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Activation of Ribonuclease I by Gamma Irradiation of 30 S Ribosomes from *Escherichia coli*

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A sensitive assay of ribonuclease activity was designed using polyuridylic acid as a substrate. The amount of the undigested polyuridylic acid was subsequently determined in a cell-free system for the polymerization of phenylalanine from *Escherichia coli*. This assay was used for the characterization of a nuclease activated by the irradiation of purified ribosomes from the same bacterium. The enzyme was identified as ribonuclease I by (1) its location on the 30 S ribosomal subunit, (2) by the absence of mononucleotides in the products of short incubation, as determined by paper chromatography, and (3) by identification of oligonucleotides as products of such an incubation using chromatography on dextran gel. Activated enzyme sedimented with 30 S ribosomes and exhibited its activity even at the concentrations of Mg^{++} which are inhibitory for the »latent« ribosomal ribonuclease.

INTRODUCTION

Three enzymes in *Escherichia coli* which break down ribonucleic acid are known. First one is »latent« ribosomal ribonuclease¹⁻³, recently named RNase I* and found to be a typical endonuclease⁴. The second enzyme, RNase II, is both endo- and exonuclease with natural substrates, but its action on polyuridylic acid is predominantly of exonucleolytic character⁴. The third enzyme is polynucleotide phosphorylase⁵, which in the presence of phosphate yields ribonucleoside-5'-diphosphates.

RNase II appears to be only loosely bound to the ribosomes⁴, while RNase I was usually considered to be a truly ribosomal enzyme, for it was found associated in a latent form with only one species of these particles, namely, the 30 S ribosomes^{6,7}. In the presence of Mg^{++} ribosomes show no »latent« RNase activity, but the disruption of ribosomes by high salt concentration², ethylenediaminetetraacetate, or urea⁷ results in the release of the enzyme. However, the ability of ribosomes to adsorb up to a 12-fold excess of partially purified RNase I⁸ cast some doubt on the true ribosomal character of the »latent« RNase. The adsorbed enzyme was in a »masked« form, i. e. it did not show its activity in the presence of Mg^{++} . The presence of this enzyme on the ribosomes is not required for their function in protein synthesis, since the ribosomes can be freed of RNase I without losing their biosynthetic activity⁹, and since the enzyme is absent from ribosomes of some strains of *E. coli* and other organisms^{10,11}.

* The abbreviations used are: RNase, ribonuclease; tris, tris(hydroxymethyl)aminomethane.

The problem of activation of »latent« ribosomal RNase came to our attention during the investigation of the inactivation of isolated *E. coli* ribosomes by gamma irradiation¹². The ribosomes irradiated after dissociation into 50 S and 30 S subunits — that is, at 10^{-4} M Mg^{++} — displayed an enormous apparent increase in radiosensitivity. Preliminary evidence has been presented^{13,14} indicating that the observed apparent high radiosensitivity has resulted from the degradation of polyuridylic acid, used in the assay of the activity of ribosomes, by an enzyme activated by irradiation of dissociated ribosomes. The present paper will describe the details of the identification of this enzyme as RNase I, as well as some properties of the enzyme in its activated state.

MATERIALS AND METHODS

Preparation of ribosomes and high speed supernatant

Ribosomes and high speed supernatant were prepared from exponentially growing *E. coli* B by differential ultracentrifugation as described earlier.¹² Three times washed ribosomes were used; for irradiation experiments they were dissociated into 50 S and 30 S subunits by overnight dialysis against two changes of 0.01 M tris — HCl buffer, pH 7.4, containing 0.0001 M magnesium acetate. They were used for the irradiation experiments immediately, without freezing.

Irradiation conditions

Irradiations were carried out in a 210 Ci ⁶⁰Co gamma ray source at a dose rate of 5×10^4 rads/hr. as determined by ferrous sulfate dosimetry. Ribosomes in suspension were cooled with an ice-water mixture during irradiation. Shortly after the end of irradiation the samples were assayed for RNase activity.

