Polyspermine-Ribonuclease Prepared by Cross-linkage with Dimethyl Suberimidate†

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ABSTRACT: Bovine pancreatic ribonuclease A, in dilute solution in phosphate buffer at pH 9.0, has been treated with dimethyl suberimidate in the presence of an excess of spermine. The reaction products were gel filtered and chromatographed on carboxymethylcellulose at pH 8.0. The most active product, obtained in 5% yield, contained an average of 8 spermine residues per molecule and had only 1 out of 10 lysine residues substituted per molecule. This polyspermine-RNase is 115 times as active as the parent enzyme toward poly(A)·poly(U) at pH 7.5 in 0.125 M NaCl, and 176 times as active toward poly(I)·poly(C). In absolute terms, the poly(U) strand in the double-stranded substrate is hydrolyzed by polyspermine-RNase at about half the rate at which the native enzyme hydrolyzes poly(U) alone. When free spermine or polyspermine was admixed with RNase A under the assay conditions with poly(A)·poly(U), the change in activity was less than 10%. The hybrid substrate poly(dA)·poly(rU) is hydrolyzed 382 times more rapidly by the polyspermine-RNase. The naturally occurring double-stranded RNAs from *Penicillium chrysogenum* virus, reovirus 3, and bacteriophage ϕ6 are hydrolyzed at similarly accelerated rates. Toward yeast RNA, polyspermine-RNase is 73 to 100% as active as RNase A; toward cyclic 2′,3′-cytidylate, the activity is 2 to 4 times that of RNase A at pH 8. When spermine is first cross-linked to give a polyspermine and the product is coupled to RNase, 20 to 40% yields can be obtained of products with 50 to 80% of the activity of the above derivative toward dsRNAs and with 2 to 3 NH_2 groups covered per molecule.

Earlier research on the abilities of RNases of the pancreatic type, including the enzyme in bovine seminal plasma, to act on double-stranded RNAs has led to the conclusion that the number of basic charges in the molecule may be an important variable (Libonati et al., 1975a,b; D'Alessio et al., 1975). The recent observation that the more basic whale pancreatic RNase A (Libonati et al., 1976) has about 40 times the activity of bovine RNase A toward poly(A)·poly(U) strengthens this view. We obtained a modest (8.5-fold) increase in activity toward this substrate by cross-linking two molecules of bovine RNase A with dimethyl suberimidate (Wang et al., 1976); this result has led us to study the properties of the enzyme when cross-linked to a polyamine, such as spermine, which has a special affinity for nucleic acids (Tabor and Tabor, 1972).

Experimental Section

Materials and Methods. Bovine pancreatic ribonuclease A (type II-A), spermine, yeast RNA (Sigma VI), yeast RNA core, cyclic 2′,3′-cytidylic acid, and cyclic 2′,3′-uridylic acid were obtained from Sigma. The [^{14}C]poly(A) (specific activity, 2700 cpm μg^{-1}) was purchased from Miles, and [^{14}C]spermine tetrahydrochloride (13.36 mCi $mmol^{-1}$) was from New England Nuclear. The double-stranded poly(A)·poly(U) and poly(I)·poly(C), the RNA·DNA hybrids poly(rC)·poly(dI) and poly(rU)·poly(dA), and the single-stranded homopolymers poly(U) and poly(C) were products of P-L Biochemicals. The double-stranded RNA of reovirus 3 (Gomatos and Tamm, 1963) was kindly donated by Dr. A. J. Shatkin, Molecular Biology Institute, Hoffmann-La Roche. The double-stranded RNAs of *Penicillium chrysogenum* virus (Wood and Bozarth, 1972) and bacteriophage ϕ6 (Van Etten et al., 1974) were generous gifts from Dr. Robert F. Bozarth, Boyce Thompson Institute for Plant Research. Dimethyl suberimidate dihydrochloride was purchased from Pierce Chemicals. Sephadex G-75 and carboxymethyl-Sephadex were products of Pharmacia Fine Chemicals. Carboxymethyl-cellulose was from Whatman. Yeast RNA was dialyzed as previously described (Wang et al., 1976).

