Biochem. J. (1986) 240, 163–170 (Printed in Great Britain)

Comparison of the primary structures of ribonuclease U_2 isoforms

Shigenori KANAYA* and Tsuneko UCHIDA†

*Biosciences Laboratory, Research Center, Mitsubishi Chemical Industries Ltd., 1000 Kamoshida-cho, Midori-ku, Yokohama, Kanagawa 227, Japan, and †Mitsubishi-Kasei Institute of Life Sciences, 11 Minamiooya, Machida, Tokyo 194, Japan

1. The primary structures of the two isoforms of ribonuclease U_2, RNAases U_2-A and U_2-B, were analysed and compared with each other. Among the chymotryptic peptides obtained from the reduced and *S*-carboxymethylated enzymes, only peptides C-3 were different from each other in terms of chromatographic behaviour on reverse-phase h.p.l.c. On the basis of chemical analyses of these peptides, it was shown that RNAase U_2-B had an isopeptide bond in which Asp-32 was linked to Gly-33 through the β-carboxy group in its side chain instead of the α-carboxy group. Deamidation of Asn-32 in RNAase U_2-A led to the formation of this unusual linkage. 2. The previously reported sequence of RNAase U_2 [Sato & Uchida (1975) Biochem. J. **145**, 353–360] was corrected by changing amino acid residues at eight different positions and by inserting an asparagine residue at position 32. The numbering of the positions of amino acid residues located downstream of Asn-32 was therefore shifted by 1. Accordingly, RNAase U_2-A was shown to be composed of 114 amino acid residues.

INTRODUCTION

RNAase U_2 (EC 3.1.27.4), which was first reported by Arima *et al.* [1,2], has more recently been shown to exist in two forms, RNAases U_2-A and U_2-B, in the culture broth of the smut fungus *Ustilago sphaerogena* [3]. Enzymically, RNAase U_2-B is identical with RNAase U_2-A in base specificity and in affinity for ApA, but possesses lower specific activity, approx. 75% of that of RNAase U_2-A. It is a slightly more acidic protein, migrating faster than RNAase U_2-A on polyacrylamide-gel electrophoresis at pH 8.3. A difference in conformation was also observed. Since there was no difference between the amino acid compositions of the two forms, it was suggested that deamidation or some other modifications caused the formation of the isoform (RNAase U_2-B) molecule. We have reported previously that the heterogeneity of non-specific RNAase T_2 from *Aspergillus oryzae* was due to the differences in the carbohydrate content [4]. However, this was not a valid possibility for the enzyme RNAase U_2, which is non-glycosylated. The presence of isoforms was found in other guanine-specific RNAases, namely RNAase T_1 from *Aspergillus oryzae* [5] and RNAase F_1 from *Fusarium moniliforme* [6]. In each case the isoform enzyme has not been isolated from the original one and its enzymic and physicochemical properties are still unclear.

Before the finding of the heterogeneity in RNAase U_2, the enzyme had been extensively studied for its enzymic and physicochemical properties [7,8], including the amino acid sequence [9]. Fortunately, the enzyme highly purified by the classical method was shown to be RNAase U_2-A [3].

Recent developments in the techniques for protein sequence determination have been considerable: the combination of reverse-phase h.p.l.c. with the automated gas-phase protein sequencer allows rapid and precise sequence determination with only microgram quantities of protein [10,11]. On the assumption that RNAase U_2-B is different from RNAase U_2-A in its primary structure, its amino acid sequence was analysed and compared with that of RNAase U_2-A.

In the present paper we describe the difference between the primary structures of RNAases U_2-A and U_2-B and the possible mechanism by which the former is converted into the latter. In addition, several corrections are made to the previously reported sequence of RNAase U_2-A [9].

MATERIALS AND METHODS

Materials

RNAases U_2-A and U_2-B were prepared by affinity chromatography with AMP–aminohexyl-Sepharose as described by Uchida & Shibata [3]. Chymotrypsin [L-7-amino-1-chloro-3-tosylamidoheptan-2-one-('TLCK'-)treated] was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Monoiodoacetic acid (Wako Chemical Co., Osaka, Japan) was dissolved in hot chloroform and crystallized with the addition of 2 vol. of light petroleum (b.p. 40–70 °C) to remove coloured material before use. Trifluoroacetic acid, acetonitrile and distilled water of h.p.l.c. grade were products of Eastman Kodak Co. (Rochester, NY, U.S.A.), Merck (Darmstadt, Germany) and Wako Chemical Co. respectively. Pre-packed columns of Sephadex G-25 (PD-10) were obtained from Pharmacia (Uppsala, Sweden). Chemicals for gas-phase sequencing were from Applied Biosystems (Foster City, CA, U.S.A.). All other chemicals were of reagent grade.

Reduction and *S*-carboxymethylation

Reduction and *S*-carboxymethylation of RNAase U_2-A or U_2-B was performed as described previously [12] except that dithiothreitol instead of 2-mercaptoethanol and guanidium chloride instead of urea were used as the

Abbreviations used: RNAase, ribonuclease; RCM-, reduced and *S*-carboxymethylated.

† To whom correspondence and requests for reprints should be addressed.

