Structure of Canine Pancreas Polysomes. Effects of Proteases on Sedimentation Behavior and Incorporation of Amino Acids into Polypeptides*

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ABSTRACT: Canine pancreas ribonucleoprotein particles have been treated with proteases, and the effects on gradient centrifugation patterns and ability to incorporate amino acids into protein were studied. Trypsin (5 μg/ml) at 37° altered purified polysomes as follows: (1) The 80 S ribosome fraction was sharply reduced in amount. (2) Subfractions (40 S and 60 S) were formed in unequal amounts. (3) The relative size of the polysome peak was first decreased, then increased. Larger amounts of trypsin resulted in almost complete disappearance of the heaviest material. The release of peptides from polysomes was demonstrated by paper chromatography. Ribonuclease treatment disrupted polysomes completely with formation of 80 S ribosomes and dimers. Chymotrypsin and papain were less effective than trypsin in inhibiting amino acid incorporation by ribonucleoprotein particles systems. Stimulation of phenylalanine incorporation with polyuridylic acid by a crude microsomal preparation (S-30) was completely prevented by low concentrations of trypsin.

The discovery of ribosomal clusters and chains (polysomes) in cells and the demonstration of their function in protein biosynthesis emphasize the relationship between individual ribonucleoprotein particles and messenger ribonucleic acid (m-RNA) strand.

Very little is known concerning the mode of association of the ribosomes with the m-RNA. The utilization of enzymes as probes in this system may furnish information on the binding forces between the components. Thus, ribonuclease (RNAase) has been shown to convert polysomes from rat liver (Wettstein *et al.*, 1963), reticulocytes (Warner *et al.*, 1963), and skeletal muscle (Breuer *et al.*, 1964) rapidly and completely to a mixture of 80 S particles. Breuer *et al.* (1964) found that rat skeletal muscle polysomes were resistant to chymotrypsin, papain, and collagenase as revealed by electron micrographs. Rabinowitz *et al.* (1964) reported that trypsin, chymotrypsin, or RNAase partially disrupted polysomes obtained from chick embryo heart muscle. Kaji and Kaji (1965) observed that pretreatment of *E. coli* ribosomes with trypsin, chymotrypsin, or papain inhibited the subsequent binding of polyuridylic acid (poly-U) or transfer RNA.

In the present work polysomes were treated with proteases. The sedimentation characteristics and ability to incorporate amino acids into protein of the particles were studied. The stimulation of phenylalanine incorporation in response to the addition of poly-U after treatment of a microsomal preparation (S-30) with trypsin and chymotrypsin was also measured.

Experimental Procedures

Materials. Trypsin (recrystallized two times), chymotrypsin, trypsinogen, and soybean trypsin inhibitor (SBTI)[1] were purchased from Worthington Biochemical Corp. They exhibited no RNAase activity. RNAase (protease-free) was bought from Sigma Chemical Co. DL-Valine-^{14}C (6 mcuries/mmole) and DL-phenylalanine (21 mcuries/mmole) were purchased from Nuclear-Chicago Corp. Poly-U was obtained from Miles Laboratories. Papain was a gift from Dr. Albert Light. It was activated by the procedure of Hill and Schmidt (1962).

Preparation of Canine Pancreas Polysomes. Freshly excised and trimmed pancreas was chilled and passed through a tissue press at 4° to remove connective tissue. The pulp was homogenized and polysomes (ergosomes) were isolated by a modification of the procedure of Wettstein *et al.* (1963) for "C" ribosomes. It was found that polyribosome isolation required the presence of the buffer salts in the 0.5 and 2.0 M sucrose solutions.[2] The pellets were rinsed in medium A, combined, and resuspended by standing overnight at 0° in 0.1 ml of this solution per tube. The suspensions were finally shaken gently for 30 min. Concentration of ribonucleoprotein was calculated from the A_{260} reading using the factor, 10 = 1 mg of ribonucleoprotein/ml.

Preparation of High Speed Supernatant Solution. Fresh rat or canine liver, or canine pancreas, was homogenized in three volumes of ice-cold medium A and centrifuged 2 hr at 86,000 × *g*. The clear portion

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[1] Abbreviations used in this work: SBTI, soybean trypsin inhibitor. ATP and GTP, adenosine and guanidine triphosphate, respectively.

[2] J. P. Breillatt and S. R. Dickman, unpublished.

