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Des-lysyl Glutamyl and Des-lysyl Pyroglutamyl Ribonucleases. III. Enzymatic Activities and Conformational Stabilities

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ABSTRACT: Comparative studies have been made on the enzymatic activities and the digestibilities by trypsin of des-lysyl glutamyl and des-lysyl pyroglutamyl ribonucleases and ribonuclease A. The des-lysyl glutamyl enzyme is slightly less active and slightly more susceptible to trypsin digestion than ribonuclease A is, but the des-lysyl pyroglutamyl enzyme is considerably less active and more susceptible to digestion. The results therefore indicate that lysine-1 plays a minor role in maintaining an active and stable conformation of ribonuclease A molecule but glutamic acid-2 plays a striking role.

The isolation and characterization of des-lysyl glutamyl and des-lysyl pyroglutamyl ribonucleases have been reported in the preceding papers (Eaker *et al.*, 1965a,b). A unique opportunity is therefore provided for a study of the effects of deletion and substitution of lysine-1 and glutamic acid-2 in ribonuclease A molecule on its function and stability. These results are described in this paper. The studies are of interest as the amino-terminal segment of ribonuclease A, comprising the first twenty-five residues, is held to the remainder of the molecule by noncovalent forces (Richards and Vithayathil, 1960), and also this segement of the molecule contains one of the two histidine residues essential for the enzymatic activity (Crestfield *et al.*, 1963).

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Experimental

Ribonuclease activities were studied as follows.

TITRIMETRIC ASSAY. Fractions were analyzed for activity toward cytidine 2′,3′-phosphate using Uziel's modification (Gundlach *et al.*, 1959) of the quantitative titrimetric procedure described by Davis and Allen (1955). Commercial samples of barium salt of cytidine 2′,3′-phosphate, contaminated with cytidylic acid, were used directly without purification. The contaminant could be estimated accurately by titration, and the calculations were made using a value of 6.04 for the pK_2 of cytidylic acid.

SPECTROPHOTOMETRIC ASSAY. The activity was measured by the change in spectrum which occurs in the enzymic depolymerization of yeast ribonucleic acid (Kunitz, 1946). For use in this assay, commercial yeast nucleic acid was deproteinized (Sevag *et al.*,

TABLE I: Comparative Activities of Ribonuclease Fractions under Different Assay Conditions.

Enzyme[a]	Specific Activity Relative to Ribonuclease A[b]		
	Hydrolysis of Cytidine 2′:3′-Phosphate[c]	Oligonucleotide Release from Yeast Nucleic Acid[d]	Spectral Change of Yeast Nucleic Acid[e]
Ribonuclease A	1.00	1.00	1.00 (1.00)
Glutamyl ribonuclease	0.99 ± 0.02	1.05	0.90 (0.98)
Pyroglutamyl ribonuclease	0.38 ± 0.01	0.57	0.61 (0.80)
Ribonuclease (Armour 381-062)	0.90 ± 0.08		
Ribonuclease (Armour 381-059)	0.95 ± 0.08		

[a] Stock enzyme solutions were prepared from samples of known moisture content. [b] Individual assays were reproducible to within 5%. [c] Assays were performed in 0.25 M Na_2SO_4 at 30° and *p*H 7.0 using a nominal substrate concentration of 0.008 M and enzyme concentrations between 3 and 25 μg/ml. The values reported are the averages of the assays with fractions isolated from four separate experiments. [d] Assays were carried out at 37° in 0.10 M sodium acetate buffer, *p*H 5.1, with 2 μg/ml of enzyme and 3.3 mg/ml of substrate. [e] Assays were carried out at 25° in 0.05 M sodium acetate buffer, *p*H 5.0, with 0.5–2.0 μg/ml of enzyme and 0.5 mg/ml of substrate. Values in parentheses are results obtained with a different preparation of substrate (see text).

1938) and was freed of oligonucleotides by repeated precipitation with acetic acid, followed by dialysis.

PRECIPITABILITY ASSAY. The activity was followed by the release of acid-soluble nucleotides from yeast ribonucleic acid, using the uranyl acetate-perchlorate procedure of Anfinsen *et al.* (1954), as modified by Kalnitsky *et al.* (1959). Yeast nucleic acid was purified as above.