Assay of the RNase activity

The assay consisted of two subsequent incubations. Mixture I (preincubation mixture) contained 10 µg. of polyuridylic acid, up to 8 µg. of ribosomes to be tested for RNase activity, 1 µmole of tris — HCl, pH 7.4 and 1 µmole of magnesium acetate in the final volume of 0.1 ml. A blank containing no ribosomes was run at the same time. The mixture was incubated for 5 minutes at 30° C. At this time 0.15 ml. of mixture II was added, containing 0.14 µmoles of tris — HCl, pH 7.4, 1.5 µmoles of magnesium acetate, 10 µmoles of NH_4Cl , 0.75 µmole of adenosine thiphosphate, 0.075 µmole of guanosine triphosphate, 2.5 µmoles of phosphoenolpyruvic acid, 5 µg. of pyruvate kinase, 2.5 µmoles of glutathione, 0.25 mg. of *E. coli* soluble ribonucleic acid, 0.02 µmole of ¹⁴C-L-phenylalanine (specific activity, 17.5 µCi/µmole), 0.05 ml. of $100,000 \times g$ supernatant, and 0.4 mg. of undissociated ribosomes. These conditions approximate the conditions used previously for the polymerization of phenylalanine.^{12,15} The samples were incubated for additional 10 minutes at 30° C, precipitated with 5% trichloroacetic acid, and extracted at 90° C for 15 minutes. Precipitates were collected on Millipore HA membrane filters, washed with an excess of 5% trichloroacetic acid, and counted in a windowless gas-flow counter.

When the fractions from density gradient ultracentrifugation (see below) were to be tested for RNase activity, 5 or 10 µl. samples were used in preincubation mixture. To express the results of these experiments, it was suitable to introduce an arbitrary unit of the enzyme activity defined as the amount of enzyme in the respective fraction causing a reduction of ¹⁴C-phenylalanine incorporation of 10,000 counts per minute under the assay conditions. The assay was linear with less than 0.5 arbitrary units of the enzyme; therefore the volume to be tested was chosen so as to contain this amount of enzyme.

Ultracentrifugation in sucrose density gradients

Samples of 1.6 mg. of dissociated ribosomes in a volume of 0.2 ml. were layered on the top of 5 to 20% exponential density gradients of sucrose containing 0.01 M tris — HCl, pH 7.4, and 0.0001 M magnesium acetate. The samples were centrifuged for 3 hours at 35,000 rpm in the SW-39 swinging bucket rotor of Spinco model L ultracentrifuge at +4° C. After centrifugation, the bottom of each tube was pierced

with a needle, and 2-drop fractions were collected. Absorbance at 260 $m\mu$ was measured in every second fraction after dilution to 1 ml. with water. The other fractions were used either for obtaining separated 50 S and 30 S ribosomes (in this case only heavier part of 50 S peak and lighter part of 30 S peak were used in order to avoid cross-contamination) or for the assay of RNase activity.

Chromatography

Paper chromatography was done on *Whatman* No. 1 filter paper by the descending technique in two solvent systems, containing 95% ethanol and 1 *M* ammonium acetate in volume ratios 75 : 30¹⁶ and 60 : 45,⁷ respectively.

Column of *Sephadex* G-75 (medium) dextran gel (1 \times 51 cm.) was equilibrated with 0.01 *M* tris — HCl buffer, *pH* 7.4, containing 0.01 *M* magnesium acetate, and eluted with the same buffer after introduction of the sample (0.5 ml.). The chromatography was carried out at +4° C. 1.0 ml. fractions were collected and their absorbance at 260 $m\mu$ measured.

Materials

Polyuridylic acid was a product of Miles Laboratory, Clifton, New Jersey, USA. *E. coli* soluble ribonucleic acid was prepared according to the procedure of von Ehrenstein and Lipmann.¹⁸ Phosphoenolpyruvate, trisodium salt, and pyruvate kinase were products of C. F. Boehringer und Soehne, Mannheim, Germany. Adenosine triphosphate and guanosine triphosphate were obtained from Pabst Laboratories, Milwaukee, Wisconsin, USA. *Sephadex* G-75 dextran gel (medium) was a product of Pharmacia Fine Chemicals, Uppsala, Sweden. ¹⁴C-L-phenylalanine was a product of Schwarz BioResearch, Inc., Orangeburg, New York, USA.

