Des-lysyl Glutamyl and Des-lysyl Pyroglutamyl Ribonucleases. II. Structural Studies*

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ABSTRACT: A comparison has been made of the tryptic peptides obtained from the oxidized derivatives of ribonuclease A and des-lysyl glutamyl and des-lysyl pyroglutamyl ribonucleases.

The results confirm the proposed structures of des-lysyl ribonucleases. Possible modes of formation of des-lysyl ribonucleases from ribonuclease A are discussed.

In the preceding paper (Eaker *et al.*, 1965a) are described the isolation and characterization of two anomalous derivatives of bovine pancreatic ribonuclease A. The structures of these two anomalous components are proposed to be des-lysyl glutamyl and des-lysyl pyroglutamyl ribonucleases. This paper reports the comparative studies of the tryptic peptides obtained from the oxidized derivatives of ribonuclease A and the des-lysyl ribonucleases. The results confirm the structures proposed for the des-lysyl ribonucleases. The possible origin of the des-lysyl ribonuclease is also discussed.

Experimental

The primary structures of the ribonucleases were compared by examining the tryptic peptides of the oxidized derivatives. Performic acid–oxidized ribonucleases (20–50 mg) were digested with trypsin at 25° in 0.2 M sodium phosphate buffer, *p*H 7.0 (Hirs *et al.*, 1956). After 6–20 hours the reaction was terminated by the addition of glacial acetic acid (1 ml) and the digest was lyophilized. A commercial preparation of two-times crystallized, salt-free trypsin (Worthington, lot 815–18) was used. To reduce the chymotryptic activity, the trypsin sample was treated with 0.2 N HCl before use (Young and Carpenter, 1961).

The lyophilized digest was dissolved in 2 ml of 25% aqueous acetic acid, then fractionated on a Sephadex G-25 column (0.9 × 310 cm) using 0.2 M HOAc as solvent. Sephadex G-25 (dry sieved, 120–200 mesh) was used for packing the column which was constructed by linking two 165-cm columns in series (King and Norman, 1962). The flow rate was *ca.* 8 ml/hr and fractions of 2 ml were collected. Effluents were analyzed by absorption at 275 mμ or by the ninhydrin procedure after alkaline hydrolysis of 100-μl aliquots (Crestfield *et al.*, 1963). Peptides were recovered from the cuts by lyophilization.

The peptides from the Sephadex cuts were next fractionated on 15- × 0.9-cm columns of cation exchange resin (Aminex MS, Q-15 blend) or 20- × 0.9-cm columns of anion exchange resin (AGI-X2 W, −400 mesh). Both resins were obtained from Bio-Rad Laboratories, Richmond, Calif. Elution was carried out at 50° with sodium citrate buffers or with volatile pyridinium formate or acetate buffers. The sodium citrate buffers are those employed for the chromatographic separation of amino acids (Moore *et al.*, 1958), with the modification that the BRIJ and thiodiglycol normally present were omitted. The compositions of the volatile buffers are given in Table I. Flow rates

TABLE I: Composition of Pyridinium Formate Buffers.

*p*H (30°)	Apparent Pyridinium Ion Concentration (M)	Pyridine (ml/liter)	99% Formic Acid (ml/liter)
3.26 ± 0.02	0.2	16.1	23.8
4.26 ± 0.02	0.2	16.1	8.5
5.26 ± 0.02	0.4	64.4	15.2

of 30 ml/hr were maintained by the use of a Milton-Roy Minipump (Spackman *et al.*, 1958). The effluents were collected in 2.0-ml fractions, and 100-μl aliquots were analyzed by the ninhydrin procedure after alkaline hydrolysis.

When pyridinium formate or acetate buffers were used, the fractions corresponding to each peak were pooled, the volume was recorded, and the pooled fractions were stored in frozen state. When sodium citrate buffers were used, the pooled cuts were first desalted by passage through a 0.9- × 150-cm column of Sephadex G-25 in 0.2 M HOAc, then stored frozen. A measured aliquot was taken from each pooled cut, evaporated to dryness, and hydrolyzed for amino acid analysis

