Subtilisin Modification of Monodeamidated Ribonuclease-A

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Limited proteolysis of RNAase-Aa₁ (monodeamidated ribonuclease-A) by subtilisin results in the formation of an active RNAase-S type of derivative, namely RNAase-Aa₁S. RNAase-Aa₁S was chromatographically distinct from RNAase-S, but exhibited very nearly the same enzymic activity, antigenic conformation and susceptibility to trypsin as did RNAase-S. Fractionation of RNAase-Aa₁S by trichloroacetic acid yielded RNAase-Aa₁S-protein and RNAase-Aa₁S-peptide, both of which are inactive by themselves, but regenerate active RNAase-Aa₁S' when mixed together. RNAase-Aa₁S-peptide was identical with RNAase-S-peptide, whereas the protein part was distinct from that of RNAase-S-protein. Titration of RNAase-Aa₁S-protein with S-peptide exhibited slight but noticeably weaker binding of the peptide to the deamidated S-protein as compared with that of native protein. Unlike the subtilisin digestion of RNAase-A, which gives nearly 100% conversion into RNAase-S, the digestion of RNAase-Aa₁ gives only a 50% conversion. The resistance of RNAase-Aa₁ to further subtilisin modification after 50% conversion is apparently due to the interaction of RNAase-Aa₁ with its subtilisin-modified product. RNAase-S was also found to undergo activity and structural changes in acidic solutions, similar to those of RNAase-A. The initial reaction product (RNAase-Sa₁) isolated by chromatography was not homogeneous. Unlike the acid treatment of RNAase-A, which affected only the S-protein part, the acid treatment of RNAase-S affected both the S-protein and the S-peptide region of the molecule.

RNAase-A† retains full enzymic activity even after the peptide bond between alanine-20 and serine-21 is cleaved by subtilisin (Richards, 1958; Richards & Vithayathil, 1959). The resulting derivative, RNAase-S, is a complex of two fragments, S-peptide (residues 1-20) and S-protein (residues 21-124), which are held together by non-covalent interactions. RNAase-S can be fractionated into inactive S-protein and S-peptide by treatment with trichloroacetic acid (Richards & Vithayathil, 1959). S-protein could be titrated with S-peptide to reconstitute enzymic activity, and the activity of the mixture is proportional to the amount of S-peptide, until

full activity is regenerated at equivalence. Richards & Vithayathil (1959) recognized that such a proportionality is due to the strong association of these fragments, which generates the enzymically active structure. Since then, many other similar systems, generally referred to as the complementing systems, have been described (Taniuchi & Anfinsen, 1969; Lin *et al.*, 1970; Corradin & Harbury, 1971; Hantgan & Taniuchi, 1976; Welling *et al.*, 1976). These complementing systems have proved very valuable in protein chemistry in understanding the structure-function relationships of enzymes and the general principles underlying the protein folding (Anfinsen & Scheraga, 1975; Richards & Wyckoff, 1971).

Since the preparation of RNAase-S, there has been considerable interest in its two components, S-peptide and S-protein. S-peptide has attracted the most attention, because it has been possible to prepare analogues of it by chemical synthesis. Semisynthetic RNAase-S' has been prepared by mixing these synthetic or modified S-peptides with S-protein (Richards & Vithayathil, 1960; Vithayathil & Richards, 1961; Potts *et al.*, 1963; Scoffone *et al.*, 1967*a,b*; Dunn *et al.*, 1974; Dunn & Chaiken, 1975). However, similar studies with S-protein are limited, apparently owing to the difficulties involved in the chemical synthesis of this larger S-protein part.

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‡ Abbreviations: RNAase-A (ribonuclease-A), the major chromatographic component of bovine pancreatic ribonuclease; RNAase-Aa₁, active monodeamidated derivative of RNAase-A (Manjula *et al.*, 1976); RNAase-S, S-protein and S-peptide are the subtilisin-modified derivatives of RNAase-A (Richards & Vithayathil, 1959).