RESULTS AND DISCUSSION

RNase activity of irradiated ribosomes

The assay used in this study was designed on the basis of the finding of Nirenberg and Matthaei¹⁹ that polyuridylic acid specifically directs the synthesis of polyphenylalanine in a cell free system from *E. coli*. Fragments of polyuridylic acid with chain length under 50 nucleotides have been reported to be relatively inactive in the polymerization of phenylalanine²⁰. Consequently, endonucleolytic breakdown of polyuridylic acid can be determined by assaying the ability of remaining polyuridylic acid to stimulate the synthesis of polyphenylalanine, as described under Materials and Methods. No activity of »latent« or »masked« ribosomal RNase could have been detected in this assay, since the preincubation mixture contained 0.01 *M* Mg⁺⁺, a concentration sufficient to make ribosomes totally inactive against natural substrates^{2,7,8}. The results presented in Fig. 1 show that under the assay conditions unirradiated ribosomes were inactive against polyuridylic acid, too. In other words, incubation of polyuridylic acid with unirradiated (control) ribosomes does not alter the ability of polynucleotide to stimulate the synthesis of polyphenylalanine in the subsequent incubation with all other components of the cell-free amino acid polymerization system. On the contrary, preincubation with as little as a few μ g. of ribosomes irradiated with 2×10^5 rads of gamma irradiation causes a marked decrease of the amount of polymerized phenylalanine. Almost linear dependence of this decrease on the concentration of ribosomes in preincubation makes it possible to use this method as a reliable assay for RNase with the amount of enzyme causing less than 50% decrease of polymerization. High sensitivity of this assay can be illustrated by the data from the same figure. Taking into account that no more than 10% of *E. coli* ribosomes contain RNase I⁷, and supposing that molecular weight of the enzyme is similar to that of pancreatic RNase, it can be calculated that 1 μ g. of ribosomes contains less

than 5×10^{-4} μg . of the enzyme. Decrease of ^{14}C -phenylalanine incorporation, caused by this amount of enzyme, is easily detectable.

Location of enzyme activity on 30 S ribosomal subunit

Data from the preceding paragraph show that an enzyme, which breaks down polyuridylic acid, is activated by gamma irradiation of purified and dissociated *E. coli* ribosomes. For the identification of the enzyme it was of interest to find out from which one of the two ribosomal subunits the enzymatic acti-

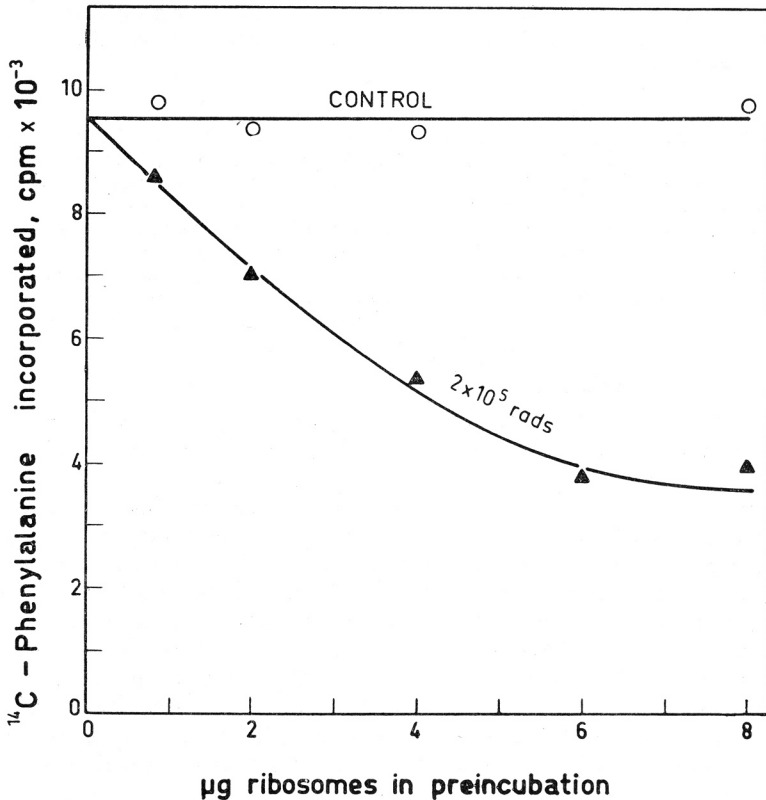


Fig. 1. Assay of the nucleolytic activity of unirradiated (control) ribosomes, and of the ribosomes irradiated with 2×10^5 rads.

vity originated. For this purpose 50 S and 30 S subunits were separated by ultracentrifugation of dissociated ribosomes in a sucrose density gradient, and irradiated with 2×10^5 rads after the separation. Control and irradiated samples were then assayed for RNase activity. Results of such an experiment are shown in Table I. It can be seen that all the activity was found associated with the 30 S subunit; negligible activity found in the 50 S fraction was probably caused by incomplete separation from the 30 S particles.

