding Ass$^{67}$ are represented in Fig. 8, based on the coordinates deposited with the Brookhaven Data Bank (Wlodawer and Sjolin, 1983). Asp$^{121}$ is hydrogen-bonded with its side chain $\text{O}_{\text{H}}$ to the NH group of Lys$^{66}$. In addition to this direct

**TABLE I**

| Substrate | Protein     | $k_{on}$ | $k_{off}$ | $10^3 \times k_{on}/K_s$ |
|-----------|-------------|----------|-----------|--------------------------|
| Cyd-2:3'P | RNase A     | 11.5     | 12.3      | 0.09                     |
|           | N67D-RNase A| 20.5     | 16.0      | 0.12                     |
|           | N67D-RNase A| 10.9     | 8.3       | 0.13                     |
|           | N67D-RNase A| 415      | 0.72      | 57.6                     |
|           | N67D-RNase A| 803      | 0.95      | 146.0                    |
|           | N67D-RNase A| 558      | 2.17      | 25.7                     |
|           | N67D-RNase A| 14.3     | 1.39      | 1.03                     |
|           | N67D-RNase A| 3.3      | 0.48      | 0.65                     |
|           | N67D-RNase A| 2.0      | 0.42      | 0.71                     |
|           | N67D-RNase A| 83.3     | 4.2       | 1.27                     |
|           | N67D-RNase A| 42.0     | 8.8       | 0.45                     |
|           | N67D-RNase A| 26.2     | 4.3       | 0.61                     |
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**DISCUSSION**

Selective deamidation is an important phenomenon in protein chemistry. It has physiological significance as a post-translational modification, marking the aging of a protein (Robinson and Rudd, 1974; Di Donato and D’Alessio, 1981; Voorter et al., 1987). Furthermore, it is a typical event in protein deterioration, occurring upon storage of the protein, upon expression of recombinant proteins packed in inclusion bodies, to be recovered through unfolding and refolding, or as an unpredictable event in the production of recombinant protein mutants.

RNase A is a classical model protein; however, its use as a model for deamidation studies has been limited, apparently because of the difficulty in defining suitably mild conditions for selective deamidation of the native protein (Wright, 1991a, 1991b). In previous investigations, carried out under rather harsh conditions (Venkatesh and Vithayatil, 1984), mono- and polydeamidated derivatives were simultaneously obtained, which could only be partially characterized. On the other hand, denatured RNase A was readily and selectively deamidated (Thannhauser and Scheraga, 1986; Wearne and Creighton, 1989). These results led to speculation (Wright, 1991a, 1991b) that structural constraints, inherent in the native conformation, hindered selective deamidation of RNase A, so that this could occur only under harsh conditions or in the unfolded protein (Wearne and Creighton, 1989). Furthermore, there has been a general consensus, following the pioneering work by Bornstein and Balian (1970) on hydroxylamine cleavage of RNase A, for a mechanism of deamidation based on the formation of a cyclic succinimide (from Asn$^{67}$ attacked at its $\alpha$-carbonyl by the NH of Gly$^{68}$). When this imide hydrolyzes, two derivatives are produced; one with an Asp and the other with an iso-Asp replacing the Asn.

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**Fig. 7.** Renaturation of denatured and reduced RNase A (●, N67D-RNase A (●), and N67D-RNase A (●). Activity values are plotted as percent of the maximal activity reached for each protein at the end of the incubation. In each case the final activity was 100 ± 5% of the original activity before denaturation. Curves have been computed with a best fit program (see "Experimental Procedures").
hydrogen bond, the $O_\alpha$ of Asp$^{121}$, which is hydrogen-bonded to His$^{119}$, is also hydrogen-bonded to a water molecule, that in turn forms a hydrogen bond to the NH of Asn$^{67}$. The carboxyl group is about 7 Å from the amide group of Asn$^{67}$. Within the loop, Asn$^{67}$ forms a C$_{10}$ β-turn-like structure, using its side chain, with a hydrogen bond between its $O_\alpha$ and the NH of Gln$^{66}$. In RNase A complexed with dinucleotide inhibitors (Wlodawer, 1985), the side chains of Asn$^{67}$, Gln$^{66}$, and Thr$^{71}$ delimitate the B2 binding site. Moreover, in the enzymically inactive derivative of RNase A, produced by cross-linking Hist$^{12}$ with a deoxythymidine derivative, Asn$^{67}$ was found to be hydrogen-bonded, either directly or through a water molecule, to O$_4$ of deoxythymidine, located very close to the B2 site (Nachman et al., 1990).

These considerations, suggesting the involvement of Asn$^{67}$ with key structural elements of the enzyme catalytic site, prompted us to investigate the enzymic properties of the isolated Asp$-$ and iso-Asp-containing derivatives. The data presented here allow a first inspection of the effects on catalysis of the replacement of Asn$^{67}$ by Asp or iso-Asp.