Enzymic Assays. The activity toward yeast RNA was determined either by the spectrophotometric assay of Kunitz (1946) or by a modification (Tarnowski et al., 1976) of the precipitation assay of Anfinsen et al. (1954). The activity toward cyclic 2′,3′-cytidylic acid was determined by the method of Crook et al. (1960) and del Rosario and Hammes (1969) under the conditions defined by Lin (1970), whereas that toward cyclic 2′,3′-uridylic acid was followed in a similar manner but at 282 nm.

The enzymic degradation of double-stranded RNA, RNA·DNA hybrids, or single-stranded homopolymers was usually determined spectrophotometrically under conditions (Wang et al., 1976) similar to those described by Libonati and Floridi (1969) with the exception that the reaction was followed at 250 nm instead of at 260 nm. The choice of 250 nm was based on the difference spectrum of poly(C) or poly(U) upon enzymic depolymerization. The use of this wavelength has the advantage that the contribution to hyperchromicity made by any dissociation (or separation) of the double-stranded RNA induced nonenzymically is much less at 250 nm than at 260 nm and, with the amount of substrate used (particularly with poly(C)·poly(I)), the absorbance was inconveniently high at 260 nm. However, the amount of pancreatic RNase A used in the assay for poly(A)·poly(U) has been increased from 10 to 30 μg per assay to give at least ΔA_{250} = 0.002 min^{-1}. Much smaller amounts of enzyme are needed when polyspermine-RNase is assayed. Poly(A)·poly(U) is preferably dissolved and stored in 0.25 M NaCl (80 μg mL^{-1}); in this way it is possible to obtain double-stranded substrate solutions with consistent properties. The solution can be kept in a freezer and remains stable after repeated freezing and thawing. Dilution with the desired buffer can be conveniently made to give 40 μg mL^{-1} at the time of assay.

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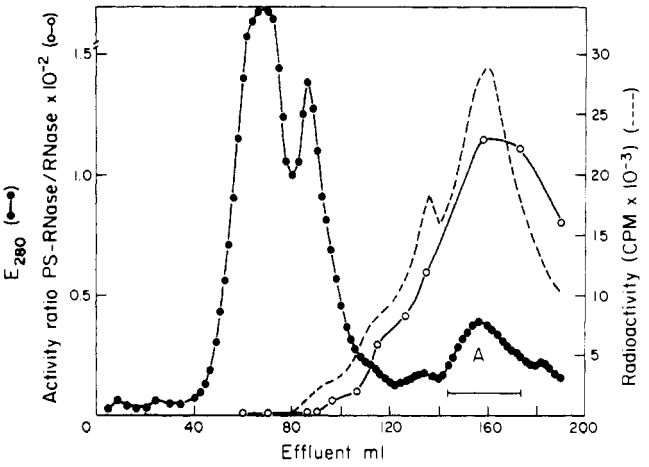

FIGURE 1: Separation of polyspermine-RNase on CM-Sephadex (C-50). Column dimension was 0.9 × 60 cm. A linear gradient of pH and NaCl consisted of 100 mL of 0.1 M phosphate buffer of pH 6.4 in the mixing vessel and 100 mL of 0.1 M phosphate buffer of pH 8.0, 0.7 M in NaCl in the reservoir. Flow rate was 8 mL per h. Fraction size was 2 mL. Activity measured toward poly(A)·poly(U).

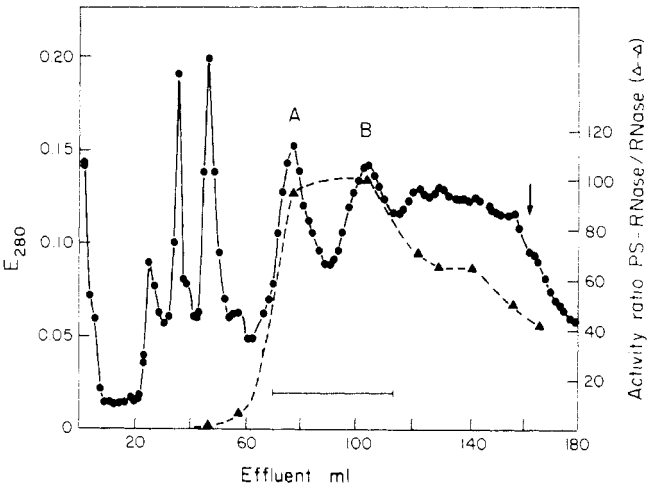

FIGURE 2: Separation of polyspermine-RNase on CM-cellulose (CM-52). Column dimension was 0.9 × 60 cm. The gradient consisted of 100 mL of 0.1 M phosphate buffer at pH 6.4 in the mixing vessel and 100 mL of 0.1 M phosphate of pH 8.0, 0.6 M in NaCl in the reservoir. At the arrow, the NaCl was increased to 0.8 M. Flow rate was 12 mL per h. Fraction size was 1.8 mL.