An alternative approach to preparing modified S-protein is the subtilisin digestion of the well-defined derivatives of RNAase-A having modifications in the S-protein region of the molecule (Fung & Doscher, 1971; Acharya & Vithayathil, 1970; Link, 1970).

We described the formation of an enzymically active monodeamidated derivative of RNAase-A by the controlled deamidation of the protein under highly acidic conditions (Manjula *et al.*, 1976). The single deamidated residue of this derivative appears to be in the segment of the polypeptide chain containing amino acid residues 67–85. This monodeamidated RNAase-A (RNAase-Aa₁) has now been subjected to subtilisin digestion with a view to studying the effect of deamidation on the susceptibility of the molecule to limited proteolysis by subtilisin. An active deamidated RNAase-S has been isolated and characterized. Further, the influence of the absence of the peptide bond 20–21 from RNAase-S on the controlled deamidation of the protein in acidic solution has also been studied. The results of these studies are presented below.

Materials and Methods

Proteinase (subtilopeptidase A, type VII, lot 64B-1470) and trypsin (type III, lot 114B-0530) were products of Sigma Chemical Co. (St. Louis, MO, U.S.A.). Preparation of RNAase-Aa₁ and antisera towards RNAase-A have been described by Manjula *et al.* (1976). Double diffusion in agar was carried out as described by Ouchterlony (1948). Chromatography of the protein samples on columns of Amberlite XE-64 resin, measurement of RNAase activity towards RNA at pH 5.1, amino acid analysis, and peptide analysis were carried out as described by Acharya & Vithayathil (1975). Paper electrophoresis of S-peptide preparations was carried out in pyridine/acetate buffer, pH 6.5 (pyridine/acetic acid/water, 10:1:89, by vol.) (Vithayathil & Richards, 1960*a,b*). All dialyses were performed with cellulose dialysis tubing (Arthur Thomas, Philadelphia, PA, U.S.A.) and were against water unless mentioned otherwise.

Subtilisin digestion

RNAase-Aa₁ was digested with subtilisin in 0.05M-ammonium carbonate buffer, pH 8.0, at 2°C. The formation of active derivatives of RNAase during the subtilisin digestion was followed by measuring the activity before and after trypsin digestion (Richards & Vithayathil, 1959). In common with RNAase-A, RNAase-Aa₁ is also resistant to trypsin digestion (Manjula *et al.*, 1976). Hence the quantity of trypsin-digestible enzymically active protein was taken to represent RNAase-S type of products in the subtilisin digest. For isolation of the

subtilisin-modified derivative, the subtilisin digestion was terminated by adjusting the pH of the digest to 3.0 with 1.0M-HCl. The solution was allowed to stand at room temperature (23°C) for 1 h to inactivate subtilisin, the pH was re-adjusted to 6.0 and the sample was directly placed on the chromatographic columns.

RNAase-S was prepared by the subtilisin digestion of RNAase-A and subsequent chromatography of the digest on CM-cellulose (Allende & Richards, 1962). Fractionation of the peptide and the protein components of the subtilisin-modified derivatives was carried out by the trichloroacetic acid-precipitation procedure (Richards & Vithayathil, 1959).

Reaction of RNAase-S with HCl

Incubation of RNAase-S (1 mM) in 0.5M-HCl at 30°C was carried out as described for RNAase-A (Manjula *et al.*, 1976), and changes in the enzymic activity were followed as a function of time. For isolation of the HCl-treated RNAase-S, the reaction mixture was diluted threefold with water, the pH of the reaction mixture was adjusted to 6.5 with 0.5M-NaOH and then the solution was dialysed against water and freeze-dried.