Susceptibilities toward trypsin digestion were measured as follows. Solutions of the three ribonucleases (2–6 mg/ml) were incubated with various concentrations of trypsin (0.1–0.4 mg/ml) for 6 to 24 hours at 25 and 40°. A commercial sample of twice-crystallized, salt-free trypsin (Worthington lot 815–18) was used directly.

Incubations were carried out in the following buffers: 0.05 M Tris-HCl, *p*H 8.05, and 0.10 M sodium phosphate, *p*H 7.26. Buffers were boiled before use and a drop of chloroform was added to allay the growth of microorganisms. Digestions were terminated by the addition of equal volumes of glacial acetic acid to the incubation mixtures. To estimate the approximate degrees of digestion, aliquots corresponding to 4–11 mg of protein were passed through a 50- × 0.9-cm column of Sephadex G-75 in 50% acetic acid at 4°. The effluent was collected in 2-ml fractions and 200-μl aliquots were analyzed by the ninhydrin procedure after alkaline hydrolysis.

Membrane diffusion experiments were carried out with 2–4 samples of protein using a dialysis cell of the type described by Craig and Konigsberg (1961). The dialysis membrane was 20/32 Visking cellophane casing. Diffusates were analyzed by the Folin procedure of Lowry *et al.* (1951). The semilogarithmic escape plots (Craig and King, 1962) were usually linear up to 60–80% escape. The diffusion rates are expressed as 50% escape times.

Results

Enzymic Activities of Ribonuclease A and the Des-lysyl Ribonucleases. In Table I are given the relative enzymatic activities of the three purified ribonucleases. The activities of two commercial preparations of ribonuclease are also reported for comparison. In the first column of the table are listed their activities toward the synthetic substrate, cytidine 2′,3′-phosphate. Although the glutamyl enzyme is fully active toward this substrate, the pyroglutamyl form is only 38% as active as ribonuclease A. Only the relative activities are reported here, as the absolute activities vary with the purity of substrates used. All samples of substrate employed in these studies contained some cytidylic acid, a competitive inhibitor of ribonuclease (Hummel *et al.*, 1961). However, the amount of inhibitor present did not affect the relative activities of these proteins. This is evidenced by the small deviations from the average values of the four sets of assays performed with substrates of different purities and enzymes from four separate isolations.

Kinetic studies were carried out at *p*H 7 using a substrate preparation which was 19% cytidylic acid and 81% cytidine 2′,3′-phosphate, with the assumption that the *p*H optima of the activities of des-lysyl ribonucleases were the same as that of ribonuclease A. Initial velocities were determined at nominal substrate concentrations of 0.007 M, 0.014 M, 0.035 M, and 0.070 M. The relative specific activities given in the first column of Table I were found to apply over this tenfold range of substrate concentration. Analysis of the data according to the method of Lineweaver and Burk (1934) indicated that the glutamyl enzyme is catalytically identical with ribonuclease A under these conditions. The pyroglutamyl enzyme has the same substrate affinity and inhibitor affinity as ribonuclease A, but

TABLE II: Digestibilities of Ribonuclease A and Des-lysyl Ribonucleases by Trypsin.[a]

Buffer	Temperature (°C)	Ribonuclease A (%)	Des-lysyl Glutamyl Ribonuclease (%)	Des-lysyl Pyroglutamyl Ribonuclease (%)
Phosphate, *p*H 7.26	40	5	11	42
Tris-HCl,	40	33		
*p*H 8.05	25	3	12	21

[a] The digestion experiments were carried out using an enzyme substrate ratio of 1:20 at the indicated temperature for 13 hours.