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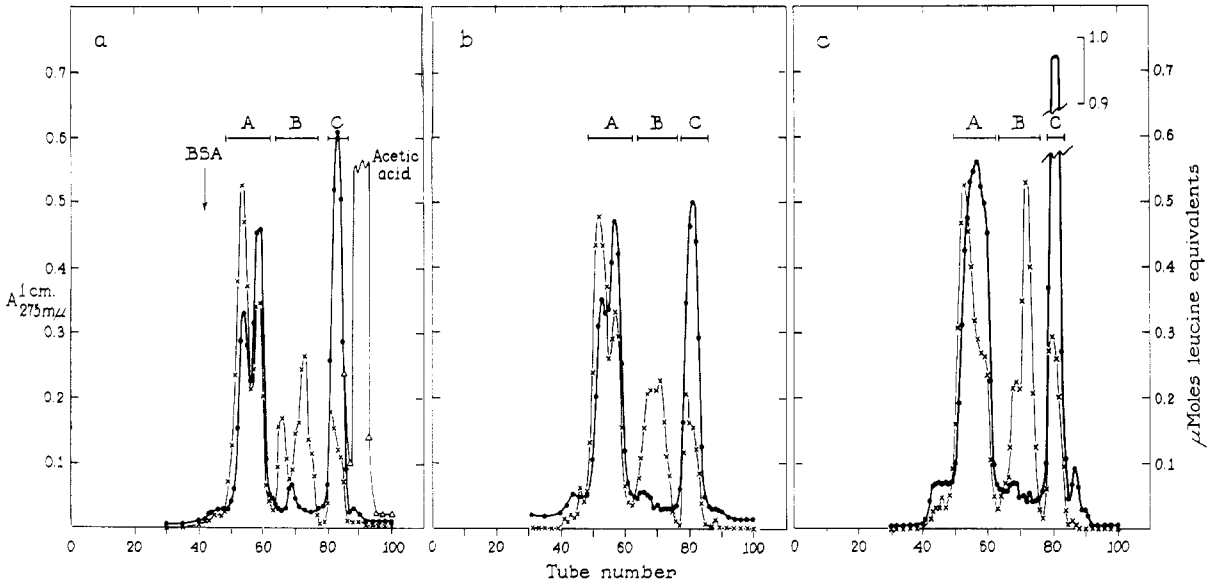

FIGURE 1: Group separation on a 310- × 0.9-cm column of Sephadex G-25 of peptides from tryptic digests of the performic acid–oxidized ribonucleases. (a) Ribonuclease A, 20 mg; (b) des-lysyl glutamyl ribonuclease, 22 mg; (c) des-lysyl pyroglutamyl ribonuclease, 37 mg. Elution was carried out at 25° with 0.2 M acetic acid and 2-ml fractions were collected. The curves denoted by solid circles were obtained by spectrophotometric analysis at 275 mμ. The curves denoted by x's were obtained by ninhydrin analysis after alkaline hydrolysis of 100-μl aliquots. The acetic acid band indicated by triangles in (a) was detected by spectrophotometric analysis at 230 mμ and is present in (b) and (c) also but is not shown. This excess acetic acid band was from the solvent used for application of the sample to column. The arrow labeled BSA in (a) shows where bovine serum albumin emerges from this column.

(Moore and Stein, 1963). Over-all yields of peptides were calculated from the amino acid analyses with proper correction for the amounts consumed during purification procedures.

The Edman degradation (Edman, 1950) on 0.2–0.5 μmole of peptide was carried out using the improved trifluoroacetic acid cyclization procedure of Konigsberg and Hill (1962). After each cycle of degradation, the reaction mixture was fractionated by passage through a 50- × 0.9-cm column of Sephadex G-25 in 50% acetic acid. As the peptides studied contain carboxyl terminal lysine, the ε-amino group of which reacts with phenyl isothiocyanate, both peptides and thiohydantoins could be located in the effluent by analysis at 267 mμ. The recovered peptides were divided into two portions, one for amino acid analysis and the other for a second cycle of degradation.

Results

Structural studies of the two des-lysyl ribonucleases were carried out according to the general scheme employed by Hirs *et al.* (1960) in their work on ribonuclease A. A modification introduced is the use of Sephadex G-25 chromatography for a preliminary separation into different size groups of the tryptic peptides obtained from the performic acid–oxidized protein. This modification is advantageous as it reduces the total number of peptides in each mixture to be separated in the subsequent step of ion-exchange chromatography.

In Figure 1 are shown the patterns obtained with tryptic digests of the three oxidized ribonucleases on the Sephadex G-25 column. All three patterns show good separation of the peptides into three main fractions. The effluent zones designated A, B, and C in the figure comprise what will be termed, respectively, the long, intermediate, and short peptide fractions. The first of the two peaks observed in zone B of the pattern shown in Figure 1a for a digest of oxidized ribonuclease A was found by paper electrophoresis to consist almost entirely of O-Tryp 10, a heptapeptide corresponding to residues 1–7 of ribonuclease A. This O-Tryp 10 peak is absent from the patterns shown in Figure 1b and 1c which contain only one rather broad peak in this region. The absence of O-Tryp 10 from the digests of the two des-lysyl ribonucleases is in accord with their proposed amino-terminal sequences. The three Sephadex patterns are otherwise similar.