The activity toward poly(A)·poly(U) was also determined by a precipitation assay of the type introduced by Anfinsen et al. (1954) with the following modification. All solutions were made up in 0.015 M Tris[1]-HCl, pH 7.5, 0.125 M in NaCl. The enzyme solution contained 2 μg of ribonuclease and 1 mg of bovine serum albumin in 500 μL. The mixture was incubated at 37 °C for 5 min. The reaction was initiated by adding an equal volume of a solution of poly(A)·poly(U) (20 μg) in the same buffer. The reaction was terminated by addition of 1 mL of cold 10% perchloric acid, 0.25% in uranyl acetate. The suspension was left on ice for 30 min before centrifugation. The absorbance of acid-soluble nucleotides in the supernatant was estimated at 260 nm directly without dilution.

The activity toward [^{14}C]poly(A) was determined according to the procedures given previously (Wang et al., 1976). The reaction mixture (incubated at 25 °C) contained: 100 μg of enzyme and 5 μg of [^{14}C]poly(A) in 1.5 mL of 0.015 M sodium citrate of pH 7.0, 0.15 M in NaCl. Aliquots of 500 μL were taken at the end of each time interval; 50 μL of bovine serum albumin (0.5%) were added and followed by 500 μL of cold 5% Cl_3CCOOH. The reaction mixture was kept on ice for 30 min before it was filtered through Millipore filter (HAWP-025-00), which was washed with 5 mL of cold 5% Cl_3CCOOH; the filter was dried and counted.

The degradation of yeast RNA core was carried out in a similar manner as described by Hilmoe (1960); the digest contained 1.7 mg of yeast RNA core, 100 μg of bovine serum albumin, and 40 μg of RNase A or 10 μg of spermine-RNase in a total volume of 0.2 ml, 0.2 M in KCl, 0.2 M in Mes (pH 7.5). The mixture was incubated at 37 °C for 30 min. The reaction was terminated by adding 0.2 mL of 25% perchloric acid, 0.25% in uranyl acetate. After the mixture was kept on ice for 30 min, the absorbance of the supernatant at 260 nm was measured after a 40-fold dilution with water.

The activity toward double-stranded RNA of virus or bacteriophage was performed in the following manner. The solution which contained 20–30 μg of dsRNA in 300 μL of 0.015 M sodium citrate, 0.15 M in NaCl, 5% in sucrose, pH 6.5, was incubated at 37 °C for 5 min. The reaction was initiated by adding 300 μL of enzyme in the same buffer. An aliquot (50 μL) was taken immediately following enzyme addition and served as the zero-time control. Samples were then taken at prescribed times and were loaded into a polyacrylamide gel. Electrophoresis was started immediately following each sample loading. The gel system was that of Loening (1967). The separation was carried out in 2.4% polyacrylamide gel at 5 mA per gel for 3 h. A 30-min prerun had been performed before application of samples in order to eliminate UV-absorbing material from the gel. The developed gels were soaked in deionized water for 1 h with occasional agitation and one change of water. The cleared sample gels were scanned at 260 nm in a recording Gilford spectrophotometer (Model 2400-S) equipped with linear transport (E_{260}, scan rate 2 cm min^{-1}, absorbance scale 0.5).

The concentration of RNase was estimated spectrophotometrically ($E_{280}^{1\%}$ 7.3; cf. Richards and Wyckoff, 1971) or by the method of Lowry et al. (1951) with RNase A as the standard. Sodium dodecyl sulfate gel electrophoresis was conducted according to Weber and Osborn (1969).

The number of spermine residues attached to RNase was estimated by the specific radioactivity of the product and expressed as moles of spermine per mole of RNase.