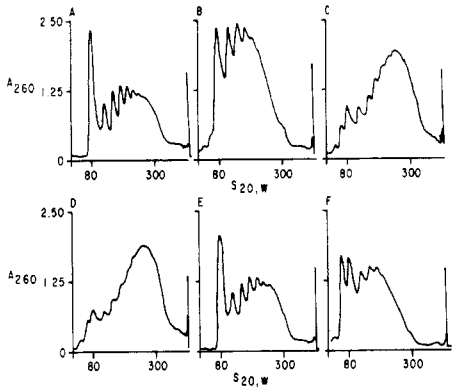

FIGURE 1: Gradient centrifugation of trypsin-treated pancreas polysomes. Polysomes were incubated at 37° as follows: (A) No addition; (B) trypsin, 5 μg/ml, 5 min; (C) trypsin, 5 μg/ml, 10 min; (D) trypsin, 5 μg/ml, 15 min; (E) trypsin, 25 μg/ml, plus SBTI, 100 μg/ml, 15 min; (F) trypsin, 25 μg/ml, 15 min.

of the supernatant solution was removed and stored at −20°.

Preincubation of Polysomes with Proteases and Incorporation of [^{14}C]Valine into Protein. Polysomes (4 mg) were preincubated for 15 min at 37° in a solution which contained (in μmoles) KCl, 60; $MgCl_2$, 2; Tris, pH 7.4, 10; β-mercaptoethanol, 2.5; ATP, 0.5; GTP, 0.25; creatine phosphate, 2.5; in 0.5 ml. In addition 5 μg of creatine kinase and proteolytic enzyme were added as indicated. The suspension was then chilled, and 0.2 ml was transferred to a test tube containing 0.3 ml of a cold mixture of KCl, $MgCl_2$, mercaptoethanol, ATP, GTP, creatine phosphate, and creatine kinase in the amounts listed above based on 0.5-ml total volume. Dog pancreas high-speed supernatant (0.1 ml), 50 μg/ml of SBTI, and DL-valine-^{14}C, 0.5 μcurie, were added, and the tube was incubated 30 min at 37°. At the end of the incubation most of the particles have been shown to occur as 80 S ribosomes.[2] The reaction was stopped with an equal volume of 10% trichloroacetic acid. The precipitate was washed by the method of Siekevitz (1952) and the final residue was dissolved in 98% formic acid, plated on Al planchets, dried, and counted in a Nuclear-Chicago automatic, windowless, gas-flow counter. All counts have been corrected for self-absorption.

Incubation of Polysomes with Proteases. Density-Gradient Sedimentation Analysis. Polysomes (4 mg/0.1 ml) in medium A were incubated for 15 min at 37° in 0.5 ml of solution which contained in μmoles: KCl, 75; $MgCl_2$, 2; β-mercaptoethanol, 5; and protease as indicated. Polysomes were found to be stable in this solution, to which nucleotides, RNA, or high-speed supernatant solution was not added. The absence of these high A_{260} components simplified the sedimentation analysis and the subsequent paper chromatography. The proteolysis was terminated by the addition of 50 μg/ml of SBTI; the solution was chilled and 0.075 ml was layered on a 10–30% linear sucrose gradient which contained medium A buffer salts. It was sedimented at 39,000 rpm for 45 min at 3 ± 1° in the SW-39 rotor of the Spinco Model L ultracentrifuge. The solution was passed through the flow cell of the Gilford automatic recording apparatus with the monochromator set at 260 mμ. The sedimentation coefficients ($s_{20,w}$) have been calculated by the equation of Martin and Ames (1961).

Paper Chromatography. The remainder of the incubated suspension (0.42 ml) was centrifuged at 60,000 × *g* for 2 hr. Aliquots of the supernatant solutions were spotted on Whatman No. 3 paper and chromatographed in 1-butanol–acetic acid–water (4:1:5) in an ascending system for 6 hr at room temperature. The paper was dried and sprayed with ninhydrin aerosol. Other portions of the supernatant solutions were diluted with H_2O and their absorbances at 260 and 280 mμ were determined.