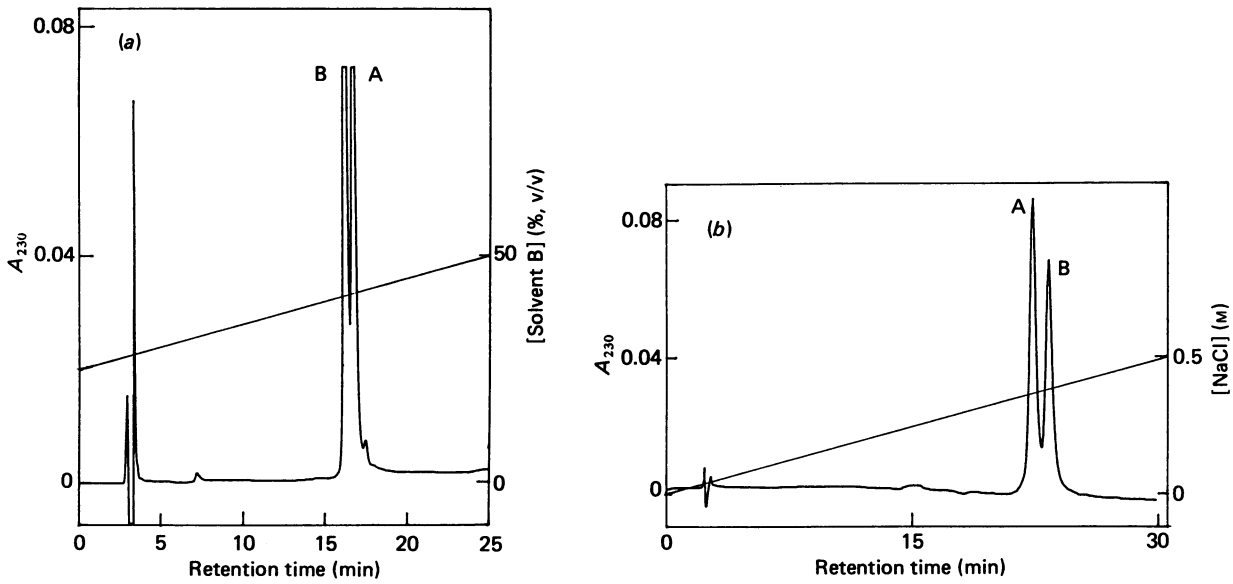


Fig. 1. Separation of RNAases U_2-A and U_2-B by (*a*) reverse-phase h.p.l.c. and (*b*) anion-exchange h.p.l.c.

(*a*) RNAases U_2-A and U_2-B (5 μg of each) were mixed and loaded on an Aquapore RP-300 column (4.6 mm × 250 mm) equilibrated with 25% (v/v) solvent B in solvent A. Elution was performed by raising linearly the concentration of solvent B in solvent A from 25% (v/v) to 50% (v/v) over 25 min. Solvents A and B were aq. 0.1% (v/v) trifluoroacetic acid and acetonitrile containing 0.05% (v/v) trifluoroacetic acid respectively. The flow rate was 1.0 ml/min. A and B in the Figure indicate RNAases U_2-A and U_2-B respectively. The straight line represents the concentration of solvent B. (*b*) RNAases U_2-A (5 μg) and U_2-B (4 μg) were mixed and loaded on an MCI GEL CQA-31S column (7.5 mm × 75 mm) equilibrated with 10 mM-Tris/HCl buffer, pH 8.0. Elution was performed by a linear gradient of 0–0.5 M-NaCl in the same buffer over 30 min. The flow rate was 1.0 ml/min. A and B in the Figure indicate RNAases U_2-A and U_2-B respectively. The straight line represents the concentration of NaCl.

reducing and denaturing agents respectively. RCM-enzyme was desalted on a PD-10 column that had been equilibrated with 0.1 M-acetic acid. The excluded fraction was collected and freeze-dried.

Chymotryptic digestion of RCM-enzymes

RCM-RNAase U_2-A or U_2-B (200 μg, approx. 20 nmol) was dissolved in 200 μl of 0.1 M-Tris/HCl buffer, pH 8.0, and digested with 10 μg of chymotrypsin at 37 °C for 6 h. The resulting peptides were separated by reverse-phase h.p.l.c. Fractions containing the peptides were collected and freeze-dried.

H.p.l.c.

Reverse-phase h.p.l.c. was carried out on an Aquapore RP-300 column (4.6 mm × 250 mm) of Brownlee Labs (Santa Clara, CA, U.S.A.), with the Altex–Beckman (Irvine, CA, U.S.A.) 110A h.p.l.c. system. Elution was performed by raising linearly the concentration of solvent B in solvent A. Solvent A was aq. 0.1% (v/v) trifluoroacetic acid and solvent B was acetonitrile containing 0.05% (v/v) trifluoroacetic acid. The initial and final concentrations of solvent B in the linear gradient chosen depended on the elution positions of the samples. The flow rate was 1.0 ml/min. Anion-exchange chromatography was carried out on an MCI GEL CQA-31S column (7.5 mm × 75 mm) of Mitsubishi Chemical Industries (Tokyo, Japan), with a Spectra Physics (San José, CA, U.S.A.) model 8700 h.p.l.c. system. Elution was performed by a linear gradient of 0–0.5 M-NaCl in 50 mM-Tris/HCl buffer, pH 8.0, over 30 min with the flow rate of 1.0 ml/min. In both cases the proteins or peptides were detected by a variable-wavelength u.v. detector set at 230 nm.