Results

Preparation of RNAase-Aa₁S

The digestion of RNAase-Aa₁ with subtilisin at pH 8.0 and 2°C did not indicate any loss in enzymic activity, at least up to 24 h (Fig. 1, inset, curve *a*). However, a gradual accumulation of active trypsin-sensitive materials in the digest was apparent on measuring the activity after trypsin treatment, showing the formation of active RNAase-S-type products. However, the formation of active RNAase-S-type products from RNAase-Aa₁ proceeded only up to a stage of 50% conversion (Fig. 1, inset, curve *b*). Under similar conditions the conversion of RNAase-A into RNAase-S is almost complete in 20 h (Fig. 1, inset, curve *c*). Even in the presence of increasing amounts of subtilisin, the formation of active RNAase-S-type products from RNAase-Aa₁ did not go to completion (Fig. 1, inset, curve *e*; subtilisin/RNAase-Aa₁ ratio 1:100, w/w). However, at this concentration of subtilisin, RNAase-Aa₁ lost activity after about 12 h (Fig. 1, inset, curve *d*). The active subtilisin derivative of RNAase-Aa₁ has been designated RNAase-Aa₁S.

The 12 h subtilisin digest of RNAase-Aa₁ was chromatographed on Amberlite XE-64 resin (Fig. 1). Most of the protein was eluted in two chromatographically distinct enzymically active fractions in peak positions corresponding to those of RNAase-Aa₁ (26 ml) and RNAase-A (39 ml) respectively. The active trypsin-sensitive protein fractions that were eluted in the position of the RNAase-A represent

Fig. 1. *Chromatographic behaviour of the subtilisin digest of RNAase-Aa₁*

The subtilisin digest of RNAase-Aa₁ (50% digestion) was chromatographed on a column (0.9 cm × 30 cm) of Amberlite XE-64 resin at pH 6.16. The protein load was 15 mg. A_{280} (○), protein concentration; A_{260} , enzymic activity before (●) and after (Δ) treatment with trypsin. The inset shows subtilisin digestion of RNAase-Aa₁ at pH 8.0 and 2°C. The protein concentration was 10 mg/ml. Curve (a) represents activities of subtilisin digests (enzyme/substrate ratio 1:500, w/w) of RNAase-Aa₁ and RNAase-A before tryptic digestion; the activities after tryptic digestion are represented by curves (b) and (c) respectively. Curve (d) represents the activity of RNAase-Aa₁ on subtilisin digestion at an enzyme/substrate ratio 1:100 (w/w), and curve (e) represents the activity of the same subtilisin digest after treatment with trypsin.

Fig. 2. *Chromatographic behaviour of the active reconstituted material generated on interchanging the protein and the peptide parts of RNAase-Aa₁S and RNAase-S*

Chromatography was carried out on columns (0.6 cm × 25 cm) of Amberlite XE-64 resin eluted with 0.2 M-sodium phosphate buffer, pH 6.06. The peak positions of RNAase-Aa₁S and RNAase-S on these columns are represented by (↓) and (↑) respectively. (a) Rechromatography of RNAase-Aa₁S (1.5 mg). A_{280} (○), protein concentration; A_{260} enzyme activity before (●) and after (Δ) digestion with trypsin. (b) Chromatography of the active material formed on mixing the peptide component of RNAase-Aa₁S (43 μg) with RNAase-S-protein (180 μg). (c) Chromatography of the active material formed by mixing the protein component of RNAase-Aa₁S (210 μg) with RNAase-S-peptide (55 μg). In (b) and (c): A_{220} (○), protein elution pattern; A_{260} (●), enzyme activity.

RNAase-Aa₁S. RNAase-Aa₁S, the protein eluted between 45 and 60 ml and completely free of RNAase-Aa₁, was isolated by dialysis against water followed by freeze-drying. On rechromatography, RNAase-Aa₁S (Fig. 2a) was eluted as a homogeneous com-

ponent in a position distinct from that of RNAase-S. The retardation of RNAase-Aa₁ on Amberlite columns after its conversion into RNAase-Aa₁S is very similar to that for RNAase-A when digested to give RNAase-S.