Results

Effect of Proteases on Canine Pancreas Ribonucleoprotein Particles

Inhibition of Protein Synthesis. Preincubation of a polysomal suspension in the presence of 10 or 50 μg/ml of trypsin resulted in inhibition of [^{14}C]valine incorporation into protein of 79 and 88%, respectively (Table I). The effect was prevented by the addition of soybean trypsin inhibitor to the protease before the polysomes were added. This result suggests that trypsin action was directly responsible for the inhibition. The addition of trypsinogen did not inhibit [^{14}C]valine in-

TABLE I: Effect of Preincubation of Canine Pancreas Polysomes with Proteases on Incorporation of [^{14}C]-Valine into Protein.[a]

Expt	Addition to Preincubation Suspension	(μg/ml)	[^{14}C]Valine Incorporated (μμmoles/mg of protein)
1	Trypsin	0	200
		10	41
		50	23
		25[b]	186
	Trypsinogen	25	176
	Papain	25	68
2	Chymotrypsin	0	173
		10	131
		25	88

[a] See text for experimental details. [b] To which had been added 100 μg of soybean trypsin inhibitor.

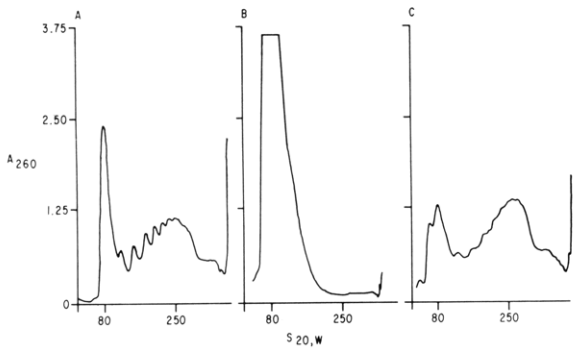

FIGURE 2: Gradient centrifugation patterns of pancreas polysomes treated with RNAase or chymotrypsin. Incubated at 37°. (A) No addition; (B) RNAase, 1 μg/ml, 15 min; (C) chymotrypsin, 50 μg/ml, 15 min.

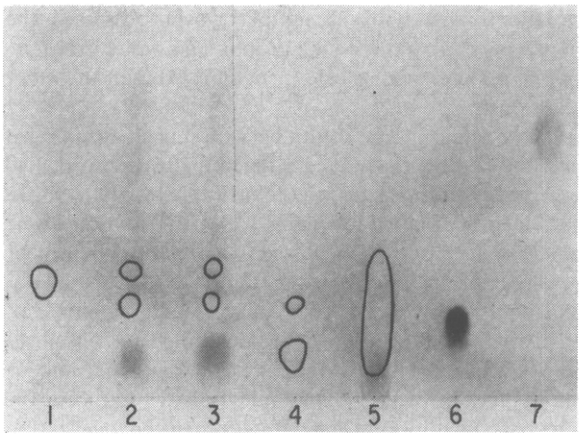

FIGURE 3: Solubilization of components from pancreas polysomes by enzymes as demonstrated by paper chromatography. Supernatant solution (0.05 ml) from polysomes, incubated for 15 min as indicated, was chromatographed. The solid dark areas indicate ninhydrin-positive material. The circumscribed areas indicate ultraviolet absorbing material. (1) No addition; (2) trypsin, 5 μg/ml; (3) trypsin, 25 μg/ml; (4) trypsin, 25 μg/ml, plus SBTI, 100 μg/ml; (5) RNAase, 0.1 μg/ml; (6) arginine standard, 70 μg; (7) phenylalanine standard, 75 μg.

corporation. Two other proteases, cyanide-activated papain and chymotrypsin, inhibited polypeptide synthesis but to a considerably lesser degree than trypsin.

Alteration in Sedimentation Characteristics. When a polysomal suspension was preincubated with trypsin for varying periods prior to density-gradient centrifugation the patterns shown in Figure 1 were obtained. The area under each peak was measured with a planimeter and the percentage of the total area was calculated. Three major effects may be noted.

(1) There was an early appearance of ribosomal subfractions of 40 S and 60 S size. The 60 S particle increased in amount with time of incubation, whereas the 40 S subfraction did not. With 25 μg/ml of trypsin acting for 15 min the amount of the 60 S subfraction was 60% as large as that of the 80 S particle.

(2) The relative concentration of the 80 S particles decreased with time of incubation with trypsin. After 15 min they were only about one-third to one-half the size of the control. The increase in subfraction material is not large enough to account for the decrease in 80 S particles.