The peptides present in Sephadex zones A, B, and C were separated by ion-exchange chromatography and were identified by their amino acid compositions. With the exception of the two des-lysyl homologs of O-Tryp 10, all of the peptides isolated correspond to peptides previously described by Hirs *et al.* (1960). Table II shows the peptide content of the three Sephadex zones obtained with digests of oxidized ribonuclease A.

The short-peptide fractions (zone C) from the two des-lysyl ribonucleases gave chromatographic patterns identical with those shown in Figure 2 for ribonuclease

A. This chromatogram was obtained with pyridinium formate buffers, but a similar result was obtained when the analogous sodium citrate buffers were used with the same elution schedule. As indicated in Table II, this short-peptide fraction contains a decapeptide, a heptapeptide, a tripeptide, and two dipeptides. If size were the only factor involved in the Sephadex separation, the decapeptide and the heptapeptide would not appear in this fraction. Both of these peptides contain two residues of tyrosine, and it is apparent that absorption due to the aromatic group has contributed to their retardation (Porath, 1962).

The large-peptide fractions (zone A) from the three ribonucleases also gave identical chromatographic patterns. A typical chromatogram is shown in Figure 3. The peptides designated O-Tryp 9, O-Tryp 16, and O-Tryp 2 proved homogeneous upon amino acid analysis. The peptide O-Tryp 4 appears in two positions in the chromatogram. The peptide O-Tryp 7–9 and the second O-Tryp 4 peptide showed about 10% cross-contamination, as would be expected from the incomplete resolution observed in Figure 3. The first O-Tryp 4 peak was also contaminated with an unidentified peptide or peptides. The appearance of O-Tryp 4 in two positions may be related to the tendency of its amino-terminal glutaminyl residue to undergo cyclization to a pyrrolidonyl moiety (Smyth *et al.*, 1962). As the contaminants present in both of these peptides precluded unambiguous estimation of their amide contents, no decision was made as to whether either of these peptides is the pyrrolidonyl form. O-Tryp 7–9 is an overlap peptide resulting from incomplete cleavage of the bond between the arginine and cysteic acid residues present

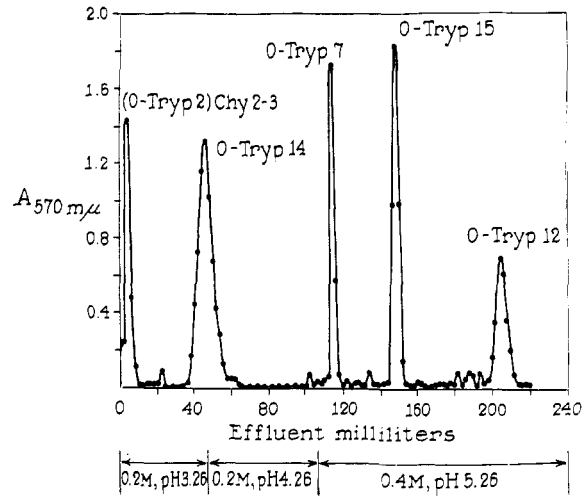

FIGURE 2: Separation on a 15- × 0.9-cm cation-exchange column (Aninex MS, Q-15 blend) of the short-peptide fraction (zone C of Figure 1a) from a tryptic digest of oxidized ribonuclease A. The column was operated at 50° and elution was carried out at 30 ml/hr with pyridinium formate buffers using stepwise buffer changes at the volumes indicated. The ordinate is the ninhydrin color obtained after alkaline hydrolysis of 100-μl aliquots from the effluent fractions.

at positions 39 and 40 of oxidized ribonuclease A, respectively (Hirs *et al.*, 1960). The resistance of this linkage to trypsin has been noted before (Anfinsen *et al.*, 1956; Hirs *et al.*, 1956; Redfield and Anfinsen,

TABLE II: Distribution of Tryptic Peptides Among Sephadex G-25 Effluent Zones.

Effluent Zone (Figure 1a)	Description of Peptides in Zones[a]			
	Peptide	Number of Residues	Number of Tyrosines	Residues in Ribonuclease
Zone A	O-Tryp 4	21	1	11–31
	O-Tryp 7–9	24		38–61
	O-Tryp 9	22		40–61
	O-Tryp 2	19	2	67–85
	O-Tryp 16	20	1	105–124
Zone B	O-Tryp 10	7		1–7
	O-Tryp 11	4		34–37
	O-Tryp 5	5		62–66
	O-Tryp 6	6		86–91
	O-Tryp 8	6		99–104
Zone C	O-Tryp 15	3		8–10
	O-Tryp 12	2		32–33
	O-Tryp 7	2		38–39
	(O-Tryp 2)Chy2–3	10	2	67–76
	O-Tryp 14	7	2	92–98

[a] The peptide nomenclature is that of Hirs *et al.* (1960).