Fractionation of RNAase-Aa₁S by trichloroacetic acid and characterization of the protein and peptide components

RNAase-Aa₁S was fractionated with trichloroacetic acid, and the resulting precipitate and supernatant were separated. The trichloroacetic acid precipitate and supernatant of RNAase-Aa₁S were inactive when assayed separately, but reconstituted nearly complete activity when they were present together. Thus the trichloroacetic acid fractions of RNAase-Aa₁S are similar in their activity behaviour to those of RNAase-S. Therefore the trichloroacetic acid precipitate and supernatant of RNAase-Aa₁S are designated RNAase-Aa₁S-protein and RNAase-Aa₁S-peptide respectively.

RNAase-S-peptide and RNAase-Aa₁S-peptide had the same electrophoretic mobility on paper at pH 6.5. The amino acid composition of the two peptides (Table 1) were nearly the same, demonstrating that the S-peptide portion of RNAase-Aa₁S is the same as that of RNAase-S. This was further confirmed by reconstitution experiments in which the peptide and the protein components of RNAase-Aa₁S and

RNAase-S were interchanged. Reconstitution was performed by incubating the protein and the peptide samples at pH 6.0 for 30 min at room temperature. The reconstituted materials were studied for their chromatographic behaviour on a column (0.6 cm × 25 cm) of Amberlite XE-64 resin at pH 6.06 (Figs. 2b and 2c). The chromatographic positions for the reconstituted derivatives, RNAase-S' and RNAase-Aa₁S', which are the same as those for RNAase-S and RNAase-Aa₁S, are indicated in Fig. 2. The chromatographic behaviour of RNAase-S protein after reconstitution with RNAase-Aa₁S-peptide (Fig. 2b) identifies the reconstituted protein to be RNAase-S'. A similar chromatographic study of RNAase-Aa₁S-protein reconstituted with RNAase-S-peptide (Fig. 2c) shows that the reconstituted protein chromatographs in the position of RNAase-Aa₁S'. Thus the chromatographic position of the reconstituted active proteins was determined by the protein component present in the reconstitution mixture. This demonstrates that the structural differences between RNAase-S and RNAase-Aa₁S lies in the 'protein' region of RNAase-Aa₁S.

Titration of RNAase-S-protein with RNAase-S-peptide showed a sharp break in the activity curve at equivalence point (Fig. 3), showing strong non-covalent interaction of the complementing fragments under the assay conditions (Richards & Vithayathil,

Table 1. *Amino acid composition of RNAase-S-peptide and RNAase-Aa₁S-peptide*

Samples were hydrolysed with 6.0M-HCl in sealed evacuated tubes for 22h at 110°C. No corrections have been applied in the calculations for either destruction or incomplete release of amino acids during acid hydrolysis. The glutamic acid content was taken as reference for calculation of amino acid composition.

Amino acid	Content (relative to glutamic acid)		
	RNAase-S-peptide		RNAase-Aa ₁ S-peptide
	Theoretical*	Observed	
Lysine	2.0	2.1	2.2
Histidine	1.0	1.1	0.9
Arginine	1.0	1.0	1.0
Aspartic acid	1.0	1.2	1.2
Threonine	2.0	1.7	1.6
Serine	3.0	3.2	2.8
Glutamic acid	3.0	3.0	3.0
Proline	—	—	—
Glycine	—	—	—
Alanine	5.0	4.4	4.4
Half-cysteine	—	—	—
Valine	—	—	—
Methionine	1.0	0.7	0.7
Isoleucine	—	—	—
Leucine	—	—	—
Tyrosine	—	—	—
Phenylalanine	1.0	0.9	0.8

* Results from Richards & Vithayathil (1959); Doscher & Hirs (1967).

Fig. 3. *Titration of the S-protein parts of RNAase-S and RNAase-Aa₁S with their corresponding peptide components*

The S-protein components (5 μg) of RNAase-S and RNAase-Aa₁S were mixed with various amounts of the corresponding peptide components and the enzymic activity generated was measured at pH 5.1 with RNA as a substrate. The measured activity is shown as a percentage of the activity of the parent protein (RNAase-S or RNAase-Aa₁S) corresponding to 5 μg of the S-protein used for titration studies. ○, Titration behaviour of RNAase-S protein with RNAase-S-peptide and ●, RNAase-Aa₁S-protein with RNAase-Aa₁S-peptide.