The number of lysine residues substituted was estimated by dinitrophenylation according to Wofsy and Singer (1963) followed by cleavage of the amidine group from lysine residues with ammonia–acetic acid (32:2 v/v) for 18 h at 25 °C (Wold, 1967). The resulting protein was hydrolyzed with 4 N HCl under reflux for 24 h. The amount of free lysine was estimated by ion-exchange chromatography (Spackman et al., 1958) performed on an analyzer described by Liao et al. (1973). The number of substituted lysine residues in polyspermine RNase was also determined in hydrolysates by amino acid analyses performed on a Durrum D-500 analyzer, with pH 6.0 for the third buffer. Samples were hydrolyzed in 6 N HCl for periods of 24, 48, and 72 h at 110 °C. Calculation of the number of residues of lysine per molecule of polyspermine-RNase was determined by reference to the recoveries of stable amino acids (aspartic acid, valine, and arginine), and after extrapolation

[1] Abbreviations used: Mes, 2-(*N*-morpholino)ethanesulfonic acid; dsRNA, double-stranded RNA; ssRNA, single-stranded RNA; PS-RNase, polyspermine-RNase; CM, carboxymethyl; Tris, tris(hydroxymethyl)aminomethane; UV, ultraviolet.

TABLE I: The Number of ϵ-NH_2 Groups of Lysine Residues Substituted in Polyspermine-RNase.

Preparations of Polyspermine Derivative	Act. Ratio (PS-RNase/RNase)[a]	No. of Lysine Residues Substituted
1 (A in Figure 1)	115	1.03[b]
2 (A + B in Figure 2)	95	2.43[c]
3 (A in an experiment similar to that in Figure 2)	66	3.22[c]

[a] Ratio of activity of the derivative to that of RNase A with poly(A)·poly(U) as the substrate. [b] Estimation was made by amino acid analysis of the free lysine following hydrolysis of the dinitrophenylated and deamidinated polyspermine-RNase. [c] Estimation was made by amino acid analysis of direct hydrolysate of the derivative.

TABLE II: Comparison of Enzymic Activities of RNase and Polyspermine-RNase at pH 7.5, 0.125 M NaCl.

Substrate	Activity ratio (PS-RNase/RNase)	
	Spectrophotometric assay	Precipitation assay
Poly(rA)·poly(rU)	115	120
Poly(rI)·poly(rC)	176	
Poly(rU)·poly(dA)	382	
Poly(rC)·poly(dI)	32	
Poly(rC)	8	
Poly(rU)	3	
Poly(rA)		4
Yeast RNA	0.73–1.0	0.73

to zero time to correct for the small percentage of hydrolysis of the ϵ-amidinated lysines (Hartman and Wold, 1967).

Results

Coupling of Spermine and RNase. In order to establish the optimum conditions for the coupling reaction between RNase and spermine by a bifunctional coupler, dimethyl suberimidate, the variables (such as pH, concentration of various reactants) were studied in terms of the yield of the spermine-RNase and its activity toward poly(A)·poly(U). The following conditions were established: RNase A (1.5 μmol) and spermine·4HCl (21.5 μmol) were dissolved in 80 mL of 0.1 M phosphate buffer, pH 9.0. The solution was stirred continuously with a magnetic stirrer as rapidly as possible without foaming. To this solution dimethyl suberimidate (65.9 μmol) was added as solid in approximately 20 equal portions over a period of 10 min. The pH of the solution was maintained by titration with 0.2 M NaOH. After an additional 5 min, 20 equiv of ammonium acetate (relative to dimethyl suberimidate) was added as 2 M solution. The reaction mixture was dialyzed in acetylated dialysis tubing (prepared from A. H. Thomas 3787-D-22 dialysis tubing according to Craig, 1960) against glass-distilled water and lyophilized. The residue was taken up in about 2 mL for gel filtration on Sephadex G-75 (0.9 × 152 cm) equilibrated with physiological saline to remove RNase dimer. The flow rate was 18 mL per h. The fractions (1.2 mL each) containing the unreacted RNase plus the spermine-RNase products eluted between 64 and 76 mL were pooled and dialyzed against water and lyophilized. The lyophilized material was taken up in 1–2 mL and chromatographed on CM-Sephadex or CM-cellulose with a linear gradient of pH and NaCl. A typical separation is shown in Figure 1. The activity toward dsRNA increased with increased retardation on the acidic exchanger. The most active product (zone A) isolated in about 5% yield contained an average of 8 residues of spermine per molecule of RNase. The yield could be increased by resynthesis using the reclaimed unsubstituted RNase. The yield of the highly active product was again 5%.