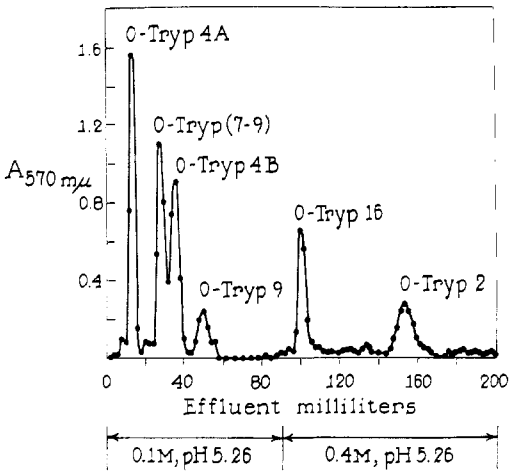

FIGURE 3: Separation on a 20- × 0.9-cm anion-exchange column (AGI-X2W) of the long-peptide fraction (zone A of Figure 1a) from a tryptic digest of oxidized ribonuclease A. In order to ensure retention of all peptides during application, the sample was applied to the column in 0.05 M pyridinium formate buffer with which the column was equilibrated at the start of the run. Elution was begun directly with the 0.1 M pyridinium formate buffer with a stepwise change to the 0.4 M buffer as indicated. The column was operated at 50° and a flow rate of 30 ml/hr. The ordinate is the ninhydrin color obtained after alkaline hydrolysis of 100-μl aliquots from the effluent fractions.

1956). Apparently, the negative charge on the cysteic acid reduces the affinity of the enzyme for the arginyl residue. When samples from the corresponding chromatographic peaks obtained with the long-peptide fractions from the three ribonucleases were mixed and rechromatographed, single symmetrical peaks were observed in all cases. As an extra carboxyl group resulting from de-amidation of a glutaminyl or asparaginyl residue would increase the retardation of a peptide on this anion-exchange column, one can conclude that the long peptides from the two des-lysyl ribonucleases have the same amide content as the corresponding peptides from ribonuclease A. This was demonstrated unambiguously for O-Tryp 9, O-Tryp 16, and O-Tryp-2, which were pure enough to permit an accurate estimation of amide content from the ammonia value obtained on amino acid analysis.

When the intermediate-peptide fractions from ribonuclease A and the two des-lysyl ribonucleases were chromatographed in either pyridinium formate buffers or sodium citrate buffers, patterns similar to those shown in Figure 4 were obtained. The patterns shown for the peptides of the des-lysyl ribonucleases were obtained with the sodium citrate system, whereas that shown for the corresponding peptides from ribonuclease A was obtained using the analogous pyridinium formate buffers. The O-Tryp 10 peak is seen to be absent from the des-lysyl patterns and is replaced in each by a new peptide. The chromatogram at the upper left shows a new peak at 116 ml. It is inferred from the results of amino acid analysis and end-group studies that this peptide corresponds to a des-lysyl version of O-Tryp 10 with the sequence shown. The absence of an equivalent of ammonia from the hydrolysate is taken to indicate that the terminal residue is glutamic acid rather than glutamine.

This peptide is absent from the pattern at the lower left but is replaced by an obvious elevation of the relatively unretarded peak at 14 ml. This des-lysyl peptide was freed of the acidic contaminant, a chymotryptic fragment of O-Tryp 2 corresponding to residues 77–85 of oxidized ribonuclease, by passing it through an anion-exchange column. The amino acid composition of the pure peptide thus obtained indicated that it was also a des-lysyl O-Tryp 10. The neutral behavior of this peptide on both the anion- and cation-exchange columns, combined with its lack of a reactive α-amino group, was taken to indicate that this peptide is a des-lysyl pyroglutamyl version of O-Tryp 10 with the sequence shown.

The absolute recoveries of these homologous amino-terminal peptides are given in Table III. No arbitrary corrections for chromatographic loss have been applied. It should be mentioned here that a peptide corresponding to normal O-Tryp 10 was isolated in 4.5% yield from the des-lysyl glutamyl digest, indicating about 5% contamination of this material with ribonuclease A.

TABLE III: Yields of Homologous Amino-Terminal Peptides.