Fig. 4. *U.v.-absorption spectrum of RNAase-Aa₁S* RNAase-S and RNAase-Aa₁S were dissolved in 0.2M-sodium phosphate buffer, pH 6.02, at a protein concentration of 0.7mg/ml, and the absorption spectrum was recorded at 23°C in a Cary 14 recording spectrophotometer. A light-path of 1cm was used. The u.v. spectra of RNAase-Aa₁S (----) and RNAase-S (—) are shown.

1959). However, such a sharp break at the equivalence point was not observed when RNAase-Aa₁-S-protein was titrated with RNAase-S-peptide. This suggests that the interaction of the peptide tail (S-peptide) of RNAase-Aa₁S with its protein part (deamidated S-protein) is not as strong as that between RNAase-S-protein and RNAase-S-peptide.

The u.v.-absorption spectra of RNAase-Aa₁S and RNAase-S in 0.2M-sodium phosphate buffer, pH 6.02 (Fig. 4), showed the overall similarity in the gross conformation of the two proteins. The slight increase seen around 285 nm in the u.v.-absorption spectrum of RNAase-Aa₁S, relative to that of RNAase-S, suggests changes in the environment of one or more tyrosine residues of the protein. This is presumably due to the weaker interaction of RNAase-S-peptide with the deamidated S-protein as compared with that in RNAase-S (Fig. 3). Also, in common with RNAase-S, RNAase-Aa₁S cross-reacted with complete identity with RNAase-A antiserum by double diffusion on agar plates, which indicated similarity in the antigenic structures of these two proteins.

Resistance of an equimolar mixture of RNAase-Aa₁ and RNAase-Aa₁S to subtilisin modification

It may be recalled that whereas the subtilisin digestion of RNAase-A (resulting in the formation of RNAase-S) proceeds to completion, the conversion of RNAase-Aa₁ into RNAase-Aa₁S did not go to completion but stopped at a stage of 50% conversion (Fig. 1, inset). To understand the apparent resistance of this 50% of RNAase-Aa₁ to subtilisin, the unchanged RNAase-Aa₁ in the subtilisin digest was isolated (Fig. 1; protein eluted between 20 and 35 ml; contains a small amount of RNAase-Aa₁S) and was again subjected to subtilisin digestion (Fig. 5, inset). Again there was a gradual formation of active trypsin-sensitive molecules and the digestion again stopped when the amount of trypsin-sensitive material (RNAase-Aa₁S) in the digest was nearly 50%. The chromatographic behaviour of this digest (Fig. 5) was similar to that of the original digest (Fig. 1). It therefore appeared that formation of an equimolar complex between RNAase-Aa₁ and RNAase-Aa₁S was preventing the subtilisin digestion from proceeding to completion. To verify this, the subtilisin digestion of RNAase-Aa₁ was carried out in the presence of an equal amount of added RNAase-Aa₁S, and the activity changes are shown in Fig. 6 along with those for the digestion of RNAase-Aa₁ and RNAase-Aa₁S. The mixture of RNAase-Aa₁ and RNAase-Aa₁S is markedly resistant to subtilisin digestion (curve c), compared with RNAase-Aa₁ (curve b). These results indicate that some non-covalent interaction between RNAase-Aa₁ and RNAase-Aa₁S is responsible for the resistance of the mixture to subtilisin digestion.