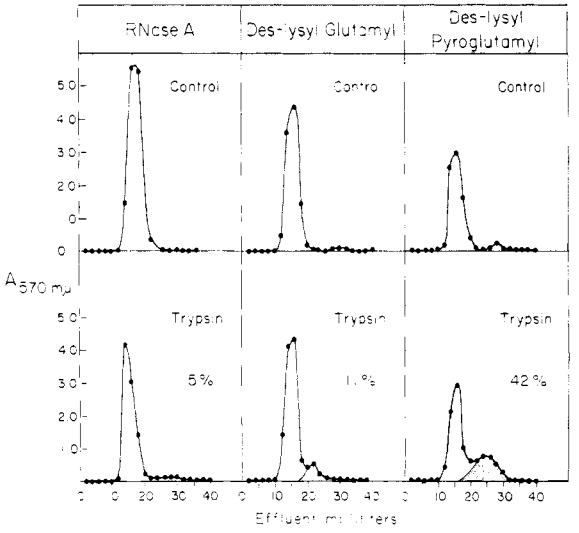

FIGURE 1: Patterns obtained by passage of incubation mixtures through a 50- × 0.9-cm column of Sephadex G-75 in 50% acetic acid (v/v) at 4° after incubation of native samples of the three ribonucleases for 13 hours at 40° in 0.1 M sodium phosphate buffer, *p*H 7.26, in the presence and absence of trypsin. The ordinate is the ninhydrin color obtained after alkaline hydrolysis of 200-μl aliquots of the effluent fractions. The percentages affixed to the lower patterns indicate that fraction of the total ninhydrin color represented by the stippled areas and give a rough measure of the extent of tryptic degradation.

its maximal velocity is only 37–38% of that found for ribonuclease A. Apparently some conformational lesion is present in the pyroglutamyl enzyme which disrupts the catalytic site slightly without altering the substrate or product binding site.

The relative specific activities of the three enzymes toward yeast nucleic acid are listed in the second and third columns of Table I. Ribonuclease A and the glutamyl enzyme are about equally active toward this substrate, as determined by two techniques, but the pyroglutamyl enzyme again shows only partial activity.

The oligonucleotide-release assays were carried out with only one substrate preparation. The spectrophotometric assays were performed with two different substrate preparations. Similar specific activities (65 and 67 Kunitz units/mg) were obtained for ribonuclease A with the two substrate preparations but, surprisingly, the relative specific activities of the three enzymes were different in the two sets of assays. The magnitude of the total decrease in absorbancy at 300 mμ obtainable upon exhaustive degradation of yeast nucleic acid with ribonuclease provides an indication of its initial degree of polymerization. By this criterion, the two preparations used in the assays differed somewhat in their degree of polymerization. The more highly polymerized of the two preparations gave the higher of the two specific activity values obtained with each of the three enzymes. Thus it would appear possible that the relative specific activity of the pyroglutamyl enzyme might approach even that of ribonuclease A if a very highly polymerized nucleic acid sample were used for the assay.

Susceptibilities of the Three Ribonucleases to Trypsin Digestion. One sensitive means to detect conformational differences is the susceptibility of a molecule of proteolytic digestion as proteolytic enzymes appear to attack only the unfolded regions of a polypeptide chain (Linderstrøm-Lang, 1952; Anson and Mirsky, 1934). This approach has been applied by other workers to locate the sites of unfolding in ribonuclease A molecule on thermal denaturation (Rupley and Scheraga, 1963; Ooi *et al.*, 1963; Ooi and Scheraga, 1964).

In Figure 1 are the patterns of trypsin-digested ribonuclease A and des-lysyl ribonucleases obtained on Sephadex chromatography. These digestions were carried out in phosphate buffer at 40°. The presence of retarded material in the patterns obtained with the trypsin-treated samples is an indication of tryptic digestion since the control samples incubated in the absence of trypsin are not retarded by the column. The number affixed to each of the lower patterns is the percentage of the total ninhydrin color present in the retarded material. This value provides a minimal estimate of the

degree of digestion, for it is assumed that the unretarded peak contains only unaltered ribonuclease. In Table II these data are compared with those obtained from incubations performed in Tris-hydrochloride buffer.

The two des-lysyl enzymes are more susceptible toward digestion than ribonuclease A in both buffers. The pyroglutamyl enzyme is particularly sensitive to digestion. The data also show that at 40° ribonuclease A was resistant to digestion in phosphate buffer but was digested considerably in the Tris-hydrochloride buffer (Ooi and Scheraga, 1964). However, ribonuclease A was quite resistant to digestion in Tris buffer at 25°. The resistance of ribonuclease A toward digestion in phosphate buffer is due to the stabilization of ribonuclease molecules by phosphate ion binding (Sela *et al.*, 1957; Rupley and Scheraga, 1960). These results indicate that the des-lysyl enzymes are slightly more disordered or can undergo the transition to a disordered state more easily than ribonuclease A. They also indicate that the des-lysyl enzymes have lower affinities for phosphate ions.

Membrane Diffusion Studies. Finally, in an attempt to determine whether the apparently lower conformational stabilities of the des-lysyl ribonucleases might cause slight changes in their diffusional volumes relative to ribonuclease A, the membrane diffusion rates of the three ribonucleases were compared using the thin-film dialysis technique.