This result prompted us to see whether the yield of the desired product might be raised by polymerization of spermine first, followed by coupling of the resulting polyspermine to RNase; 30 mg of RNase (2.2 μmol) was dissolved in 2 mL of 0.1 M phosphate buffer with a final pH of 9.0. Spermine·4HCl (120 μmol) was dissolved in 2 mL of 0.2 M triethanolamine and the pH was adjusted to 9.7. To this solution, dimethyl suberimidate·2HCl (124 μmol) was added with stirring. The pH of this solution was maintained at ca. 9.7. The polymerization reaction was allowed to proceed for 1 min. This solution was then added to the above RNase solution with continuous stirring. The coupling reaction was allowed to proceed for 5 min. The reaction was terminated by the addition of 1.5 mL of 2 M ammonium acetate. The mixture was processed in the manner described above for gel filtration and ion-exchange chromatography. A typical elution profile from CM-cellulose is given in Figure 2.

The yield of polyspermine-RNases, in this case, was greater than 80%. The most active products were located in two major peaks (A and B in Figure 2) and together constituted approximately 40% of the total yield. Derivatives which showed greater retardation than the two most active ones were increasingly less active.

In contrast to the results described above, if the procedure is reversed by treating RNase in about 1% solution with dimethyl suberimidate first, followed by coupling the resulting RNase-amidinyl suberimidomethyl ester with spermine, little or no polyspermine-RNase of the most active type could be detected, and the major product was a monospermine substituted RNase which exhibited very low activity toward dsRNA; its activity toward poly(A)·poly(U) was only twice that of RNase A.

The results on lysine residues substituted in the main products are summarized in Table I. The most active material from zone A in Figure 1 has only 1 out of 10 lysine residues modified. Sodium dodecyl sulfate gel electrophoresis of the material at pH 7.0 gave a single but diffused zone as to be expected for a mixture of closely related products with varying chain lengths in the polyspermine moiety. The decreased activity of more modified products is consistent with earlier observations on the degree of modification of NH_2 groups and the activities of cross-linked RNase dimers (Wang et al., 1976).

The Activity of Polyspermine-RNase toward Poly(A)·Poly(U) (Table II). The most active derivative fraction hydrolyzes poly(A)·poly(U) at pH 7.5 more than 100 times as rapidly as does RNase A both spectrophotometric and precipitation assays. There is a variation of the activity ratio of these two enzymes with time in the acid precipitation assay with poly(A)·poly(U) as the substrate. As the incubation time is increased, interfering factors, such as substrate limitation and end-product inhibition, begin to exert their influence on the hydrolysis by the more active polyspermine-RNase. If, however, the curve is extrapolated to zero time, the activity ratio (Table II) is the same as that found by the spectrophotometric assay. The pH optima for the actions of the derivative

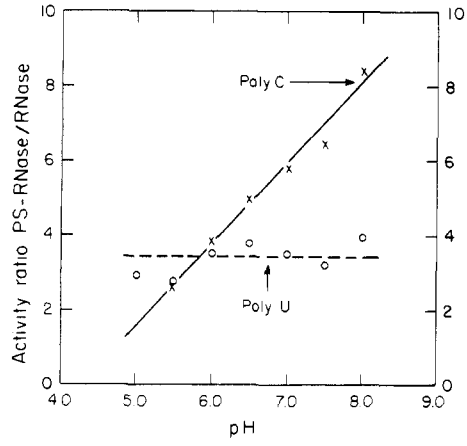

FIGURE 3: The ratios of the activities of polyspermine-RNase and RNase A toward poly(C) (X—X) or poly(U) (O—O) as a function of pH.