TABLE I

Effect of preincubation of polyuridylic acid with irradiated 50 S and 30 S ribosomes on the incorporation of ^{14}C -phenylalanine

Ribosomes in preincubation	Concentration ($\mu\text{g./sample}$)	c. p. m. of ^{14}C -Phenylalanine incorporated after preincubation with	
		control ribosomes	irradiated ribosomes (2×10^5 rads)
30 S + 50 S prior to separation	2	1964	1329
30 S	2	1810	807
50 S	5	1914	1823
Control — no ribosomes in preincubation		1915	

Endonuclease type of action

Location of the enzyme activity on 30 S ribosomes suggests that the enzyme in question is RNase I. Polynucleotide phosphorylase was excluded by the fact that no phosphate is required for the reaction. RNase II, which is an exonuclease with polyuridylic acid⁴, was excluded by the examination of the products of preincubation by paper chromatography. No mononucleotides could have been detected by this technique. The endonuclease type of action was further supported by the chromatography of preincubation mixture on *Sephadex G-75*. The results are shown on Fig. 2. The elution peak of polyuridylic acid incubated with unirradiated ribosomes is in the fraction number 15. In the second run on the same column polyuridylic acid incubated with irradiated ribosomes gave a broad peak with maximum at the fraction number 30. For comparison, a mixture of polyuridylic acid and uridylic acid (UMP) was chromatographed on the same column. It is evident that the products of degradation of polyuridylic acid are not mononucleotides; they are oligonucleotides smaller in size than the original polyuridylic acid. Therefore, the enzyme in question is an endonuclease. and, taking into account its location on the 30 S ribosome, it is very probably RNase I.

Sedimentation of activated enzyme with ribosomes

The methods described up to now for the activation of latent ribosomal RNase^{2,7} led to the desintegration of the ribosome, or at least to the dissociation of the enzyme from the particles. Moreover, ribosomes are able to adsorb and hence »mask« partially purified RNase I⁸. It was therefore surprising to find that the ribosomes were not able to adsorb and inhibit RNase activated by irradiation. A possible explanation was that irradiation led to irreversible dissociation of the enzyme from 30 S ribosomes. To test this idea, dissociated ribosomes were first irradiated with 2×10^5 rads, and then sedimented by ultracentrifugation in sucrose density gradients. As shown on Fig. 3, all enzyme activity was found to sediment with the 30 S peak. Consequently, the enzyme was not irreversibly dissociated from the particle, but irreversibly activated on it.

Comments

The mechanism of the activation of RNase I by gamma irradiation of purified and dissociated *E. coli* ribosomes is far from being understood. Irradiation of lyophilized ribosomes, permitting only direct effect of irradiation, does not lead to the activation of RNase I¹². Consequently, it could be supposed that the activation of the enzyme is caused by the indirect effect of irradiation, (*i. e.* effect of ionizations in the surrounding medium). This is in a good agreement with the view that RNase I is located on the surface of the 30 S ribosome.