(3) The quantity of the larger polysomes (greater than tetramers) varied with time of incubation. There was a decrease after 5-min treatment with trypsin followed by an increase in the 10- and 15-min samples. The data suggest that aggregation of a large portion of the altered 80 S particles occurred, and this phenomenon is responsible for the increase in heavy material. This material was ultimately unstable in the presence of 25 μg/ml of trypsin, since the heavier polysomes have been degraded in this sample (Figure 1, F). As Figure 1 (E) shows, soybean trypsin inhibitor completely prevented this effect of trypsin on polysomal preparations.

The effect of RNAase is quite distinctive and totally different from that of trypsin (Figure 2, B). Nuclease treatment completely destroyed the polysome region with formation of monomeric 80 S particles primarily and small amounts of dimers. No trace of subfractions was evident. Chymotrypsin treatment (50 μg/ml) produced a pattern quite similar to that from the 5 μg/ml trypsin-treated sample. These results clearly demonstrate that the effects of the proteases on the particles can be readily distinguished from those of RNAase.

The data of Table II show that RNAase action liberated over four times as much A_{260} material as the control or the trypsin-treated sample. The somewhat lower absorbances in the ultraviolet spectral range of the supernatant solution of this latter sample compared to the untreated was unexpected. Secondary reactions of the protease-formed products may be responsible for this result. It is also noteworthy that the

TABLE II: Absorbance of Supernatant Solutions after Incubation of Polysomes with Enzymes.[a]

Enzyme	(μg/ml)	A_{260}	A_{280}	A_{260}/A_{280}
Trypsin	0	0.117	0.069	1.70
Trypsin	5	0.101	0.062	1.63
Trypsin	25	0.069	0.046	1.50
Trypsin + SBTI	25 + 100	0.095	0.057	1.67
RNAase	0.1	0.442	0.249	1.77

[a] Polysomes were incubated 15 min at 37°, then centrifuged at 60,000 × *g* for 2 hr. The supernatant solutions were diluted 1:100 with H_2O for the absorbance readings.

sample treated with 25 μg of trypsin possessed the lowest A_{260}/A_{280} ratio of the group. This value is indicative of a lower nucleic acid/protein ratio than the other solutions.

These supernatant solutions were chromatographed and the sheet was sprayed with ninhydrin. It was also examined during exposure to ultraviolet light. These results are presented in Figure 3. In confirmation of the absorbance data it can be seen that trypsin treatment resulted in the release of ninhydrin-positive material as well as small amounts of nucleotides. The effects of RNAase action were likewise not limited to solubilization of components from its specific substrate since a small amount of peptidelike material was found in the supernatant solution. The differences in the localization of the ribosomal hydrolytic products on chromatography suggest distinctive mechanisms in the solubilization phenomena. The nucleotides released by trypsin migrated as two separate zones, neither of which overlapped the ninhydrin-positive area. Those nucleotides solubilized by RNAase, on the other hand, formed a larger and more diffuse area, the slower migrating portion of which definitely overlapped the peptide spot near the origin.

TABLE III: Effect of Preincubation of Canine Pancreas S-30 with Proteases on Stimulation of Incorporation of [^{14}C]Phenylalanine or [^{14}C]Amino Acid Mixture into Protein by Poly-U.[a]

Additions to Preincubation Medium	(μg/ml)	Addition of Poly-U Incubation Medium	[^{14}C]Polypeptide Synthesis [^{14}C]-Phenylalanine (μμmoles/mg protein)	[^{14}C]-Amino Acid Mixture (cpm/mg protein)
Trypsin	0	−	6	85
		+	380	435
	5	−	4	70
		+	20	155
	10	−	4	
		+	4	
	25	−	4	
		+	4	
	50	−	4	
		+	4	
Chymotrypsin	10	−	4	
		+	112	
	25	−	4	
		+	60	

[a] S-30 (Gardner *et al.*, 1962, 10 mg) was preincubated, then incubated as described in the text except that 0.5 μcurie of DL-[^{14}C]phenylalanine (21 mcuries/mmole) or 0.5 μcurie of algal protein hydrolysate was added. Dog pancreas high-speed supernatant (0.1 ml) and 200 μg/ml of SBTI were added to the incubation media. Poly-U (125 μg/ml) was included as indicated.