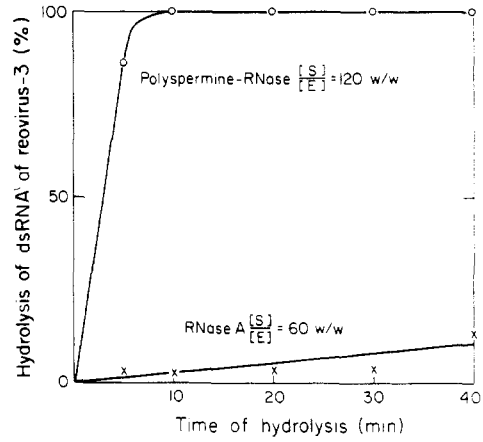

FIGURE 4: Hydrolysis of dsRNA of reovirus-3 by RNase A and polyspermine-RNase. Details are given under Experimental Section.

and RNase A toward poly(A)·poly(U) are pH 6.5 and pH 6.0, respectively, a result that depends in part upon the tendency of the double-stranded substrate to be dissociated at lower pHs.

The Activity of Polyspermine-RNase on DNA-RNA Hybrids. The DNA–RNA hybrid, poly(dA)·poly(rU), was degraded by a covalently cross-linked dimer at a rate 8.5 times greater than that with RNase monomer (Wang et al., 1976), whereas polyspermine-RNase shows an activity 382 times that of the native enzyme (Table II). The derivative showed only a 32-fold increase in activity toward poly(dI)·poly(rC).

The Activity of Polyspermine-RNase on Poly(C) and Poly(U). The activity ratios of polyspermine-RNase/RNase for these two ssRNAs at various pHs as presented in Figure 3 are quite different. Toward poly(C) (with a potential NH_3^+ group on the pyrimidine ring), the derivative was much more efficient than the native enzyme at higher pH and the difference in activity between these two enzymes diminished as the pH dropped. In contrast, the difference in nucleolytic activity between these two enzymes toward poly(U) is relatively pH independent. The pH optimum for activity of either enzyme toward poly(C) and poly(U) was pH 7.0. At pH 7.5 RNase hydrolyzed poly(U) (40 μg/mL) at a rate of 27 *A* units min^{-1} mg^{-1}, whereas polyspermine-RNase hydrolyzed poly(A)·poly(U) (80 μg/mL) at 12 *A* units min^{-1} mg^{-1}. Thus the derivative hydrolyzes the poly(U) complementary strand of the dsRNA at a rate approximately 50% of that with which RNase A hydrolyzes poly(U).

The Activity of Polyspermine-RNase toward Yeast RNA and Yeast RNA Core. Yeast RNA core, which had been derived by exhaustive digestion by RNase A, gave essentially no hydrolysis with RNase A under the conditions of our assay. However, polyspermine-RNase showed a specific activity of 29 units/mg (Hilmoe, 1960). When yeast RNA was the substrate, polyspermine-RNase was only 73–100% and 73% as active as RNase A at pH 7.5 and at pH 5.0, respectively, by the spectrophotometric method, and 73% as active at pH 7.5 by the acid precipitation method.

The Activity of Polyspermine-RNase toward Poly(A). Although pancreatic RNase is normally specific for pyrimidine nucleotides of RNA, the polynucleotide of adenylate is hydrolyzed at a very slow rate at high enzyme concentration (Beers, 1960; Libonati, 1971; Wang et al., 1976). In neutral and alkaline aqueous solutions and ambient temperature, poly(A) occurs as a single-stranded, uncomplexed polymer, but with highly ordered helical secondary structure which is maintained by base-stacking interactions (Felsenfeld and Miles, 1967; Holcomb and Tinoco, 1965; Van Holde et al., 1965; Leng and Felsenfeld, 1966; Sarker and Young, 1965; Poland et al., 1966; Brahms et al., 1966). Aside from the base specificity of RNase, this secondary structure may indeed also contribute to the resistance of poly(A) to the action of this enzyme. Polyspermine-RNase has a slightly enhanced nucleolytic activity toward this substrate; when the amount of enzyme was 20 times the amount of substrate, in 60 min RNase A solubilized 5% of the poly(A) and polyspermine-RNase solubilized 20%. At the low enzyme:substrate ratios used in a typical assay toward the dsRNA poly(A)·poly(U), no significant hydrolysis of the poly(A) moiety can be expected.

The Activity of Polyspermine-RNase on Viral dsRNAs. The nucleolytic activity of polyspermine-RNase toward dsRNA of *Penicillium chrysogenum* virus was 60 times as great as that of RNase A. The polyspermine-RNase is thus more active toward this substrate than the RNase dimer (Wang et al., 1976).