Peptide	Yield (%)
O-Tryp 10	97
Des-lysyl glutamyl O-Tryp 10	62
Des-lysyl pyroglutamyl O-Tryp 10	72

The peak observed in the center of all three chromatograms shown in Figure 4 corresponds to a mixture of two hexapeptides, O-Tryp 6 and O-Tryp 8. These peptides are separated easily by rechromatography on the same column if the *p*H 3.26 buffer is omitted and elution is begun directly with the *p*H 4.26 buffer, as illustrated in the lower half of the figure.

Although the sodium citrate and the analogous pyridinium formate buffers produced similar elution patterns with the intermediate-peptide fraction from oxidized ribonuclease A when used with the same elution schedule, the two buffer systems are not completely equivalent. The pyridinium formate buffers did not accomplish the separation of the des-lysyl O-Tryp 6 or O-Tryp 8 in a single run with any elution schedule tried. For simplicity, the patterns obtained with the

citrate system are shown in Figure 4 for the intermediate-peptide fractions of the des-lysyl ribonucleases.

In Table IV are shown the initial amino acid compositions of the three homologous amino-terminal peptides and the compositions of the residual peptides after each of two stages of the Edman degradation. The total recovery is better than 80% in all cases, indicating that very little manipulative loss has been incurred. O-Tryp 10 loses only lysine in the first stage of the degradation and glutamic acid in the second, in accord with its known sequence (Hirs *et al.*, 1960). As the COOH-terminal lysine residues of all three peptides are converted to their ε-phenylthiocarbamyl derivatives in the first coupling reaction, lysine is not determined in the subsequent amino acid analyses.

The des-lysyl glutamyl peptide loses glutamic acid in the first stage and threonine in the second stage in accord with the structure proposed. Furthermore, glutamic acid phenylthiohydantoin was recovered from the Sephadex column after the first stage, indicating that the peptide linkage between the glutamyl and threonyl residues is of the normal α-type. If the glutamyl residue were linked to the threonyl residue by its γ-carboxyl group, no hydantoin would have been liberated in the first stage and the α-amino group of the threonyl residue would not have been available for the second coupling reaction.

The amino acid composition of the des-lysyl pyroglutamyl peptide is not altered after two stages of the Edman degradation, indicating that no α-amino group is present. An *N*-acyl glutamyl residue could account for the negative end-group results but would not explain the chromatographic properties of this peptide. Its neutral behavior on both anion- and cation-exchange resins requires that the γ-carboxyl group of the glutamyl residue be blocked. These observations permit an unambiguous assignment of the terminal pyrrolidone structure to this peptide, although the free pyrrolidonecarboxylic acid was not isolated. The structures of these homologous amino terminal peptides are given at the bottom of Table IV.

With the exception of these amino terminal peptides, all of the peptides isolated from the tryptic digests of the oxidized des-lysyl ribonucleases were indicated by their chromatographic properties and amino acid compositions to be identical with the corresponding peptides from ribonuclease A. Neither of the des-lysyl ribonucleases has a shortage of amide groups. Therefore, as regards primary structure, the two des-lysyl ribonucleases differ from ribonuclease A only in the nature of their amino terminal residues.

TABLE IV: Edman Degradation of Homologous Amino-Terminal Tryptic Peptides.

	O-Tryp 10				Des-lysyl Glutamyl O-Tryp 10				Des-lysyl Pyroglutamyl O-Tryp 10			
	Composition of Residual Peptide				Composition of Residual Peptide				Composition of Residual Peptide			
Amino Acid	Initial	Stage 1	Stage 2	Total Recovery	Initial	Stage 1	Stage 2	Total Recovery	Initial	Stage 1	Stage 2	Total Recovery
Lysine	2.13				1.05				0.98			
Glutamic acid	1.01	1.06	0.1		1.01	0.03	0.05		1.02	1.06	0.91	0.82
Threonine	0.91	1.06	1.02	0.93	0.93	0.90	0.20		0.91	0.94	0.88	0.87
Alanine	3.00	3.00	3.00	0.81	3.00	3.00	3.00	0.84	3.00	3.00	3.00	0.85

O-Tryp 10: H_2N-Lys-Glu-Thr-Ala-Ala-Ala-Lys-OH
Des-lysyl glutamyl O-Tryp 10: H_2N-Glu-Thr-Ala-Ala-Ala-Lys-OH
Des-lysyl pyroglutamyl O-Tryp 10: pyroglu-Thr-Ala-Ala-Ala-Lys-OH

