ACTIVITY AND ANTIGENICITY OF RIBONUCLEASE HYBRIDS

Gjalt W. WELLING, Johannes A. LENSTRA and Jaap J. BEINTEMA
Biochemisch Laboratorium, Rijksuniversiteit, Zernikelaan, Groningen, The Netherlands

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1. Introduction

Since the preparation of RNase-S* by Richards [1] there has been a considerable interest in its two components, S-peptide and S-protein. The S-peptide (residues 1–20) has attracted most attention, because it has been possible to synthesize analogs of it. By mixing it with S-protein, the influence of amino acid modifications, replacements or removal on activity and binding, could be assayed. It has turned out that there are residues which are important for binding (Glu-2, Phe-8, Arg-10, Gln-11, Met-13, Asp-14) and for enzymic activity (His-12) [2,3]. Except for Met-13, which is replaced by Val or Ile in a few ribonucleases, all these residues are invariant in pancreatic ribonucleases of 23 animal species [4]. A number of these ribonucleases, too, were capable of cleavage by subtilisin and the S-peptides were isolated [5]. It seemed of interest to determine what effect the substitutions which have occured during molecular evolution of a certain ribonuclease have on enzymic activity and binding. Natural S-peptides from cow, dromedary and kangaroo ribonuclease were used. Rat ribonuclease cannot be cleaved by subtilisin [5,6], so synthetic rat S-peptides (13 and 17 residues long) were used instead. S-peptides were combined with a constant amount of S-protein from dromedary or bovine ribonuclease and the activity was measured with cytidine 2′,3′-cyclic phosphate as a substrate.

*Abbreviations: RNase-S, native ribonuclease cleaved by subtilisin. The resulting active complex can be separated into its two inactive components: S-peptide and S-protein. Upon remixing equimolar amounts of S-peptide and S-protein, active RNase-S′ is formed.

Immunologic techniques have proved to be sensitive probes of the surface structure of proteins in solution [7]. RNase-S, S-peptide and S-protein have been subject of such studies [8,9]. In this study, the surface structure of ribonuclease hybrids consisting of cow, dromedary, kangaroo and rat S-peptides combined with bovine S-protein is scanned by utilising antiserum directed against RNase-S.

2. Materials and methods

Bovine pancreatic ribonuclease was obtained from Miles-Seravac Ltd. (Maidenhead). Kangaroo and dromedary ribonucleases were isolated by affinity chromatography as described by Wierenga et al. [10]. Rat ribonuclease was isolated as described by Beintema et al. [11]. Bovine S-protein was from Sigma Chemical Company (St Louis) or prepared from RNase-S [5]. which was purified on SE-Sephadex C-25 (Pharmacia, Uppsala) as described by Dunn et al. [12]. Bovine RNase S and cytidine 2′,3′-cyclic phosphate (sodium salt) were purchased from Sigma Chemical Comp. (St Louis). Dromedary S-protein and S-peptides were isolated as described [5]. Rat S-peptides were synthesized by M. Voskuyl-Holtkamp and others (M. Voskuyl-Holtkamp, C. Schattenkerk and E. Havinga; to be published). Agarose was a product from BDH (Poole, Dorset). All other reagents were analytical grade products from Merck AG (Darmstadt).

Amino acids analysis, high-voltage paper electrophoresis, dansylation and dansyl-Edman degredation were performed as described [10,13]. Concentrations of S-peptide and S-protein solutions were determined by amino acid analysis.