Both the native enzyme and N67D- and N67D-RNase A derivatives were found to have the same specificity constant values ($K_{cat}/K_m$, see Table I) with a nucleoside cyclic phosphate as a substrate. However, for N67D-RNase A this is engendered by the combined effects of weaker substrate binding and a doubling of $k_m$. This is somewhat surprising, and difficult to interpret, as Asn$^{67}$ does not appear to be involved with the B1 site (see above). Considering that the position of the crucial His$^{119}$ residue is not unique to the structure of RNase A (Borkakoti et al., 1983; Martin et al., 1987), one could speculate that these effects are mediated by a different positioning of His$^{119}$, occurring as a consequence of the replacement of Asn$^{67}$ by Asp.

It has been shown (Walker et al., 1975) that at high substrate concentrations RNase A hydrolyzes cCMP with non-hyperbolic kinetics. We also proposed that deamidation(s) occurring during the purification and/or upon storage could be the basis of this phenomenon (Walker et al., 1975). Our data (cf. Fig. 6) clearly rule out this possibility, as both derivatives of deamidated RNase A display nonhyperbolic saturation curves identical with that of the native enzyme. This finding favors instead other interpretations (Piccoli and D'Alessio, 1984), suggesting that the nonhyperbolic effect observed for RNase A in the substrate concentration range 20–40 mM, analogous to that observed for naturally dimeric seminai RNase at much lower concentrations, may be mediated by the substrate, inducing the dimerization of the protein.

As for the effects of deamidation on catalysis of the first transphosphorolytic reaction step, this was investigated with dinucleotide substrates and with yeast RNA. The lower specific activity found with RNA as a substrate, for both N67D and N67D derivatives, correlates well with the kinetic data obtained with small substrates, showing a general decrease in catalytic power when at position 67 Asn is replaced by Asp or iso-Asp. The only exception to this generalization is that observed for N67D-RNase A and a substrate such as UpA, with a purine at the B2 site. In this case, a large increase in $k_m/K_m$ is observed, generated both by a higher $k_m$ and by a lower $K_m$. For the increase in binding energy it can be speculated that when D substitutes for N at position 67 the enzyme-substrate hydrogen bond interactions are reinforced by the presence of a negative charge. This effect is lost in N67D-RNase A, probably because of the repositioning of the -COO$^-$ from the β to the α position and because of the insertion of an extra -CH$_2$ in the main chain. Furthermore, the increased flexibility in the main chain would induce a widening of B2, hence a higher $K_m$.

The availability of homogeneous derivatives in which Asn$^{67}$ is replaced by Asp or iso-Asp also provided the opportunity to address another problem of interest, that of the role of Asn$^{67}$ and of the loop formed by residues 63–75 in the protein folding pathway.

It has been proposed that the loop 63–75 is one of the chain-folding initiation sites in the refolding of RNase A and that it may affect the refolding of the proposed primary chain-folding initiation site located within residues 103–124 (Némethy and Scheraga, 1979; Montelione et al., 1984, 1989). In fact, Venkatesh and Vithayathil (1985) found lower refolding rates for the denatured deamidation products of RNase A obtained by treatment with strong acid. Using homogeneous products from a single deamidation event as described here, we observed a lower refolding rate, with a 2-fold lengthening of the renaturation tip, for N67D-RNase A, i.e. the derivative containing the isopeptide linkage between residues 67 and 68. This finding confirms that Asn$^{67}$ and the loop region comprising it are crucial structural elements in the main refolding pathway of RNase A. On the other hand, it indicates that the presence of a negative charge as such in the microenvironment has no effect on refolding. Rather, the lengthening of the backbone chain, with the presence of an extra -CH$_2$, may impair the ability of the chain to find its proper folding pathway, because it may affect the pairing of the 65–72 disulfide. This is the first of the four disulfides to pair in refolding RNase A (Takahashi et al., 1976) and has been considered important, if not essential, for the correct refolding.

![Figure 8: Three-dimensional structure of the residues surrounding Asn$^{67}$ of RNase A, based on the coordinates deposited with the Brookhaven Data Bank (Wlodawer and Sjolin, 1983). For the one-letter code symbols, see the legend to Fig. 3.](image-url)
of the 63–75 loop (Milburn and Scheraga, 1988; Altmann and Scheraga, 1990).

The results presented here may also help shed light on the importance of primary versus higher order structures in determining deamidation rates in proteins. Although they confirm the importance of both the quality of nearest neighbors in the sequence (Robinson and Rudd, 1974; Wright, 1991b), and of the tertiary determinant (Kossiakoff, 1988), our results also suggest that other determinants must have a role. This is especially evident in a comparison between the data obtained here for RNase A and previous results on selective deamidation of Asn67 in seminal RNase (Di Donato et al., 1986). This comparison reveals that Asn67 is deamidated in BS-RNase at a much faster rate, comparable with that of denatured RNase A. Yet RNase A and the BS-RNase subunit share more than 80% of their residues, and in particular in the loop region containing Asn67, the only substitution is that of Ala in BS-RNase for Thr66 of RNase A, a substitution that should not be relevant in this context (Mazzarella et al., 1987). It should be added that Asn67 deamidates selectively in BS-RNase in vivo, quite probably spontaneously, as it occurs also in the recombinant protein produced with different expression systems. An approach to this intriguing question can only be based on surmising a role for BS-RNase dimeric structure in determining the deamidation rate of Asn67.

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