Amino acid analysis

Samples (0.1–1.0 nmol) were hydrolysed with 6 M-HCl at 105 °C for 24 h in evacuated sealed tubes. Amino acid compositions were determined with a Waters Associates (Milford, MA, U.S.A.) amino acid analysis system with a model 510 h.p.l.c. pump.

Sequence determination

Amino acid sequence analyses were performed by stepwise Edman degradation [13] in a gas-phase automated sequencer (Applied Biosystems model 470A). Samples in the range 0.1–1.0 nmol were used. The amino acid phenylthiohydantoin derivatives were analysed on a Toyosoda (Tokyo, Japan) TSK-GEL ODS-80TM column (4.6 mm × 150 mm) with a Spectra Physics model SP8100 h.p.l.c. system. They were eluted from the column isocratically as described previously [14].

RESULTS

Isolation of the chymotryptic peptide that distinguishes RNAase U_2-A from RNAase U_2-B

The h.p.l.c. chromatographic behaviour of native enzymes or RCM-enzymes was first examined. As shown in Fig. 1, native RNAases U_2-A and U_2-B were clearly separated from each other by h.p.l.c. with either the reverse-phase column (Aquapore RP-300) (Fig. 1*a*) or the anion-exchange column (MCI GEL CQA-31S) (Fig. 1*b*). From the latter column RNAases U_2-A and U_2-B

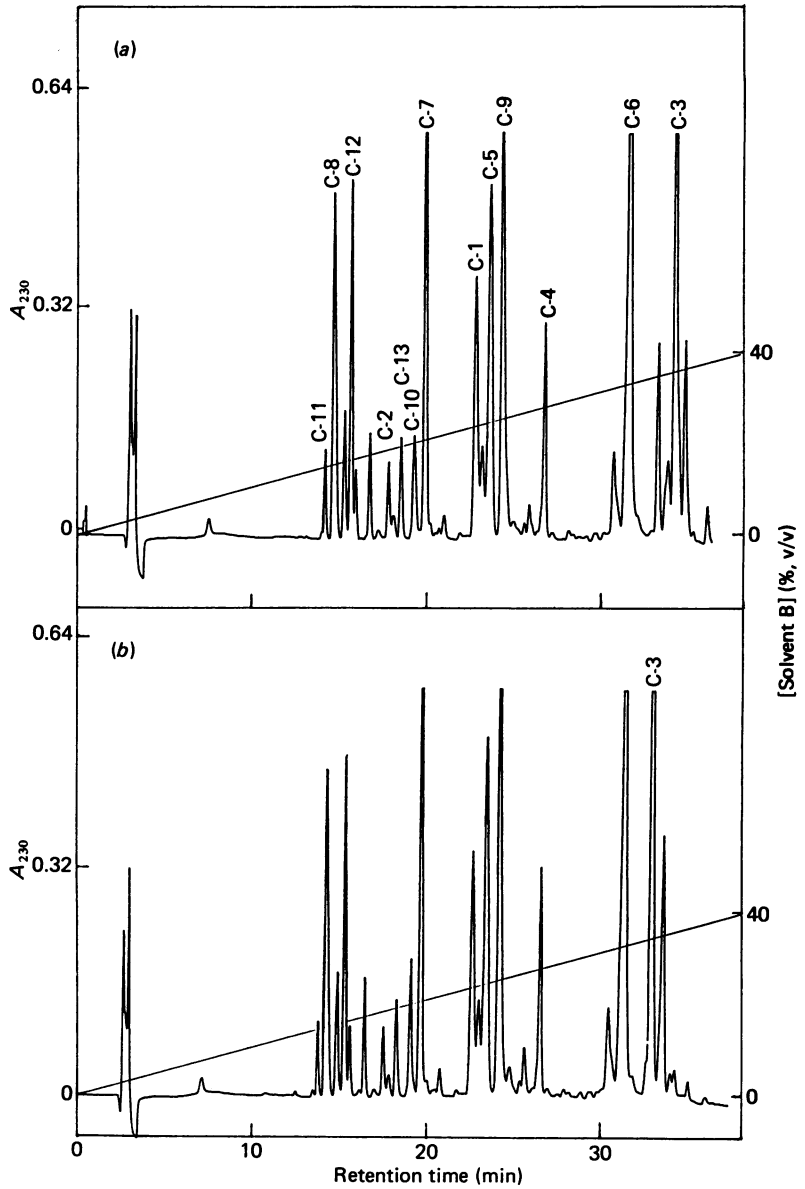


Fig. 2. Separation of peptides from chymotryptic digests of RCM-RNAases U_2-A and U_2-B

Chymotryptic digests (20 nmol) of RCM-RNAases U_2-A (*a*) and U_2-B (*b*) were directly loaded on an Aquapore RP-300 reverse-phase column (4.6 mm × 250 mm) equilibrated with solvent A. Elution was performed by raising linearly the concentration of solvent B in solvent A to 45% (v/v) over 45 min. Solvents A and B are described in the legend to Fig. 1(*a*). The flow rate was 1.0 ml/min. All the peaks were collected and analysed for amino acid composition. The positions of the chymotryptic peptides C-1 to C-13 are shown in (*a*). Only peptide C-3, which was eluted at different positions in the digests of RCM-RNAases U_2-A and U_2-B, is designated in (*b*). The straight line represents the concentration of solvent B.