Discussion

The des-lysyl glutamyl and pyroglutamyl ribonucleases are a pair of subtle structural homologs unique in protein chemistry. The minor structural alterations are found to exert profound influences on the conformational stabilities and the catalytic efficiencies of these two proteins, as will be reported in an accompanying paper (Eaker *et al.*, 1965b). It is, therefore,

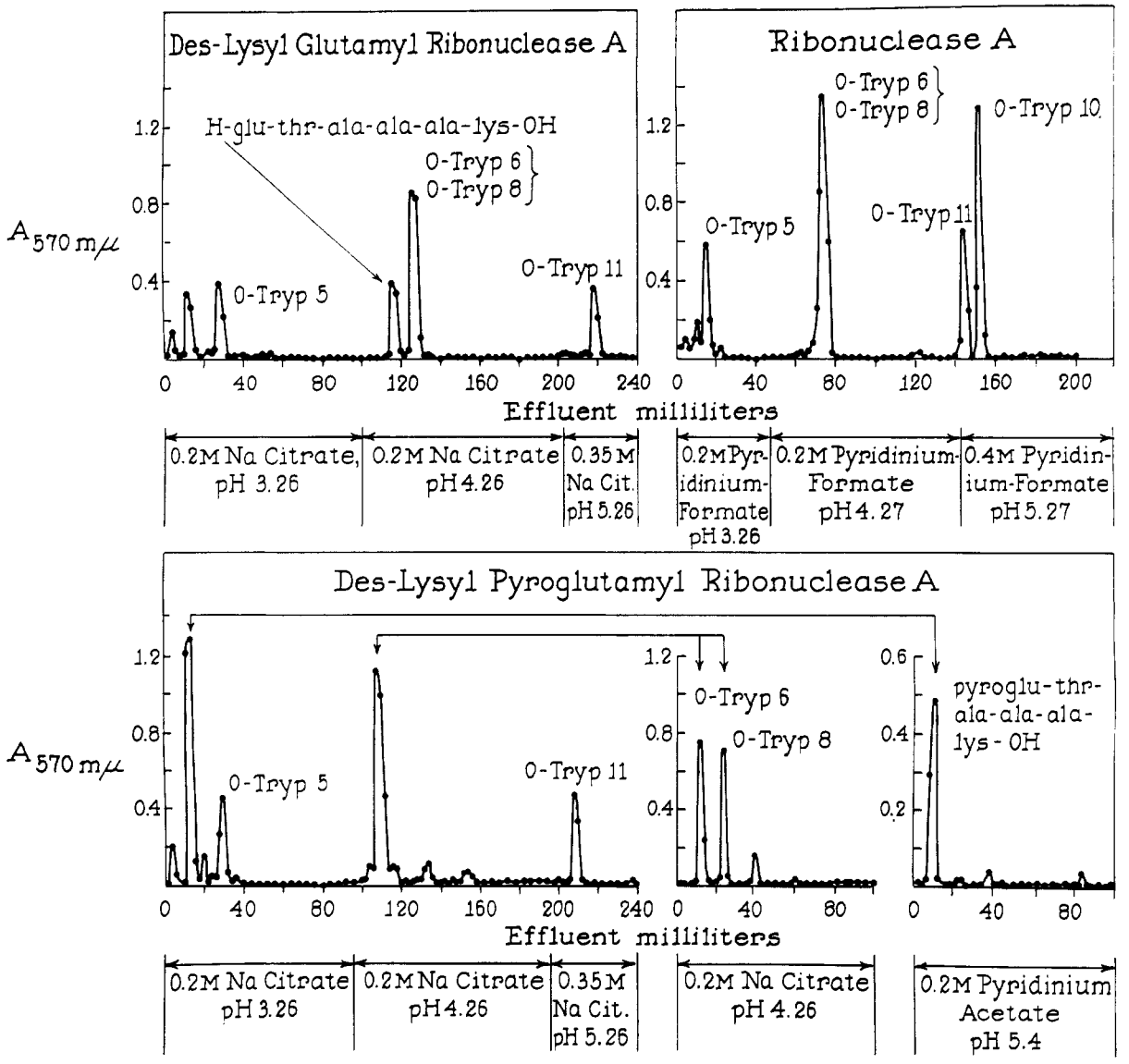

FIGURE 4 : Separation on a 15- × 0.9-cm cation-exchange column (Aminex MS, Q-15 blend) of the intermediate-peptide fractions from tryptic digests of oxidized ribonuclease A and the two des-lysyl ribonucleases (zones B of the patterns in Figure 1). The column was operated at 50° and elution was carried out at a flow rate of 30 ml/hr with pyridinium formate or sodium citrate buffers using stepwise buffer changes at the volumes indicated. The ordinate is the ninhydrin color obtained after alkaline hydrolysis of 100-μl aliquots from the effluent fractions. At the bottom are shown the separation of O-Tryp 6 and 8 obtainable by rechromatography on the same column with a different elution schedule, and the rechromatography of the pyroglutamyl peptide on an anion-exchange column (AGI-X2W). The amino acid sequences of the two des-lysyl homologs of O-Tryp 10 are affixed to the appropriate peaks.

relevant to consider the possible origins of these homologs.