A similar result was obtained when dsRNA of reovirus 3 was subjected to the action of these two enzymes (Figure 4). In terms of structural stability, the reovirus dsRNA is much less resistant than that of *Penicillium chrysogenum* virus to the action of polyspermine RNase. The dsRNA of bacteriophage ϕ6 was degraded by polyspermine-RNase at a rate similar to dsRNA of reovirus, under similar conditions. The specific activity of the derivative toward reovirus dsRNA was 113 times that with RNase A, a result identical with that observed with poly(A)·poly(U).

The Activity of Polyspermine-RNase toward Cyclic 2',3'-Cytidylate. In 0.025 M NaCl at pH 8, polyspermine-RNase was about twice as active as RNase A toward cyclic 2',3'-cytidylate; in 0.225 M NaCl the derivative was four times as active as RNase toward the cyclic substrate.

The Effect of Free Spermine or Polyspermine on the Activity of RNase. Polyamines, such as spermidine and spermine, are known to interact with nucleic acids to form nucleic acid–polyamine complexes (Cohen, 1971; Tabor and Tabor, 1964, 1972). Spermine has been shown to stimulate or inhibit nucleases (Frank et al., 1975; Gabbay and Shimshak, 1968; Schmukler et al., 1975; Holbrook et al., 1975). In order to eliminate the possibility that the enzymic activity shown by polyspermine-RNase could be duplicated by an admixture of RNase A and free polyamine, control experiments on the effect of free spermine and the cross-linked polyamine on the degradation of various substrates were carried out. At concen-

trations between 1 μM to 0.2 mM spermine (or spermine in the polymerized form) exerted only a slight (less than 10%) enhancement of RNase activity toward poly(A)·poly(U). At higher concentrations (0.72 to 2.87 mM) spermine inhibited the enzyme. A similar effect of this polyamine was also observed on the degradation of poly(C) by RNase A, except that the concentration necessary to yield these effects was shifted about 40-fold downward.

Discussion

The method of synthesis and the chromatographic purification give a product which is a mixture of closely related isomers which can vary in the length of the polyspermine side chain and in the position of attachment to a lysine residue. The number of basic charges introduced is a factor in the enhancement of the activity of the enzyme since monospermine-RNase has very low activity relative to polyspermine-RNase. The result of the polyspermine attachment is an increased ability of the enzyme to hydrolyze highly structured RNAs. Such structure is dependent upon salt concentration (Michelson et al., 1967); most of the assays have been conducted in 0.125 M NaCl and near neutral pH. Polyspermine-RNase is thus active toward a variety of double-stranded substrates at physiological pH and ionic strength values, a type of activity that is of current concern in research on bacterial ribonucleases (Robertson et al., 1968; Dunn, 1976) and mammalian ribonucleases (Bardón et al., 1976; Sarngadharan et al., 1975; Ohtsuki et al., 1977). The interest in derivatives of RNase extends to the study of their actions on tumor cells, as examined with the cross-linked dimer by Tarnowski et al. (1976).

Acknowledgments

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Postproline Cleaving Enzyme: Identification as Serine Protease Using Active Site Specific Inhibitors†