were eluted in that order, which was consistent with their properties in that RNAase U_2-B is more acidic than RNAase U_2-A. On the other hand, they were eluted in the reversed order from the former column. Reduction and *S*-carboxymethylation slightly delayed the elution position of the enzyme from the reverse-phase column (results not shown). Since RCM-RNAases U_2-A and U_2-B were also separated from each other by h.p.l.c. as the native enzymes were, it became obvious that RNAase U_2-B was neither a disulphide-linkage isoform nor a conformationally denatured form of RNAase U_2-A.

The RCM-enzymes were digested with chymotrypsin, and the digests were analysed by reverse-phase h.p.l.c. (Fig. 2). Peaks were collected and subjected to amino acid analyses. All the chymotryptic peptides that were identified previously [9] were recovered, although peptides containing cysteine residues were recovered as RCM-peptides instead of reduced and *S*-aminoethylated peptides. The Sato & Uchida [9] nomenclature of the peptides was followed, in which the peptides were prefixed by C and numbered in order of their position in the primary sequence. Only the two peptides C-3 were eluted at different positions from the reverse-phase column, as shown in Fig. 2. When both peptides obtained from RCM-RNAases U_2-A [C-3(A)] and U_2-B [C-3(B)] were subjected together to reverse-phase h.p.l.c., they were eluted from the column in the order peptides C-3(B) and C-3(A). Since this order of the elution

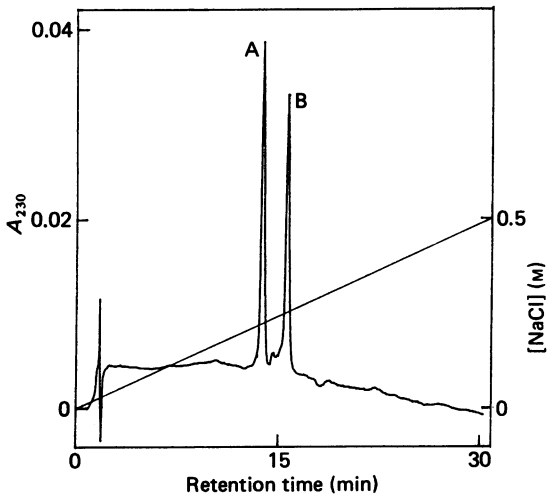


Fig. 3. Separation of chymotryptic peptides C-3(A) and C-3(B) of RNAases U_2-A and U_2-B

The peptides C-3 derived from RCM-RNAases U_2-A [C-3(A)] and U_2-B [C-3(B)] (1 μg of each) were mixed and analysed by h.p.l.c. with the MCI GEL CQA-31S column (7.5 mm × 75 mm). Elution was performed as described in the legend to Fig. 1(*b*). The straight line represents the concentration of NaCl. Peptides C-3(A) and C-3(B) are simply designated by A and B respectively in the Figure.

Table 1. Amino acid composition of chymotryptic peptides C-3 of RNAases U_2-A and U_2-B

	Composition (mol of residue/mol of peptide)		
	C-3(A)	C-3(B)	Reference*
Asp	9.64	10.0	9
Thr	1.07	1.05	1
Ser	1.10	1.12	1
Glu	2.23	2.30	2
Pro	2.21	2.12	2
Gly	2.23	2.18	2
Ala	3.07	3.00	3
Val	1.07	1.00	1
Ile	1.95	1.79	2
Leu	1.00	1.10	1
Tyr	1.30	1.14	2
His	1.40	1.50	1
Arg	0.90	0.90	1
Total			28

* Data calculated from the previously reported sequence [9].

positions was the same as that of the original enzymes or RCM-enzymes, it was concluded that the difference between RNAases U_2-A and U_2-B could be attributed to that between peptides C-3(A) and C-3(B). To confirm this, both peptides were also analysed by h.p.l.c. with the MCI GEL CQA-31S column (Fig. 3). Peptide C-3(B) behaved as a more acidic peptide than peptide C-3(A), which reflected the character of the respective native enzymes. The amino acid compositions of peptides C-3(A) and C-3(B) were identical (Table 1). However, the number of aspartic acid residues in each peptide seemed to be one more than that calculated from the previously reported sequence [9], suggesting that one aspartic acid or asparagine residue had been missed previously. The poor recovery of tyrosine was due to degradation during the hydrolysis in 6 M-HCl. The high apparent content of histidine might be due to the contribution of the 'noise' peak eluted by the shock of the buffer change on h.p.l.c.