The possibility that the presence of these two homologs is due to genetic variants need not be considered seriously. Since commercial preparations of ribonucleases represent materials isolated from large pools of animals, it is unlikely that one preparation is heavily represented by certain genotypes of animals and the other one is not. The simple chemical relationship between the two homologs also suggests this not to be the case.

An alternative possibility is that the homologs are formed by chemical modifications of a common precursor. The following observations, however, must be taken into consideration. First, the lysyl-glutamyl bond in ribonuclease A is not susceptible to cleavage by a wide range of proteolytic enzymes such as trypsin (Hirs *et al.*, 1960), chymotrypsin (Rupley and Scheraga, 1963), pepsin (Anfinsen, 1956), and subtilisin (Richards, 1958). Second, there is no chemical evidence that an acyl-glutamyl peptide bond should be more labile than other peptide bonds toward acid- or base-cata-

lyzed hydrolysis (Witkop, 1961). Unless there is an increase in reactivity due either to a neighboring group assistance in the ribonuclease molecule or to a specific metal ion catalysis, it is difficult to visualize a preferential cleavage of the lysyl-glutamyl linkage. Third, the glutamyl and pyroglutamyl homologs do not interconvert at a significant rate in neutral solutions at room temperature. Extrapolating from the data on the equilibration of glutamic and pyroglutamic acids (Wilson and Cannan, 1937), glutamyl ribonuclease would have to be boiled at *p*H <4 or >8 for many hours in order to give the observed 2:1 ratio of the two homologs. Either one of these conditions would be expected to lead to extensive degradations of the ribonuclease molecule.

From these considerations, it is thought unlikely that ribonuclease A can serve as the precursor of the two homologs, as drastic treatments are required to effect the necessary chemical modifications. However, Shapira and Parker (1960) have shown that brief heating of the chromatographically purified ribonuclease A at 100° produced minor components which are chromatographically identical with certain of the minor components observed in unfractionated starting material. As these authors have not carried out chemical studies of the minor components so produced, it is therefore not possible to draw any conclusions.

A plausible precursor, satisfying part of the above requirements, is a ribonuclease A molecule in which the second residue is glutamine rather than glutamic acid. With this hypothetical precursor one could expect the formation of a des-lysyl glutamyl ribonuclease by limited proteolysis with trypsin, as trypsin is known to cleave the amino-terminal lysyl residue when not followed by an acidic acid. For example, trypsin readily cleaves the lysyl-glutaminyl bond at the amino terminus of a hexapeptide (Fischer *et al.*, 1959). The resulting des-lysyl glutaminyl ribonuclease could cyclize to form the pyroglutaminyl derivative with the loss of ammonia. The tendency of amino-terminal glutamines in peptides to undergo cyclization is well recorded (Sanger and Thompson, 1953; Sanger *et al.*, 1955). However, it is difficult to account for the formation of the des-lysyl glutamyl ribonuclease from the hypothetical precursor molecule, for there is no reason to suspect that the amide group present in the glutaminyl ribonuclease should be particularly labile toward hydrolysis. The pyroglutamyl ribonuclease, once formed, will be quite stable and can be converted to the glutamyl derivative only under extreme conditions.

On the other hand a modified ribonuclease A, in which the γ-carboxyl group of the second residue, glutamic acid, is esterified, can account satisfactorily for the formation of both des-lysyl proteins. Such a modified ribonuclease could also be digested by trypsin to yield a des-lysyl derivative. While the des-lysyl glutaminyl ribonuclease would form only the pyroglutamyl protein, this derivative could yield both des-lysyl homologs, as an ester group is generally more readily hydrolyzed than is an amide.