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ABSTRACT: Postproline cleaving enzyme (PPCE) (EC 3.4.21.-) is an endopeptidase which has a strong specificity for catalyzing the cleavage of the Pro-X peptide bond. The enzyme also exhibits esterase activity. Highly purified PPCE is not inhibited by EDTA or 1,10-phenanthroline and partial inhibition is obtained only with high molar ratios of *p*-hydroxymercuribenzoic acid, sodium tetrathionate, 5,5′-dithiobis(2-nitrobenzoic acid), or 2-iodoacetamide. Both peptidase and esterase activities are lost upon treatment with diisopropyl phosphorofluoridate (iPr_2P-F). Titration with tritiated inhibitor, [^{3}H]iPr_2P-F, reveals that this inhibition is irreversible and occurs on a 1:1 molar basis. PPCE inhibited by treatment with [^{3}H]iPr_2P-F retains the p*I* of 4.8 of unmodified PPCE and has a similar subunit molecular weight (58 000) on sodium dodecyl sulfate electrophoresis as unmodified PPCE, and identical chromatographic properties on the affinity column Z-Pro-D-Ala-poly(Lys)-Sepharose 4B. The pH dependence of incorporation of [^{3}H]iPr_2P-F is similar to that of the enzymatic hydrolysis of the standard substrate Z-Gly-Pro-Leu-Gly. Increasing concentrations of the competitive inhibitor Z-Gly-Pro progressively reduce the amount of [^{3}H]iPr_2P-F incorporated into PPCE and none of the [^{3}H]iPr_2P-F is incorporated when PPCE is preincubated in the presence of 8 M urea or subjected to heat treatment. These results suggest, albeit based on indirect evidence, that a single serine residue plays a vital role in the catalytic process of PPCE. Chloromethyl ketone derivatives of Tos-Pro, Z-Pro, Tos-Gly-Pro, Z-Gly-Pro, and Z-Gly-Gly-Pro inhibit PPCE following a pseudo-first-order rate constant ($k_{obsd}/[I]$ = 0.43, 0.35, 10.3, 55.6, and 109 M^{-1} s^{-1}, respectively), but these inhibitors do not affect trypsin, α-chymotrypsin, elastase, and papain. Pretreatment of PPCE with Z-Gly-ProCH_2Cl prevents the incorporation of [^{3}H]iPr_2P-F into the enzyme. These data are taken as indirect evidence that the chloromethyl ketone inhibitors interact with a critical histidine residue of PPCE. On the basis of the results presented, it is hypothesized that the active site of PPCE has a Asp-His-Ser triad, analogous to other serine proteases.

Postproline cleaving enzyme (PPCE)[1] was first discovered in human uterus, when it was found that preparations of this target organ of oxytocin cleave the prolyl[7]-leucyl[8] peptide bond of the nonapeptide hormone (Walter et al., 1971). Subsequent studies seem to suggest that this enzymatic activity is ubiquitous in vertebrates, but is present only at low levels (Walter, 1973). The enzyme has been purified from lamb kidney and characterized as the first known endopeptidase with a high specificity for cleaving the -L-Pro-X-peptide bond except for the -L-Pro-L,D-Pro- bond, which for all practical purposes is not hydrolyzed (Walter, 1976; Koida and Walter, 1976).

In this paper, information on the active site of PPCE is obtained by inhibition and kinetic studies, and the catalytic mechanisms of both the peptidase and esterase activities of the enzyme are investigated. While reagents known to inhibit sulfhydryl proteases (Glazer and Smith, 1971) and metal-activated enzymes (Mildvan, 1970) only reduce the activities of PPCE at exceedingly high molar ratios, the synthetic organophosphorus inhibitor, diisopropyl phosphorofluoridate (Jansen et al., 1949), stoichiometrically reacts with PPCE; this finding may be the first indication that PPCE is a serine protease since this agent is known as a rather specific inhibitor of all serine proteases, such as α-chymotrypsin, trypsin, elastase, and subtilisin BPN′ (Cohen et al., 1967; Hartley, 1960; Walsh and Wilcox, 1970). PPCE was also allowed to react with amino acid and peptide chloromethyl ketones, which have been important in implicating by specific alkylation the functional role of a histidine residue located in the active center of the above serine proteases (Petra et al., 1965; Schoellmann and Shaw, 1963; Thompson and Blout, 1973; Morihara and Oka, 1970). Whereas chloromethyl ketone derivatives of phenylalanine and lysine were ineffective in the case of PPCE, newly synthesized proline-containing chloromethyl ketone derivatives showed a high degree of specificity and only inhibited PPCE irreversibly. The results presented lead to the tentative conclusion that PPCE is a representative member of the well-characterized family of serine proteases.

Materials and Methods

PPCE was purified over 10 000-fold from lamb kidney as described by Koida and Walter (1976). (9-[1-^{14}C]Glycinamide)arginine-vasopressin was from the same batch prepared by Walter and Havran (1971) used previously. α-Chymotrypsin, trypsin, elastase, papain, N^{α}-benzyloxycarbonyl-

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[1] Abbreviations used follow the tentative Rules and Recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature for amino acids and peptides ((1972), *J. Biol. Chem. 247*, 977) and for enzyme inhibitors as circulated by the Office of Biochemical Nomenclature on July 17, 1975. All optically active amino acids are of L configuration unless otherwise stated. Additional abbreviations used are: PPCE, postproline cleaving enzyme; Nbs, 5,5′-dithiobis(2-nitrobenzoic acid); iPr_2P-F, diisopropyl phosphorofluoridate.