Sequence determination

All chymotryptic peptides derived from RCM-RNAase U_2-A were first analysed for their amino acid sequences. Even the amino acid residue located at the *C*-terminus of each peptide could be identified if a sufficient amount (approx. 1 nmol) of sample was used (Fig. 4). By comparing the amino acid sequence of RNAase U_2-A with the previously reported sequence [9], Asn-2, Glu-5, Pro-56, Ser-58 and Arg-Pro-Asp-Gly (residues 32–35) were corrected to Asp-2, Gln-5, Ser-57, Pro-59 and Asn-Gly-Asp-Arg-Pro (residues 32–36) respectively. The numbers of the positions of serine and proline were shifted by 1 owing to the insertion of the asparagine residue at position 32. Because asparagine and aspartic acid residues are abundant in the enzyme (21 out of 114 residues) it was difficult to decide by amino acid analysis whether the number of these residues should be 20 or 21. The peptide C-3(A) was shown to be composed of 29 amino acid residues from Ser-15 to Tyr-43 instead of 28 residues, which is consistent with the value determined by amino acid analysis. Although the amino acid sequence of the peptide C-3(A) was completely determined from Ser-15 (*N*-terminus) to Tyr-43 (*C*-terminus), that of peptide C-3(B) was only partially determined. This was because the sequential Edman degradation stopped at Ala-31 and did not proceed beyond this residue even if a large quantity of the peptide was analysed. In the region from Ser-15 to Ala-31 the two sequences were identical. Other chymotryptic peptides derived from RCM-RNAase U_2-B were also analysed for their amino acid sequences. Except for the region comprising amino acid residues 32–43 the amino acid sequence of RNAase U_2-B was perfectly matched with that of RNAase U_2-A (Fig. 4). The region from residue 32 to residue 43 is located in the *C*-terminal part of C-3(B). Cleavage of peptide C-3(B) by the proline-specific endopeptidase from *Flavobacterium* sp. (Seikagaku Kogyo Co., Tokyo, Japan) allowed us to confirm the amino acid sequence from Asp-37 to Tyr-43 (results not shown). Finally, the sequence from residue 32 to residue 36 in RNAase U_2-B was left undetermined. Since no difference in amino acid composition between peptides C-3(A) and C-3(B) was found, and the corresponding residue at position 32 in C-3(A) is asparagine, the evidence suggested that C-3(B) has an unusually modified asparagine residue at position 32.

Conversion of RNAase U_2-A into RNAase U_2-B

Deamidation of an asparagine residue followed by the formation of a β-aspartyl peptide bond is known as one of the reactions of the amide side chain of asparagine in proteins [15–17]. It is postulated that peptide C-3(B) contains a β-aspartylglycyl sequence at positions 32 and 33 on the basis of the following criteria. (1) The localization of the difference in the primary sequences between RNAases U_2-A and U_2-B is limited to the region comprising residues 32–36. (2) Peptide C-3(B), which contains this sequence, is more acidic than peptide C-3(A), which reflects the nature of the native enzymes,

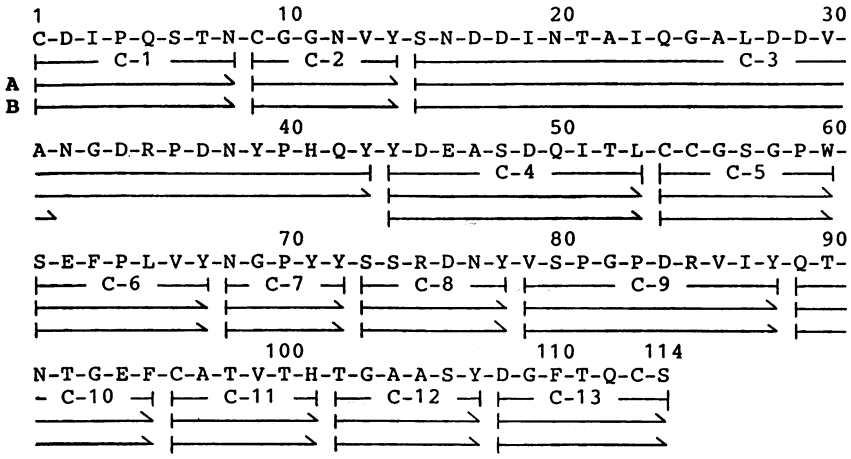


Fig. 4. Amino acid sequences of RCM-RNAases U_2-A and U_2-B

Chymotryptic peptides of RCM-RNAases U_2-A and U_2-B are indicated by ⊢⊣ and designated in accordance with Sato & Uchida [9]. The amino acid sequences positively determined by automated gas-phase sequencer are denoted by ⊢⇀. A and B represent RCM-RNAases U_2-A and U_2-B respectively. All cysteine residues were identified as RCM-cysteine. Repetitive yields of amino acid phenylthiohydantoin derivatives were about 95%. The one-letter notation of amino acids is used.

indicating the presence of additional acidic group(s) in it. The appearance of a free α-carboxy group at position 32 would fulfil this condition. (3) The sequence downstream of Ala-31 is -Asn-Gly- in peptide C-3(A), for which the β-carboxy linkage was reported to be formed primarily [15]. (4) Sequential Edman degradation, a procedure that only works with α-amino acids, did not proceed beyond Ala-31 in peptide C-3(B), although it did so in peptide C-3(A), containing Asn-32. The β-linked aspartate was not removed in the form of a phenylthiohydantoin derivative [18]. To confirm this, peptide C-3(A) was subjected to conditions under which the formation of the β-aspartyl peptide bond is known to take place [16].