The diffusion rates of all three proteins are higher at 40° than at 30°, as would be expected from the solvent viscosity change. The rates are seen to remain the same or even decrease as the temperature is increased to 50°, indicating that a swelling of the protein occurs which counteracts the effect of the decrease in viscosity over this temperature interval. Ordinarily, differences such as those observed between the three proteins would be significant, but the analytical difficulties encountered with the small amounts of material employed in these experiments impose an indeterminacy of ±10% on these results, so it is difficult to decide whether the small differences observed between the three proteins are significant or not. These experiments must be repeated with somewhat larger samples before conclusions may be drawn with certainty, but it is safe to say that neither of the two des-lysyl ribonucleases are seriously swollen relative to ribonuclease A. Indeed, they even appear to dialyze somewhat faster.

Discussion

As the des-lysyl glutamyl ribonuclease is nearly fully active, one can conclude that lysine-1 of ribonuclease A is not required for activity, in agreement with the findings of Van Vunakis *et al.* (1960) that the amino-terminal lysyl residue could be deaminated selectively to an α,ε-dihydroxycaproyl moiety without reducing the activity of the enzyme. Also Gordillo *et al.* (1962) have shown that the S-peptide derived from the subtilisin-modified glutamyl ribonuclease recombined with S-protein to regenerate full activity.

The des-lysyl pyroglutamyl ribonuclease is only 40% as active as ribonuclease A when assayed with cytidine 2′,3′-phosphate as substrate, but it is 60–80% as active when assayed with yeast nucleic acid. These activity properties resemble those of the urea-denatured and the polyalanylated ribonucleases (Anfinsen and White, 1961; Anfinsen *et al.*, 1962), and they are assumed to indicate an altered catalytic site which can be compensated for by a substance having a high affinity for the enzyme. The altered catalytic site must be a direct consequence of the replacement of a glutamyl residue by a pyroglutamyl residue, as the glutamyl enzyme is fully active. By inference then, glutamic acid residue 2 in ribonuclease A plays a role in maintaining the active conformation of the molecule.

The increased susceptibilities of the des-lysyl ribonucleases to trypsin digestion as compared to ribonuclease A demonstrate that they may have more conformational freedom. Furthermore, phosphate ions do not provide the des-lysyl enzymes, particularly the pyroglutamyl form, with the degree of protection against trypsin digestion observed with ribonuclease A. Ribonuclease A is protected by phosphate ions against proteolytic digestion (Rupley and Scheraga, 1960), because the conformation of the protein is stabilized by the binding of the ions (Sela *et al.*, 1957). Thus, the lack of protection by phosphate ion as well as their decreased activities may also be taken to indicate that the des-lysyl ribonucleases have more conformational freedom than ribonuclease A.

TABLE III: Relative Dialysis Rates of Ribonuclease A and the Des-lysyl Ribonucleases.[a]

Sample	E_{50} (hours) 30°	40°	50°
RNAase A	4.1	2.9	2.9
Des-lysyl glutamyl RNAase A	4.1	2.0	2.6
Des-lysyl pyroglutamyl RNAase A	3.8	2.2	2.6

[a] Solvent, 0.01 M acetic acid.

Other than these differences, the des-lysyl ribonucleases are identical with ribonuclease A with respect to their optical rotatory dispersions, ultraviolet spectra, diffusional volumes as estimated by dialysis, and substrate binding affinities. Therefore it is reasonable to conclude that the des-lysyl enzymes have nearly the same conformation as that of ribonuclease A but they have greater degrees of conformational freedom. The increased conformational freedom may be the result of a decreased binding or an imperfect orientation of the amino-terminal segment to the remainder of

the ribonuclease molecule, as this segment is not cross-linked by disulfide bonds to the remainder of the molecule (Spackman *et al.*, 1960) and is held tightly to the other portions by noncovalent forces (Richards and Vithayathil, 1960).

From the recent findings of Finn and Hofmann (1965) on the reconstitution of ribonuclease activities with S-protein and synthetic S-peptides, it would appear that the decreased binding is the cause. An analog of S-peptide corresponding to amino acid residues 1–13 in ribonuclease A regenerates 50% of the maximal activity when the molar ratio of S-peptide to S-protein is 3, but the analogs corresponding to residues 2–13 and residues 3–13 require respectively molar ratios of 41 and 2000. These findings lend support to our result that lysine-1 plays a minor role in maintaining an active and stable conformation of ribonuclease A molecule but glutamic acid-2 plays a striking role.

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