2.1. *Activity measurements*

The hydrolysis of cytidine 2′,3′-cyclic phosphate was measured with a Zeiss PMQ II spectrophotometer equipped with a recorder (absorbance scale 0 to 0.05) at 284 nm at 25 ± 1°C as described by Crook et al. [14]. Peptide, protein, and substrate solutions were prepared in 0.1 M Tris-NaCl pH 7.13 [14]. At a given S-protein concentration (1.5×10^{-7} M for bovine S-protein; 2.0×10^{-7} M for dromedary S-protein), the slopes at a number of peptide concentrations were determined, giving activity values in ΔE/min. To calculate the apparent dissociation constant for the different complexes, the free S-peptide concentration $[\text{peptide}]_{\text{free}}$ should be known. This can be found as follows:

The reaction velocity a is proportional to the concentration of RNase S′,

$$\text{so } \frac{a}{a_{\max}} = \frac{[\text{RNase S}']}{[\text{RNase S}']_{\max}} = \frac{[\text{peptide}]_{\text{bound}}}{[\text{protein}]_{\text{added}}}, \quad (1)$$

where $a_{\max}$ is the velocity at saturating S-peptide concentration.

$$\text{Since } [\text{peptide}]_{\text{free}} = [\text{peptide}]_{\text{added}} - [\text{peptide}]_{\text{bound}}, \quad (2)$$

we obtain, combining (1) and (2):

$$[\text{peptide}]_{\text{free}} = [\text{peptide}]_{\text{added}} - \frac{a}{a_{\max}} \times [\text{protein}]_{\text{added}}. \quad (3)$$

A plot of $1/a$ against $1/[\text{peptide}]_{\text{free}}$ yields the reciprocal of the apparent dissociation constant as intercept on the abscissa (see Results and discussion). This procedure yields the same results as the method which Berger and Levit [15] developed for determination of dissociation constants in the RNase-S′ system from activity data, but it dispenses with complicated calculations.

K_M values for different hybrids were determined with S-protein which was fully saturated with S-peptide at substrate concentrations ranging from 0.06 to 0.77 mM.

2.2. *Immunologic methods*

Antiserum to bovine RNase-S was produced in rabbits as described by Westendorp Boerma et al. for Hb [16] with some modifications. A first course antiserum (S1) and a second course antiserum (S2) were used for the experiments.

Double immunodiffusions were performed in 2% agarose gel with antigens at a concentration of about 25 nmol/ml in 0.1 M Tris-NaCl pH 7.13.

Quantitative precipitation was performed by mixing equal volumes (200 or 50 μl) of antiserum and antigen in 0.1 M Tris-NaCl pH 7.13 and subsequent incubation at 37°C for 1 h. After 4 days at 4°C and carefully mixing each day, the solutions were centrifuged at 2000 *g* (1 h) and washed twice with 500 or 50 μl of cold buffer. The precipitate was dissolved in 1 ml or 250 μl of 0.5 M NaOH and absorbance was read at 280 nm using 1 cm or 1 mm quartz cuvettes for the macro and micro procedure, respectively.

3. Results and discussion

Bovine and dromedary S-protein were titrated with cow, dromedary, kangaroo and rat S-peptides, of which the amino acid sequences are shown in fig.1. The activity was determined with cytidine cyclic 2′3,′-phosphate as a substrate and the profiles of these titrations are shown in figs.2 and 3. No large differences in activities were observed when titrating bovine S-protein with cow, kangaroo, dromedary and rat S-peptides (fig.2). Titration of dromedary S-protein with cow and rat S-peptides showed that with excess S-peptide, the activity was about 90% of the homologous combination.

These data were used to calculate an apparent dissociation constant. The following scheme was assumed:

$$\text{S-protein} + \text{S-peptide} \rightleftharpoons \text{E}, \text{ and}$$

$$\text{E} + \text{S} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{ES} \xrightarrow{k_2} \text{E} + \text{P}$$

in which E denotes RNase-S′, S the substrate, and P the product. The dissociation constant K_d is equal to:

$$K_d = \frac{[\text{protein}] \times [\text{peptide}]}{[\text{E}]}, \quad (4)$$

	1								10									20
Cow	Lys	Glu	Thr	Ala	Ala	Ala	Lys	Phe	Glu	Arg	Gln	His	Met	Asp	Ser	Ser	Thr	Ser	Ala	Ala
Dromedary	Ser	Glu	Thr	Ala	Ala	Glu	Lys	Phe	Glu	Arg	Gln	His	Met	Asp	Ser	Tyr	Ser	Ser	Ser	
Kangaroo	Glu	Thr	Pro	Ala	Glu	Lys	Phe	Gln	Arg	Gln	His	Met	Asp	Thr	Glu	Thr	Ser	Thr	Ala	Ser
Rat 13 res.	Glu	Ser	Ser	Ala	Asp	Lys	Phe	Lys	Arg	Gln	His	Met	Asp							
Rat 17 res.	Gly	Glu	Ser	Arg	Glu	Ser	Ser	Ala	Asp	Lys	Phe	Lys	Arg	Gln	His	Met	Asp			

Fig.1. Amino acid sequences of cow, dromedary, kangaroo and synthetic rat S-peptides [4]. The numbering of bovine ribonuclease was used.

in which [protein] and [peptide] denote the concentration of free S-protein and S-peptide, respectively. The Michaelis constant

$$K_M = \frac{k_{-1} + k_2}{k_1}, \text{ which in the steady state equals}$$

$$K_M = \frac{[E] \times [S]}{[ES]} \qquad (5)$$

The initial velocity is proportional to the concentration of the ES complex, so if V_{max} is the maximal velocity (which is reached if no free enzyme or S-protein is left),

$$a = \frac{[ES]}{[ES]_{max}} \times$$

$$V_{max} = \frac{[ES]}{[ES] + [E] + [protein]} \qquad (6)$$

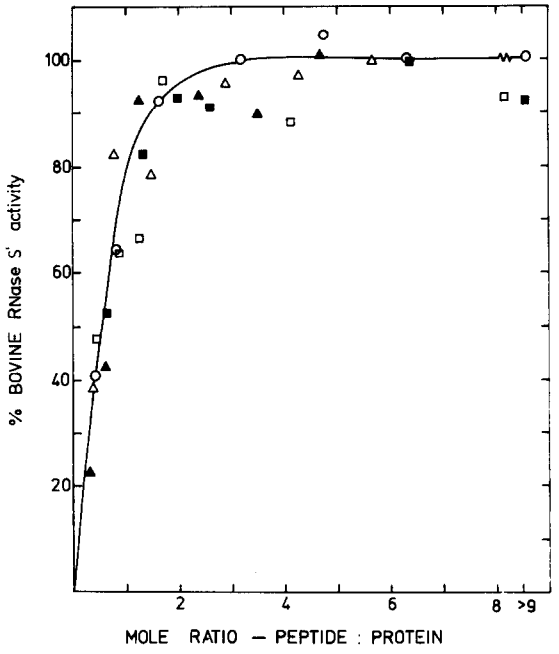

Fig.2. Activity of different S-peptide-bovine S-protein complexes using cytidine 2',3'-cyclic phosphate as substrate.(○) Cow S-peptide; (△) kangaroo S-peptide; (▲) dromedary S-peptide; (□) rat S-peptide (17 residues); (■) rat S-peptide (13 residues).

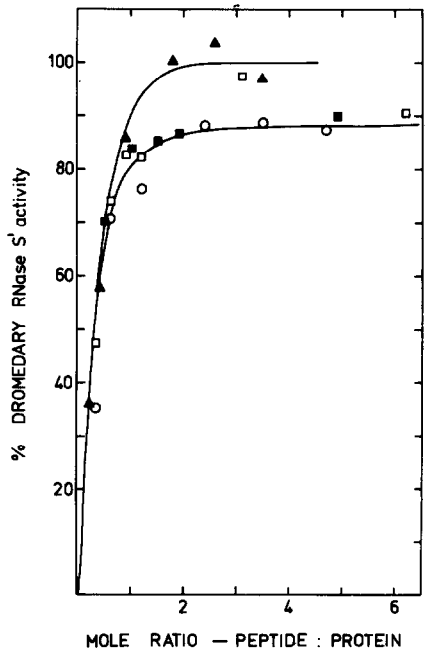

Fig.3. Activity of different S-peptide-dromedary S-protein complexes using cytidine 2',3'-cyclic phosphate as substrate. (○) Cow S-peptide; (▲) dromedary S-peptide; (□) rat S-peptide (17 residues); (■) rat S-peptide (13 residues).