HCl treatment of RNAase-S

With a view to examining the possibility of obtaining RNAase-Aa₁S by the controlled deamidation of RNAase-S, the latter was subjected to acid treatment under conditions similar to those used for RNAase-A (Manjula *et al.*, 1976). There is a marked difference in the kinetics of inactivation of RNAase-S and RNAase-A (Fig. 7, inset). The activity curves of both RNAase-A and RNAase-S have an initial period during which the enzymes maintain their original activity and a later stage where the enzymes undergo progressive inactivation. For RNAase-A, the initial period of full activity is about 12 h, whereas for RNAase-S full activity is maintained only up to about 6 h. In the subsequent period, the rate of inactivation of RNAase-S was faster than that of RNAase-A.

On chromatography on Amberlite XE-64 (Fig. 7), RNAase-S after 10 h treatment with HCl was resolved into at least two major fractions, which were eluted in the peak positions of RNAase-Aa₁S (70 ml) and RNAase-S (128 ml) respectively. (The protein eluted

Fig. 5. Susceptibility of unchanged RNAase-Aa₁ (see Fig. 1) to subtilisin digestion

The unchanged RNAase-Aa₁ (the trypsin-resistant activity) present in the subtilisin-digested RNAase-Aa₁ (see the text and Fig. 1) was isolated by chromatography on Amberlite XE-64 resin and was redigested with subtilisin at pH 8.0 and 2°C at an enzyme/substrate ratio 1:500 (w/w) for 12 h. Again 50% of the starting activity became trypsin-sensitive (see the inset). This material (2.8 mg) was chromatographed on a column (0.6 cm × 25 cm) of Amberlite XE-64 resin at pH 6.05. A_{280} (○), protein concentration. The enzymic activity (A_{260}) before (●) and after (△) tryptic digestion is shown. The inset shows the changes in the activity of unchanged RNAase-Aa₁ during its subtilisin digestion. The activity of the above subtilisin digest has been measured before (○) and after (●) tryptic digestion to follow the formation of RNAase-Aa₁S.

Fig. 6. Resistance of an equimolar mixture of RNAase-Aa₁ and RNAase-Aa₁S to subtilisin modification

RNAase-Aa₁, RNAase-Aa₁S and an equimolar mixture of RNAase-Aa₁ and RNAase-Aa₁S (all at a protein concentration of 10 mg/ml) were incubated with subtilisin at an enzyme/substrate ratio 1:500 (w/w) at pH 8.0 and 2°C. Curve (a) represents the enzymic activity, before tryptic digestion, of the subtilisin digest of RNAase-Aa₁ and RNAase-Aa₁S as well as the equimolar mixture of RNAase-Aa₁ and RNAase-Aa₁S. Curve (b) represents the activity of subtilisin digest of RNAase-Aa₁ after tryptic digestion. The activity of the subtilisin digest of the equimolar mixture of RNAase-Aa₁ and RNAase-Aa₁S after tryptic digestion is shown by curve (c).

at 30 ml was inactive, whereas the other two major peak fractions were active.) The yield of the first fraction from 10 h HCl-treated RNAase-S is considerably greater than the yield of RNAase-Aa₁ from 10 h HCl-treated RNAase-A. This RNAase-S derivative (fractions eluted between 45 and 81 ml) was isolated by dialysis and freeze-drying. Since the chromatographic-peak position of this RNAase-S derivative bears the same relation to the position of RNAase-S as does the position of RNAase-Aa₁ to that of RNAase-A, this derivative has been designated RNAase-Sa₁. On rechromatography, RNAase-Sa₁ was eluted as an unsymmetrical peak (Fig. 8a). The absorbance peak preceded the activity peak, indicating the heterogeneity of the material under this peak.