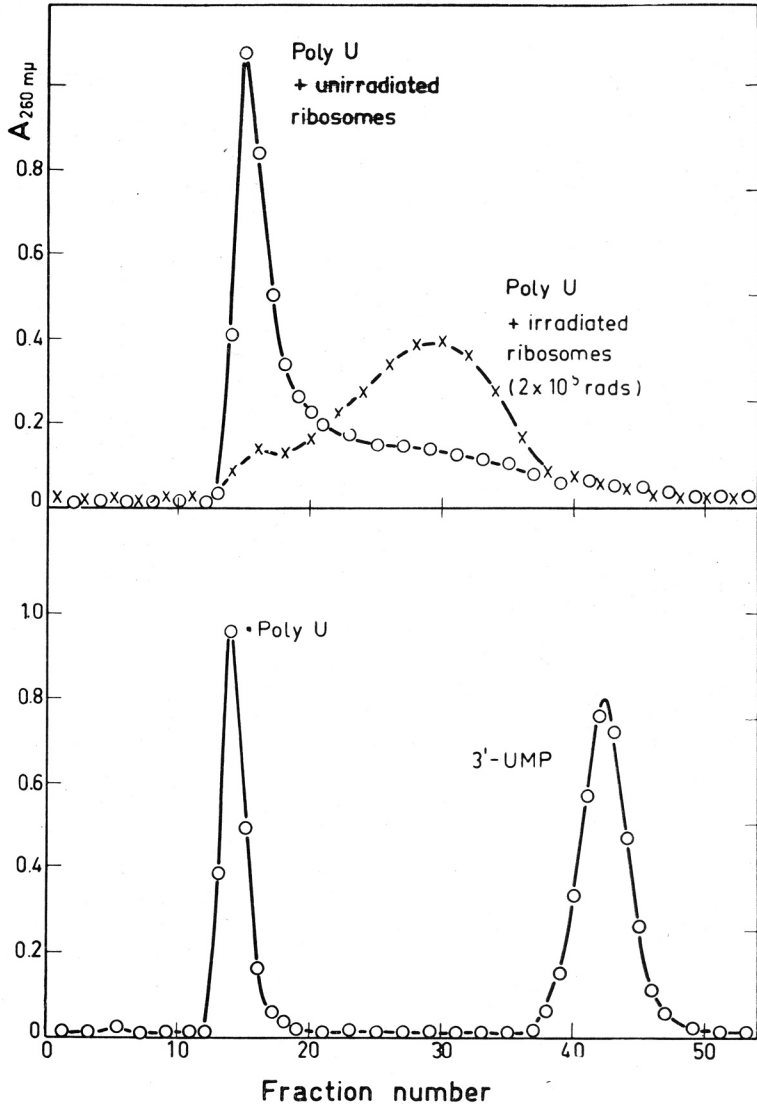


Fig. 2. Chromatography of polyuridylic acid, incubated with unirradiated ribosomes or with the ribosomes irradiated with 2×10^5 rads, on a column of *Sephadex G-75*. For comparison, elution pattern of a mixture of polyuridylic acid and uridylic acid is shown on the lower part of the graph.

However, from the present data, it cannot be concluded whether the indirect effect on the 30 S ribosome or on the adsorbed enzyme is responsible for the activation.

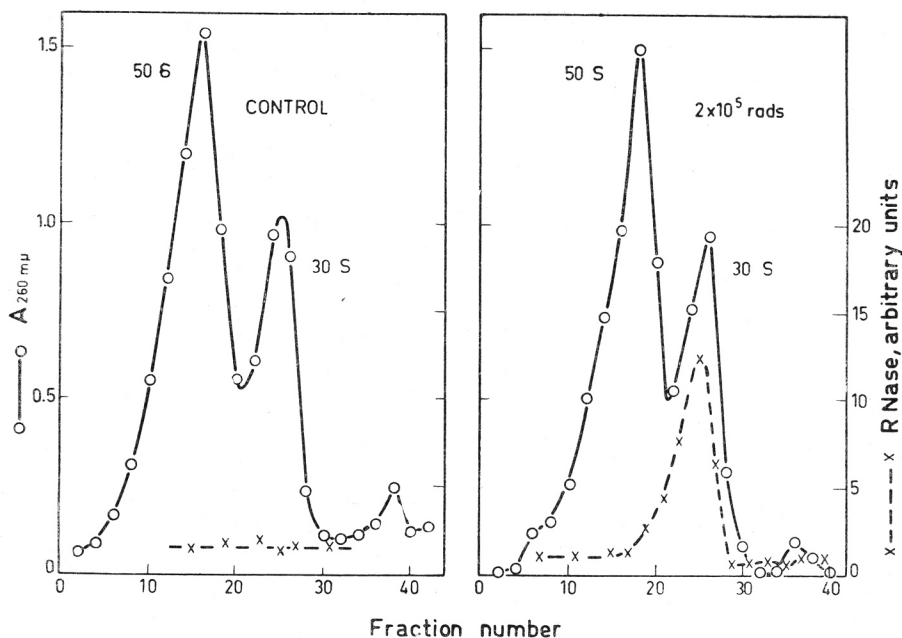


Fig. 3. Ribonuclease activity of the fractions obtained by sucrose density gradient ultracentrifugation of unirradiated (control) ribosomes and ribosomes irradiated with 2×10^5 rads.

The observed activation of RNase I could have a role in the *in vivo* breakdown of ribonucleic acid in irradiated *E. coli*. The work at this Institute²¹ has shown that a part of ribonucleic acid is degraded even after relatively small doses of X-irradiation. Our preliminary evidence²² indicates that an endonucleolytic process is going on in this case, too.

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IZVOD

Aktivacija ribonukleaze I gama zračenjem 30 S ribosoma bakterije *Escherichia coli*

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Razrađena je vrlo osjetljiva metoda za mjerenje aktivnosti ribonukleaze uz upotrebu poliuridilne kiseline kao supstrata. Količina preostale poliuridilne kiseline mjerena je zatim u *in vitro* sistemu za polimerizaciju fenilalanina iz bakterije *Escherichia coli*. Ta je metoda upotrebljena za karakterizaciju nukleaze aktivirane zračenjem pročišćenih ribosoma iz iste bakterije. Enzim je bio identificiran kao ribonukleaza I na bazi (1) njegove lokacije na 30 S ribosomskoj podjedinici, (2) odsutnosti mononukleotida u produktima kratke inkubacije, što je ustanovljeno kromatografijom na papiru, te (3) na bazi utvrđivanja oligonukleotida kao produkta takve reakcije pomoću kromatografije na dekstranskom gelu. Aktivirani je enzim sedimentirao s 30 S ribosomima i ispoljavao svoju aktivnost čak i kod koncentracija Mg^{++} koje potpuno inhibiraju »latentnu« ribosomsku ribonukleazu.

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