Combining (4), (5) and (6) and taking the reciprocal value, results in:

$$\frac{1}{a} = \frac{1}{V_{max}} \left(1 + \frac{K_M}{[S]} + \frac{K_M \times K_d}{[S] \times [peptide]}\right). \qquad (7)$$

If, at a constant concentration of substrate and added S-protein, $1/a$ is plotted against $1/$ [peptide] then K_d follows from the value of $1/$[peptide] at the intercept of the abscissa:

$$[peptide]_{1/a = o)} = -\frac{K_d}{1 + \frac{[S]}{K_M}} = -K_d', \qquad (8)$$

in which K_d' is the apparent dissociation constant at a given substrate concentration. From equation (8) we conclude that the substrate concentration may have a marked influence on K_d'. However, if $[S] \ll K_M$, $1 + S/K_M \approx 1$ and $K_d' \approx K_d$. To find the right substrate concentration, K_M values were determined. K_M values for the five ribonuclease hybrids containing bovine S-protein were about equal, 1.3 to 2.0 mM. A similar behaviour was observed for the four hybrids containing dromedary S-protein, which showed values

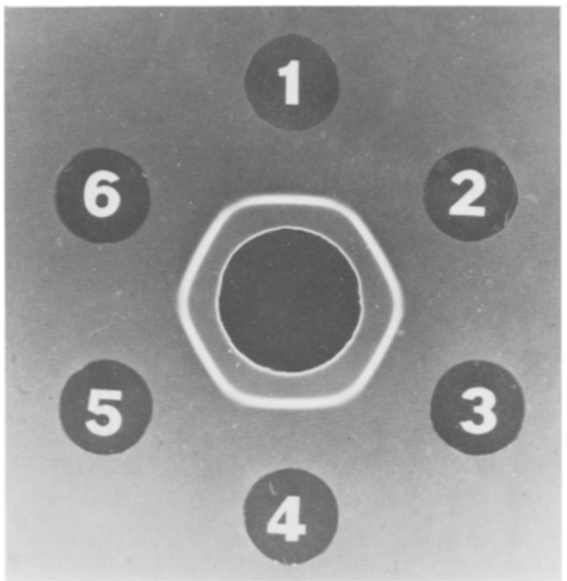

Fig.4. Immunodiffusion study in which undiluted antiserum directed against bovine RNase-S (S2) was developed against bovine S-protein (wells 1 and 4) and against complexes of S-peptides with bovine S-protein. S-peptides used: dromedary (2), rat 13 residues (3), kangaroo (5) and cow (6). Antigen concentration was 0.5 mg/ml.