A 25 μg portion of peptide C-3(A) was dissolved in 100 μl of 0.1 M-NH_3 and incubated at 30 °C. At appropriate intervals a 20 μl sample was withdrawn, mixed with 6 μl of 1 M-acetic acid to lower the pH to 5.0 and analysed by reverse-phase h.p.l.c. (Fig. 5*a*). Peptide C-3(A) was shown to be converted into peptide C-3(B) with a yield of approx. 50% in 1 h and approx. 80% in 2.5 h. Other minor peaks that appeared might be denaturation or degradation products, but have not been characterized. The conversion of native RNAase U_2-A into RNAase U_2-B by alkali treatment was also observed (Fig. 5*b*). This observation removed any possibility that the existence of the two forms might be due to sequence isomerism in the unsequenced region comprising residues 32–36.

DISCUSSION

It has been shown that RNAase U_2-B is a deamidated form of RNAase U_2-A that has a β-carboxy linkage between Asp-32 and Gly-33. Although this unusual linkage has not been directly identified, all chemical analyses strongly supported the presence of this β-linkage in RNAase U_2-B. According to Wold [15], hydrolysis of the cyclic imide that is formed as an intermediate associated with the deamidation of asparagine may generate either an α- or a β-carboxy linkage. However, no RNAase U_2 derivatives containing aspartic acid at position 32 have been obtained, indicating that the α-carboxy amide bond of the cyclic imide was preferentially cleaved by hydrolysis. This finding is consistent with the prediction by Bornstein & Balian [19] that the β-linked peptide should be the more abundant product. The Bornstein & Balian [19] mechanism for the production of the β-linked peptide bond in RNAase U_2-B is shown in Scheme 1.

The identification of the β-aspartyl linkage in RNAase U_2-B contributed to some extent to an understanding of the structure–activity relationship of the enzyme. The formation of the β-linkage in the enzyme caused the decrease in activity, but did not affect the affinity for ApA, suggesting that a catalytically essential amino acid residue might be located close to this linkage. RNAase U_2, like RNAase T_1, forms an active site with two histidine residues, one arginine residue and a crucial glutamic acid residue [8,9]. Glutamic acid and histidine are involved in the catalytic site, and arginine in the binding site of the phospho group. However, it was unclear how the two histidine residues located at positions 41 and 101 (previously designated as 40 and 100 respectively) are concerned in the enzymic activity [20,21]. Since it is possible that the replacement of the α-linked peptide bond by a β-linked bond at position 32 might influence the conformational arrangement of His-41 more significantly than that of His-101 [located apart in the molecular structure on the basis of X-ray analysis (T. Matsuzaki & T. Uchida, unpublished work)], some involvement of His-41 in the catalytic site is expected. This hypothesis is supported by the n.m.r. studies (Y. Shibata, T. Uchida & T. Miyazawa, unpublished work). Comparison of the pH titration curves of the n.m.r. resonance of the histidine residues among RNAases U_2-A, U_2-B and T_1 suggested that the micro-environment of His-41 (His-40 in RNAase T_1) is mutually different among both forms of RNAase U_2 and RNAase T_1, but that of His-101 (His-92 in RNAase T_1) was similar in these enzymes. His-101 is thus considered to be directly implicated in catalytic action, and His-41 to participate indirectly in the enzymic activity. Lending support to this conclusion is the finding by X-ray analysis of RNAase U_2-A that His-41 is located close to