Effect of Proteases on Response of Canine Pancreas S-30 to Poly-U. A pancreas S-30 preparation, after preincubation, exhibited a large increase in phenylalanine incorporation in response to the addition of poly-U (Table III). This stimulation by poly-U was completely blocked when 10 μg/ml of trypsin was included in the preincubation suspension. Small amounts of trypsin (5 μg/ml) or of chymotrypsin (10 μg/ml) resulted in 95 and 30% inhibition, respectively. These results indicate that the membranous components of the S-30 do not protect the ribosomes from trypsin degradation. It appears that the ribosomes of this preparation have been disrupted as readily as those of the polysome suspension, and poly-U is unable to be attached to them in a functional manner.

Discussion

At present, information on the detailed structure of ribosomes is lacking. It is well known that 80 S ribonucleoprotein particles are unstable when placed in Mg^{2+}-free solutions and dissociate into 40 S and 60 S subunits. It may be noted that T'so and Vinograd (1961) observed that the 40 S subunit was considerably less stable than the 60 S when rabbit reticulocyte ribosomes were placed in solutions which contained no Mg^{2+}. The possibility must be considered that the two types of subfractions which are formed by proteolysis are different from the subunits produced in the absence of Mg^{2+}. It is for this reason that we refer to those formed by trypsin action as subfractions.

The variation in the size of the polysome peak with length of trypsin incubation makes assessment difficult whether individual 80 S particles are more or less resistant to protease attack than those associated with the m-RNA strand. Definite changes in both the single as well as the polysomal forms of the 80 S ribosomes are evident after 5-min incubation with 5 μg/ml of trypsin. These results suggest that association with the m-RNA strand does not confer additional stability.

Another aspect of the effects of proteases on polysomes concerns the components of the various peaks in the range 110–300 S. From the paper chromatographic patterns, it appears that more peptidelike material from these samples was solubilized than in the control. However, the solubilization of total A_{260} material is lower in the trypsin-treated (25 μg/ml) sample supernatant than that of the control. These data suggest that much of the rapidly sedimenting material in the treated samples is not composed solely of trypsin-resistant polysomes but is, in fact, altered ribosomes and subfractions randomly aggregated with RNA. It can be further postulated that this material, adsorbed nonspecifically to the various polysomes, is responsible for the greater overlapping of these peaks in the patterns obtained after trypsin treatment. Further work is necessary to determine the cause of the transient increase in the heavy material.

The proteins of ribosomes possess alkaline isoelectric

points, and they contain a high proportion of basic amino acids (Petermann, 1964). Thus, it is worth noting that an enzyme with a specificity for hydrolysis of peptide bonds adjacent to arginine or lysine residues (trypsin) is considerably more damaging to the ribosomes than either chymotrypsin or papain. The amino acid composition and size of the various free peptides which were formed by trypsin action remain to be determined.

The almost complete conversion of polysomal material to 80 S ribosomes by RNAase with these pancreas preparations is similar to the results of Wettstein *et al.* (1963) with liver and of Breuer *et al.* (1964) with rat skeletal muscle polysomes, but differs from those of Rabinowitz *et al.* (1964) on embryo heart polysomes. Perhaps the polysomal structure of many tissues is held together solely by RNA while in at least one instance other types of chains assist in its maintenance. On the other hand, Breuer *et al.* (1964) found no discernible effect of chymotrypsin or papain on the sedimentation properties or appearance in the electron microscope of polysomes from rat skeletal muscle.

Ribosomes in a "natural" milieu, attached to membranes and in the presence of other components of the high-speed supernatant, are more strongly inhibited by trypsin treatment in their response to added poly-U than is the direct incorporation of amino acids into protein by purified polysomes. The latter are largely disrupted to 80 S particles under the conditions of incorporation of free amino acids into protein.[2] This process might be expected to occur in the S-30 preparation as well. A significant difference in the two systems lies in the relationship of the individual ribosomes to the m-RNA strand. With the S-30, the incorporation data furnish a measure of newly formed associations of the particles with the added messenger (poly-U). With the 80 S ribosomes, the incorporation may primarily reflect the finishing of incompleted polypeptide chains mediated by the m-RNA already associated with the particles. Reattachment of individual ribosomes to long strands of m-RNA in the sample may play but a small role in amino acid incorporation under these conditions.

These results thus suggest a differential effect of proteolysis on ribosomal function. The initiation of complex formation between the particles and m-RNA appears to be the most protease-sensitive step in the over-all series of reactions in the biosynthesis of proteins. This conclusion does not imply, however, that ribosomal protein and m-RNA bind directly. It will be interesting to determine whether the "m-RNA association site" on the ribosomes will consist of protein, RNA, or of both components.

Acknowledgment

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