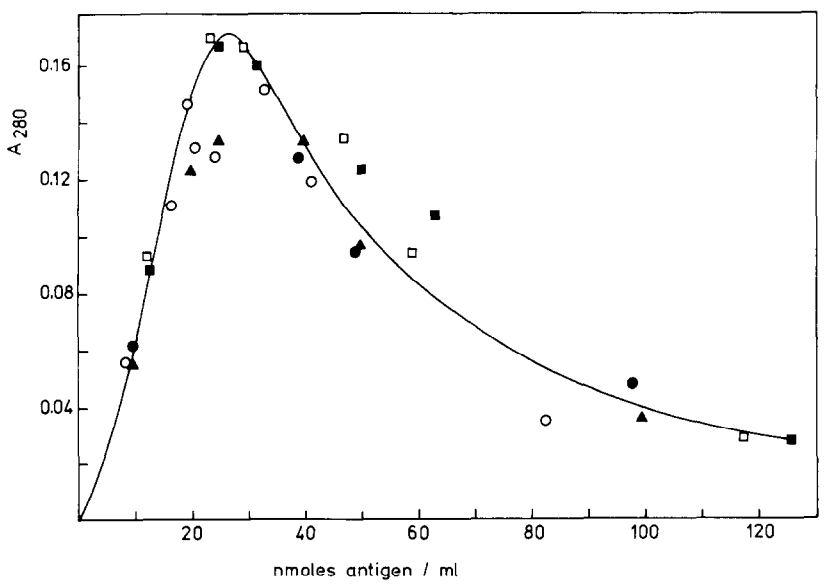

Fig.5 Quantitative precipitation (micro-method, see 2.2.) in which antiserum directed against bovine RNase-S (S1), was allowed to react with bovine S-protein (●) and complexes of bovine S-protein with different S-peptides from cow (○), dromedary (▲), rat 17 residues (□) and rat 13 residues (■).

ranging from 0.7 to 1.1 mM. Activity measurements were made at a substrate concentration of 0.16 mM, so K_d should be 1.1 to 1.2 K_d'. In this way, K_d' values for the hybrids containing bovine S-protein were found to be 1.0–4.5 × 10^{-8} M and those for the hybrids containing dromedary S-protein, 0.5–1.3 × 10^{-8} M. These values are in reasonably good agreement with those determined by others using cytidine 2',3'-cyclic phosphate as a substrate (1 × 10^{-7} [17]; 1 × 10^{-8} [18]; 5 × 10^{-7} [19]; 3.6 × 10^{-8} [20]). The dissociation constants found by using direct binding assays [21] are consistently three orders of magnitude higher, which is most probably due to aggregation of S-protein at the high protein concentrations used [19,22]. However, the value found with agarose-bound S-protein is also rather high (2.5 × 10^{-6} M) [19], indicating that in some way agarose may influence the binding.

An immunologic comparison has been made for the ribonuclease hybrids containing bovine S-protein. In fig.4 an example is shown of an Ouchterlony immunodiffusion test. For all hybrids, and also for free bovine S-protein, complete identity was observed. Quantitative precipitation (micro-adaption, see Materials and methods), too, failed to reveal any difference between the various hybrid ribonucleases and bovine S-protein (fig.5). In this case antiserum S1 was used. To enhance possible differences, the macro-test was performed using antiserum S2 and as antigens, bovine S-protein, and cow and rat S-peptide (17 residues) combined with bovine S-protein. Again no differences were observed (fig.6). The reaction of free S-protein indicates that it has a similar structure as the whole complex. However, antibodies fitting to the hybrid molecule may force an S-protein into the correct shape.

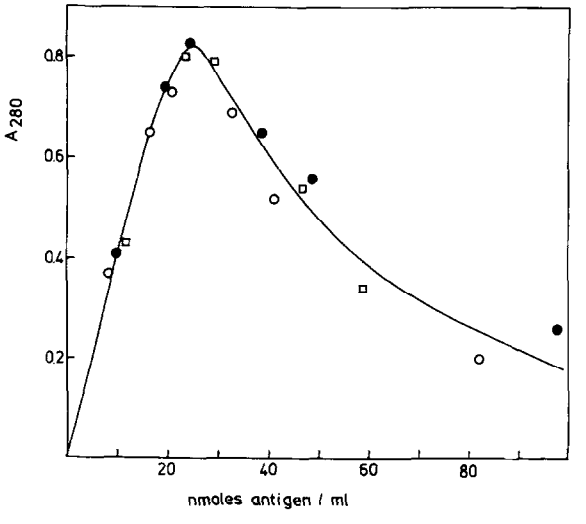

Fig.6. Quantitative precipitation (see 2.2.). Antiserum to bovine RNase-S (S2) was allowed to react with bovine S-protein (•), and complexes of bovine S-protein with cow S-peptide (○) and rat S-peptide (17 residues) (□).

In conclusion, the results indicate that despite the many differences in primary structures of the S-peptides which have originated during molecular evolution, there are no important differences between the RNase hybrids with respect to binding constant, maximal activity, Michaelis constant of the catalysed reaction and antigenic properties.

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