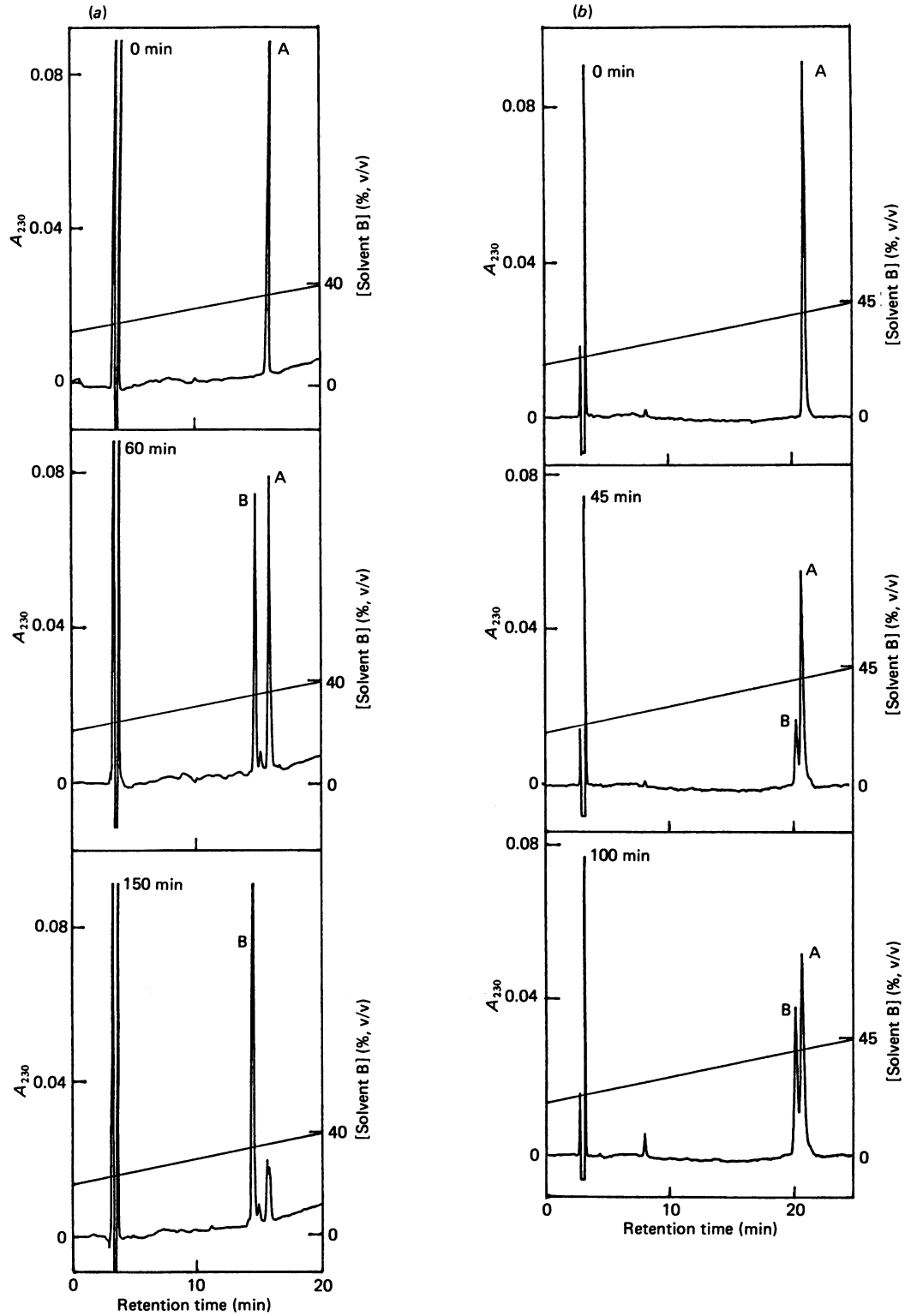
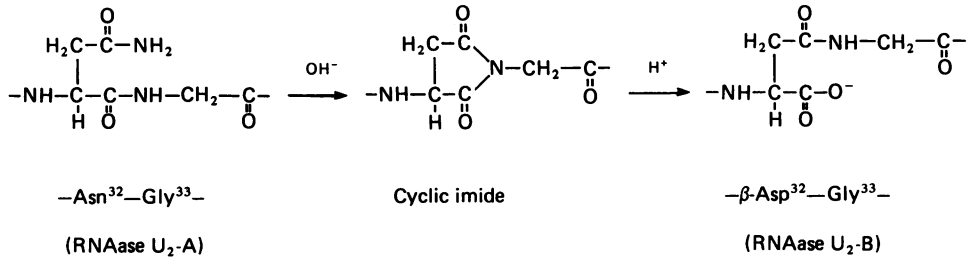


Fig. 5. Alkali treatment of peptide C-3(A) and RNAase U_2-A

Time courses of the conversions of peptide C-3(A) into peptide C-3(B) (*a*) and of RNAase U_2-A into RNAase U_2-B (*b*) are shown. A 5 μg portion of peptide C-3(A) or RNAase U_2-A was incubated in 0.1 M-NH_3 at 37 °C for the period shown in each Figure and loaded on an Aquapore RP-300 reverse-phase column (4.6 mm × 250 mm) equilibrated with 20% (v/v) solvent B in solvent A. Elution was performed as described in the legend to Fig. 1(*a*), except that the concentration of solvent B in solvent A was linearly raised from 20% (v/v) to 40% (v/v) over 20 min (*a*) or from 20% (v/v) to 45% (v/v) over 25 min (*b*). The straight line represents the concentration of solvent B. Minor peaks detected by h.p.l.c. with the incubation time might be denatured or degraded peptide products (not yet identified). Peptide C-3(A) and RNAase U_2-A are simply designated by A, and C-3(B) and RNAase U_2-B by B, in the Figure.



Scheme 1. Proposed mechanism for the formation of the β-carboxy linkage in RNAase U_2-A

The mechanism shown here is basically that proposed by Bornstein & Balian [19]. The superscript denotes the position of the residue in RNAase U_2-A or RNAase U_2-B.

the glutamic acid residue that is crucial to catalytic action.

The secondary structure of RNAase U_2-B was reported to be different from that of RNAase U_2-A [3], namely a portion of α-helical structure and β-pleated sheet structure in RNAase U_2-A appears to be unfolded in RNAase U_2-B. The preliminary results of X-ray-diffraction analysis of RNAase U_2-A suggest the presence of α-helical structure in the region from residue 18 to residue 30. The location of β-aspartate at position 32 may be close enough to affect this structure. Determination of the tertiary structure of RNAase U_2 will facilitate understanding of the difference in conformation between RNAases U_2-A and U_2-B and of the arrangement of the amino acid residues forming the active site.