Characterization of the peptide and protein components of RNAase-Sa₁

RNAase-Sa₁ was fractionated by trichloroacetic acid into the peptide and the protein components. These peptide and protein components (inactive by themselves) reconstituted enzyme activity in the presence of each other. The protein and the peptide parts of RNAase-Sa₁ were examined by studying their chromatographic behaviour in the presence of

Fig. 7. *Chromatography of RNAase-S maintained in 0.5M-HCl at 30°C for 10h*

RNAase-A and RNAase-S, both at a concentration of 1 mM, were incubated in 0.5M-HCl at 30°C and samples of the protein solutions were withdrawn after various periods of incubation and placed in a suitable volume of 0.1M-sodium acetate buffer, pH 5.1, for measurement of enzymic activity. The inset shows the activity changes of RNAase-A (○) and RNAase-S (●). For the chromatographic study, a sample of RNAase-S treated with 0.5M-HCl for 10h was isolated as described in the Materials and Methods section. The protein (80mg) was chromatographed on a column (1.7cm×25cm) of Amberlite resin at pH 6.12. A_{280} , protein concentration; A_{260} , enzymic activity.

Fig. 8. *Chromatographic behaviour of the active material formed on mixing the protein and peptide components of RNAase-Sa₁ with RNAase-S-peptide and RNAase-S-protein*

The chromatography of the protein samples was carried out on columns (0.6cm×25cm) of Amberlite XE-64 resin at pH 6.06. The peak positions of RNAase-S and RNAase-Aa₁S are indicated by (↑) and (↓) respectively. (a) Rechromatography of a sample of 1.2mg of RNAase-Sa₁. A_{280} , protein concentration; A_{260} , enzyme activity. (b) Chromatography of active material formed on mixing the protein part of RNAase-Sa₁ (500µg) with RNAase-S-peptide (130µg). (c) Chromatography of the active material formed on mixing the peptide component of RNAase-Sa₁ (145µg) with RNAase-S-protein (750µg). In (b) and (c): A_{220} , protein concentration; A_{260} , enzymic activity.

RNAase-S-peptide and RNAase-S-protein respectively (Figs. 8b and 8c). When the protein part of RNAase-Sa₁ was reconstituted with RNAase-S-peptide, the enzymically active material generated

was eluted in the position of RNAase-Aa₁S' (Fig. 8b). Unlike RNAase-Sa₁ (Fig. 8a), the reconstituted material was eluted as a symmetrical peak, and the specific activity was nearly the same as that of

Fig. 9. *Electrophoretic behaviour of RNAase-Sa₁ peptide*
The electrophoresis of the peptide components of RNAase-Sa₁ and RNAase-S was carried out at pH 6.5 (pyridine/acetic acid/water, 10:1:89, by vol.) on Whatman no. 1 paper at a voltage gradient of 10V/cm for 5 h. The load on the paper was approx. 175 μ g. After electrophoresis the peptides were located by staining with Bromophenol Blue/HgCl₂ reagent (Lederer, 1957). (a) RNAase-S-peptide; (b) peptide component of RNAase-Sa₁. The vertical bars represent the origin.

RNAase-S. Hence the protein part of RNAase-Sa₁ is distinct from that of RNAase-S, and appears to be the same as that of RNAase-Aa₁S.

When the peptide part of RNAase-Sa₁ was reconstituted with RNAase-S-protein, the enzymically active material formed was resolved into two chromatographically distinct fractions (Fig. 8c), indicating the heterogeneity of this peptide. Nearly 70% of the protein was eluted at the position of RNAase-S', and had the same specific activity as that of RNAase-S. About 30% of the material was eluted at the position of RNAase-Aa₁S, with a specific activity 50–60% of that of RNAase-S. Since the protein portion of these two active RNAase-S-type species is RNAase-S-protein, the material eluted at the RNAase-S' position accounts for the unmodified S-peptide of RNAase-Sa₁ and that eluted at the RNAase-Aa₁S' position accounts for the modified S-peptide. The difference in the elution positions of RNAase-S' and RNAase-Aa₁S' represents the monodeamidation of RNAase-S; the 30% of material eluted at the RNAase-Aa₁S' position presumably represents monodeamidated RNAase-S. Apparently in this case the deamidation is in the S-peptide region of the complex.

