PROTEOLYTIC ACTIVITY OF PARTLY PURIFIED RIBONUCLEASE INHIBITOR FROM RAT LIVER

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SUMMARY

Increasing interest has been focused on ribonuclease inhibitor from rat liver as protecting agent in studies on polyribosomes. Most investigators use either the crude 100 000 $\times$ g supernatant or a preparation partly purified on DEAE-Sephadex. Whereas partially purified material protects the integrity of the polysomes, it contains a component that interferes with peptide formation. The interfering activity is of proteolytic nature and can be removed by gel filtration on Sephadex G-100.

Evidence is provided that at least part of the enzymic activity has to be ascribed to a potent leucine aminopeptidase.

Recently we reported the isolation and purification of a polysome stabilizing factor from rat liver^{1,2} identical with the ribonuclease inhibitor, first described by ROTH³. We use this factor as protecting agent in the isolation procedure for intact polysomes, ribosomal subunits and the preparation of run-off ribosomes, which is performed under conditions of amino acid incorporation. For the isolation of intact polysomes from leukemic spleen the presence of the factor was a prerequisite⁴, and when ribosomal subunits from bovine eye lens tissue had to be prepared, we frequently observed that an almost complete degradation of the 40-S subunit occurred when the stabilizing factor was omitted.

In various other laboratories, too, the crude 100 000 $\times$ g supernatant or the inhibitor partly purified on DEAE-cellulose according to SHORTMAN⁵ has been used to obtain intact polysomes from different sources⁶⁻⁸.

It may also be advantageous to use the ribonuclease inhibitor in amino acid incorporation experiments. However, while studying protein biosynthesis in the eye lens system, we observed that on the one hand the inhibitor partly purified on DEAE-Sephadex prevented polyribosomes from degradation, but on the other hand the amino acid incorporation was diminished. We found that further purification of the DEAE-Sephadex material on Sephadex G-100 (Fig. 1) was necessary to obtain optimal incorporation of amino acid (Fig. 2).

The inhibitory effect of both the DEAE-Sephadex material and of Peak I from Sephadex G-100 is evident. During the first 10 min of incubation there was only a slight difference in effect on amino acid incorporation of either of the fractions tested. This may mean that the accessibility of the newly formed peptide chains

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Fig. 1. Gel filtration of the partly purified ribonuclease inhibitor on Sephadex G-100. ●---●, inhibitor activity.

is involved in this phenomenon. If this is true proteolytic activity might explain the inhibitory effect on amino acid incorporation. To test this assumption we incubated the different ribonuclease inhibitor fractions with ^{14}C -labeled hemoglobin, prepared according to LOCKARD *et al.*⁹ by incubation for 2 h of a reticulocyte lysate system with ^{14}C leucine. After removal of the ribosomes the supernatant was passed through a Sephadex G-25 column and the void volume was designated as radioactive hemoglobin. The results are listed in Table I. From Fig. 2 it can be concluded that the gel filtration step removes the "inhibitory" activity on amino acid incorporation whereas Table I shows that this activity is due to some kind of proteolysis, as the hot tri-

TABLE I

DEGRADATION OF RADIOACTIVE HEMOGLOBIN AFTER INCUBATION IN THE PRESENCE OF CERTAIN RIBONUCLEASE INHIBITOR FRACTIONS

The undegraded hemoglobin used in the incubation experiment corresponded with 8500 disint./min. Incubation was for 2 h. The buffer consisted of 0.05 M Tris-HCl (pH 7.6), 0.07 M KCl, 0.005 M magnesium acetate and 0.001 M dithiothreitol.

Addition	Radioactivity (disint./min)
Buffer only	8300
DEAE-Sephadex material (700 μg protein)*	5300
Sephadex G-100 Fraction I (525 μg)	5500
Sephadex G-100 inhibitor (15 μg)*	8200
Serum albumin (control for self absorption) (700 μg)	8300

* 175 units.

Fig. 2. $[^{14}\text{C}]$ Leucine incorporation activity of calf lens polyribosomes in the presence of different protein fractions. Calf lens polyribosomes¹¹ (100 μg RNA) were incubated during different time intervals with calf lens supernatant (2 mg of protein). The incubation mixtures contained 1.25 μmoles phosphoenolpyruvate, 0.125 μmole ATP, 0.063 μmole GTP, 0.69 mg 2-mercaptoethanol, 12.5 μg pyruvate kinase (EC 2.7.2.40), 10 μmoles DL $[^{14}\text{C}]$ leucine (specific activity 34 mC/mmole) a mixture of unlabeled amino acids (excluding leucine) 10 nmoles per amino acid. All components were dissolved in Medium B, containing 0.05 M Tris-HCl buffer (pH 7.6), 0.008 M MgCl_2 and 0.025 M KCl. The final volume was 0.25 ml. Incubation was performed for 45 min at 37°. At zero time 25 μl of the following fractions were added: x---x, buffer, O—O, Sephadex G-100 ribonuclease inhibitor fraction (11 μg of protein, 102 units); ▲—▲, Sephadex G-100 first protein peak (328 μg of protein); Δ—Δ, Sephadex G-100 second protein peak (328 μg of protein); ●—●, DEAE-Sephadex ribonuclease inhibitor fraction (410 μg of protein, 102 units). After different time the reaction was stopped by the addition of trichloroacetic acid. With reticulocyte polyribosomes similar results were obtained.

chloroacetic acid-precipitable radioactive material diminished about 30 % after incubation with crude DEAE-Sephadex inhibitor or Sephadex G-100 Fraction I. In gel filtration on Sephadex G-50 the degradation products of hemoglobin emerged in the region of free amino acids.

We provide evidence that a potent leucine amino peptidase is, at least partly, responsible for the proteolytic activity. According to BERNT AND BERGMAYER¹⁰, L-leucyl- β -naphthylamine hydrochloride is a highly specific substrate for this enzymic activity. 1 mg of the DEAE-Sephadex material contains as much as 0.3

I.U. of the enzyme. The proteolytic activity that originates from the soluble enzyme fraction also attacks growing peptide chains. This was demonstrated by replacing the radioactive hemoglobin by ribosome-bound peptides.

These findings have to be kept in mind during studies on protein synthesis in a cell-free system in which rat liver cell sap is used. In particular, it will be difficult to draw unequivocal conclusions concerning the terminal NH_2 of newly formed polypeptides in the homologous system from rat liver *in vitro*.

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