It is noteworthy that another -Asn-Gly- sequence exists in RNAase U_2, and this is located at positions 68 and 69. The asparagine residue at position 68 was resistant to deamidation by alkali treatment, indicating that this asparagine residue might be buried in the enzyme. Preliminary results of X-ray-diffraction analysis support this hypothesis: whereas the -Asn-Gly- sequence at positions 32 and 33 seems to be exposed, that at positions 68 and 69 is buried. The -Asn-Gly- sequence was searched for in RNAase T_1 and RNAase F_1, both of which were reported to have isoforms of the enzyme like RNAase U_2-B [5,6]. Since neither enzyme contains this sequence, the heterogeneity caused by the deamidation followed by the formation of β-linkage seems to be characteristic for RNAase U_2-A in these RNAases. However, the possibility that the formation of the β-aspartyl peptide bond causes the heterogeneity in RNAase T_1 or F_1 cannot be ruled out, because the α/β shift on deamidation may occur when glycine is replaced by other residues.

The primary sequence of RNAase T_1 was recently corrected by Takahashi [22] at the region that has been shown to be critical for enzymic activity [23]. The previously reported sequence of RNAase U_2 [9] has also now been corrected, as described in the Results section. Corrections were made at several positions. Nevertheless the sequence homology with RNAase T_1 was not greatly affected, and the analogous sequences between RNAase U_2 (RNAase U_2-A) and RNAase T_1 shown by Sato & Uchida [9] were still conserved.

The physiological role for the deamidation of RNAase U_2-A is unclear. It seems unlikely that the formation of this atypical β-carboxy linkage is required to provide a methylation site, as reported in corticotropin [16], since there have been no reports describing the methylation of RNAases. Since the β-linkage could be formed as an artifact, RNAase U_2-B might be regarded as a denatured form of RNAase U_2-A. Exposure of the enzyme to alkaline solution during the cultivation of *Ustilago sphaerogena* and the purification procedures might cause spontaneous deamidation followed by formation of the β-carboxy linkage. Since the pH of the culture medium of *Ustilago sphaerogena* increased beyond pH 7 after late exponential phase, the generation of RNAase U_2-B, with the lower catalytic activity may be related to this.

We are grateful to Mr. T. Tagawa for the amino acid analyses and Mrs. H. Shibata for the preparation of RNAases U_2-A and U_2-B. We also thank Dr. T. Hishida and Dr. T. Kobayashi of Mitsubishi Kasei Chemical Industries Co. for their interest and encouragement.

REFERENCES

1. Arima, T., Uchida, T. & Egami, F. (1968) Biochem. J. **106**, 601–608
2. Arima, T., Uchida, T. & Egami, F. (1968) Biochem. J. **106**, 609–613
3. Uchida, T. & Shibata, Y. (1981) J. Biochem. (Tokyo) **90**, 463–471
4. Kanaya, S. & Uchida, T. (1981) J. Biochem. (Tokyo) **90**, 473–481
5. Kanaya, S. & Uchida, T. (1981) J. Biochem. (Tokyo) **89**, 591–597
6. Yoshida, H., Fukuda, A. & Hashiguchi, M. (1980) J. Biochem. (Tokyo) **88**, 1813–1818
7. Uchida, T. & Egami, F. (1971) Enzymes 3rd Ed. **4**, 205–250
8. Egami, F., Oshima, T. & Uchida, T. (1980) Mol. Biol. Biochem. Biophys. **32**, 250–277
9. Sato, S. & Uchida, T. (1975) Biochem. J. **145**, 353–360
10. Hunkapiller, M. & Hood, L. (1983) Methods Enzymol. **91**, 486–493
11. Hunkapiller, M., Kent, S., Caruthers, M., Dreyer, W., Firca, J., Giffin, C., Horvath, S., Hunkapiller, T., Tempst, P. & Hood, L. (1984) Nature (London) **310**, 105–111
12. Crestfield, A. M., Moore, S. & Stein, W. H. (1963) J. Biol. Chem. **238**, 622–627
13. Edman, P. & Begg, G. (1967) Eur. J. Biochem. **1**, 80–91
14. Tsunasawa, S., Kondo, J. & Sakiyama, F. (1985) J. Biochem. (Tokyo) **97**, 701–704
15. Wold, F. (1985) Trends. Biochem. Sci. **109**, 4–6
16. Aswad, D. W. (1984) J. Biol. Chem. **259**, 10714–10721
17. Murray, E. D., Jr. & Clarke, S. (1984) J. Biol. Chem. **259**, 10722–10732
18. Gráf, L., Bajusz, S., Patthy, A., Barát, E. & Cseh, G. (1971) Acta Biochim. Biophys. Acad. Sci. Hung. **6**, 415–418

19. Bornstein, P. & Balian, G. (1977) Methods Enzymol. **47**, 132–145
20. Uchida, T. & Sato, S. (1973) in Ribosome and RNA Metabolism (Zelinka, J. & Balan, J., eds.), pp. 453–472, Publishing House of the Slovak Academy of Sciences, Bratislava
21. Sato, S. & Uchida, T. (1975) J. Biochem. (Tokyo) **77**, 795–800
22. Takahashi, K. (1985) J. Biochem. (Tokyo) **98**, 815–817
23. Nishizawa, S., Fujimoto, K., Yanase, K., Tanaka, T., Uesugi, S., Ohtsuka, E. & Ikehara, M. (1985) Nucleic Acids Symp. Ser. **16**, 287–290

Received 7 April 1986/6 June 1986; accepted 24 July 1986