The possibility of such a proposed tryptic cleavage is also suggested by the recent experiments of Riehm *et al.* (1965) on the trypsin digestion of an oxidized and esterified preparation of ribonuclease A. Separation of the digest by chromatography on Dowex-50 revealed that the amino-terminal peptide O-Tryp 10 from the oxidized and esterified ribonuclease is less retarded than the corresponding peptide from ribonuclease A. As pointed out by these authors, esterification of the carboxyl group of glutamic acid-2 in O-Tryp 10 would lead to a one-unit increase in positive charge of the peptide, and it should be more retarded than the unmodified peptide; this was not found. The observed chromatographic position of their O-Tryp 10 is immediately following that of the peptide O-Tryp 8, and this position is close to that of the O-Tryp 10 from des-lysyl glutamyl ribonuclease which is immediately preceding that of O-Tryp 8 (Figure 4). The chromatographic position of their O-Tryp 10 could be explained if its structure were Glu-Thr-Ala-Ala-Ala Lys in which the γ-carboxyl group of Glu has been esterified. The possibility of a tryptic cleavage of the bond between Lys-1 and esterified Glu-2 was not considered by Riehm *et al.* (1965), though the amino acid composition of their O-Tryp 10 did not agree entirely with that expected.

A partially esterified ribonuclease A could easily be formed during the procedures used for its isolation. According to the patents issued to the Armour Co. (Maxwell and Thompson, 1956), commercial preparations of ribonuclease are extracted from beef pancreas which have been freed of insulin by acid-alcohol treatment. The insulin-free beef pancreas, containing 65% ethanol at *p*H 2.85, is diluted with water and vacuum distilled at 16–22° to remove the alcohol. The concentrate is then fractionated by ammonium sulfate precipitation to yield the crude ribonuclease fraction which is further purified by crystallization. Partial esterification of ribonuclease might have easily occurred during the extraction or evaporation steps as a result of slight changes in the procedures. The limited proteolysis and hydrolysis of the esterified protein could have taken place during the crystallization steps. Since detailed information concerning the preparation of this lot of ribonuclease is not available, one can only speculate that this is the most probable source of these des-lysyl proteins.

References

Anfinsen, C. B. (1956), *J. Biol. Chem. 221*, 405.

Anfinsen, C. B., Sela, M., and Tritch, H. (1956), *Arch. Biochem. Biophys. 65*, 156.

Crestfield, A. M., Moore, S., and Stein, W. H. (1963), *J. Biol. Chem. 238*, 622.

Eaker, D. L., King, T. P., and Craig, L. C. (1965a), *Biochemistry 4*, 1473 (this issue; preceding paper).

Eaker, D. L., King, T. P., and Craig, L. C. (1965b), *Biochemistry 4*, 1486 (this issue; following paper).

Edman, P. (1950), *Acta Chem. Scand. 4*, 277; 283.

Fischer, E. H., Graves, D. J., Snyder-Crittenden, E. R., and Krebs, G. (1959), *J. Biol. Chem. 234*, 1698.

Hirs, C. H. W., Moore, S., and Stein, W. H. (1956), *J. Biol. Chem. 219*, 623.
Hirs, C. H. W., Moore, S., and Stein, W. H. (1960), *J. Biol. Chem. 235*, 633.
King, T. P., and Norman, P. S. (1962), *Biochemistry 1*, 709.
Konigsberg, W., and Hill, R. J. (1962), *J. Biol. Chem. 237*, 2547.
Maxwell, L. C., and Thompson, W. M. (1956), U.S. Patent No. 2,751,329; U.S. Patent No. 2,758,055.
Moore, S., Spackman, D. H., and Stein, W. H. (1958), *Anal. Chem. 30*, 1185.
Moore, S., and Stein, W. H. (1963), *Methods Enzymol. 6*, 819.
Porath, J. (1962), *Advan. Protein Chem. 16*, 209.
Redfield, R. R., and Anfinsen, C. B. (1956), *J. Biol. Chem. 221*, 385.
Richards, F. M. (1958), *Proc. Natl. Acad. Sci. U.S. 44*, 162.
Riehm, J. P., Broomfield, C. A., and Scheraga, H. A. (1965), *Biochemistry 4*, 760.
Rupley, J. A., and Scheraga, H. A. (1963), *Biochemistry 2*, 421.
Sanger, F., and Thompson, E. O. P. (1953), *Biochem. J. 53*, 366.
Sanger, F., Thompson, E. O. P., and Kitai, R. (1955), *Biochem. J. 59*, 509.
Shapira, R., and Parker, S. (1960), *Biochem. Biophys. Res. Commun. 3*, 200.
Smyth, D. G., Stein, W. H., and Moore, S. (1962), *J. Biol. Chem. 237*, 1845.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.
Wilson, H., and Cannan, R. (1937), *J. Biol. Chem. 119*, 309.
Witkop, B. (1961), *Advan. Protein Chem. 16*, 221.
Young, J. D., and Carpenter, F. H. (1961), *J. Biol. Chem. 236*, 743.

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.*,