The electrophoretogram of the peptide part of RNAase-Sa₁ showed that it is indeed a mixture of two peptides (Fig. 9). The major component (nearly 75%) has the same electrophoretic mobility as that of RNAase-S-peptide, and hence appears to be identical with RNAase-S-peptide. The cathodic mobility of the second component (nearly 25%) is less than that of RNAase-S-peptide, showing that

this derivative has a lower net positive charge at pH 6.5 than that of RNAase-S-peptide. RNAase-S-peptide has a single amide function, namely glutamine-11. On the basis of this electrophoretic behaviour and the chromatography of the reconstituted material, it has been tentatively concluded that this material is the deamidated S-peptide.

Discussion

RNAase-Aa₁, the monodeamidated derivative of RNAase-A, is acted on by subtilisin in much the same way as RNAase-A to yield an active RNAase-S-type of product, namely RNAase-Aa₁S. This derivative has the same specific activity as that of RNAase-S and an antigenic conformation similar to that of RNAase-A. However, some difference in the environment of tyrosine residues of RNAase-Aa₁S compared with that of RNAase-S was apparent in the u.v.-absorption spectrum. A noticeable difference in the interaction of deamidated S-protein and RNAase-S-peptide was also apparent in the titration of these two constituent fragments. Just as in RNAase-Aa₁S, the interaction between the peptide and the protein part in RNAase-Aa₁ is probably not as strong as that in RNAase-A. Such a decrease in strength of the binding of peptide tail to the protein may very well be responsible for the slight changes previously observed in the thermal-transition behaviour of RNAase-Aa₁ (Manjula *et al.*, 1976).

The subtle differences in the structures of RNAase-A and RNAase-Aa₁ resulting from the monodeamidation is also apparent in the resistance of an equimolar mixture of RNAase-Aa₁ and RNAase-Aa₁S to subtilisin digestion. The nature of the interaction between RNAase-Aa₁ and RNAase-Aa₁S responsible for such resistance is not clear at present. Presumably these non-covalent interactions are not very strong, since under the conditions of Amberlite chromatography (0.2M-phosphate buffer, pH 6.0–6.3) no such interactions are apparent. Even the preliminary studies on the gel-filtration behaviour of such a subtilisin-resistant mixture on Sephadex G-75 in the phosphate buffer system (Crestfield *et al.*, 1962) indicated that no stable complex of the type of dimers of RNAase-A is present in the mixture of RNAase-Aa₁ and RNAase-Aa₁S. However, it remains to be seen whether the phosphate buffer used in Amberlite chromatography and the gel-filtration studies reverses the interactions originally present between RNAase-Aa₁ and RNAase-Aa₁S in the (NH₄)₂CO₃ buffer system used for subtilisin digestion. Further, a fully active derivative of RNAase-A formed by exposing the native enzyme to an aqueous environment at pH 11.0 and at 25°C (Vithayathil *et al.*, 1967) is not stable under conditions of CM-cellulose chromatography (Taborsky, 1959), and dissociated to give 50% RNAase-A and

an inactive species. On the other hand, it chromatographed on Amberlite columns as a complex showing signs of dissociation. More recently it has been observed that phosphate helps to reverse the 'trapped conformation' present in acid-treated RNAase-A (Das, 1975).

RNAase-S also underwent structural and activity changes similar to those of RNAase-A during the initial stages of acid denaturation, and the primary reaction appeared to be the deamidation of the S-protein part. In addition there was modification in the S-peptide region as well, presumably deamidation at the single glutamine residue at position 11. By synthetic procedures it has been shown that this glutamine residue can be replaced by a glutamic acid residue without a major effect on the activity (Finn & Hofmann, 1965; Scoffone *et al.*, 1967b). In the studies of Scoffone *et al.* (1967b), arginine-10 of S-peptide was replaced by an ornithine residue, and glutamine-11 by a glutamic acid residue. Such a peptide showed an activity of 37% in the presence of equimolar amounts of S-protein, whereas when used in tenfold molar excess over S-protein it exhibited 57% activity. The deamidated derivative isolated in the present study appeared to exhibit nearly 50–60% activity at equivalence.

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