ON THE LOCALIZATION OF RIBONUCLEASES IN BACTERIA

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1. Introduction

Enzymatic activities with specificities similar to those of ribonuclease (RNAase) I (EC 2.7.7.1.6), II and II of *E. coli* were found thus far in all bacterial species examined (15 species; unpublished work). Since these enzymes are likely to have, in the different bacteria studied, a similar role in RNA metabolism their cellular localization might give some indication on their possible metabolic function.

It is an accepted practice to separate bacterial cell free extracts into a supernatant and a ribosomal pellet, and to determine to what extent the protein in question sediments with the ribosomal pellet and to suggest from it a cellular localization. The experiments performed here suggest that finding a protein in the ribosomal pellet does not necessarily suggest that it is localized on the ribosome in the intact cell.

2. Results

The enzyme RNAase I, in extracts of *E. coli* prepared by alumina grinding, is found in the ribosomal pellet [1], specifically on the 30 S ribosomes [2, 3]. About 50% of RNAase II activity [4] and almost all of the RNAase III activity [5] are also found in the ribosomal pellet in such extracts. In order to find out whether or not there is a localization of any of these enzymes (RNAase II and III) to a particular ribosomal subunit, extracts from strain AB301 which contain all the ribonucleases and strain D10 which is deficient in RNAase I [6] were prepared. The ribosomal pellets were separated to 50 S and 30 S ribosomal subunits by centrifugation in sucrose gradients. As can be seen in fig. 1 neither of these two enzymes, RNAase II or RNAase III, sticks specifically to any of the ribosomal subunits. There is a small amount of RNAase II which sediments with the 30 S ribosomes (see Discussion), while as expected RNAase I was bound to the 30 S ribosomes. These results were obtained with strain AB301, similar results with respect to RNAase II and III were found with the ribosomal pellet from strain D10.

That specificity of localization is not indicated by finding a ribonuclease in the ribosomal pellet was further illustrated from the comparative studies with other two bacteria, *Enterobacter aerogenes* and *Pseudomonas aeruginosa.* With these organisms the distribution of the enzymes RNAase I, II and III were studied in different cellular fractions. Cell free extracts were prepared (S-30) and they were separated after a 150,000 *g* centrifugation to a supernatant and a ribosomal pellet. The ribosomal pellet was washed with a buffer containing 0.2 M NH_4Cl and recentrifuged. Enzymatic activities were measured in all these fractions. As can be seen in table 1, as expected, RNAase III in *E. coli* and *E. aerogenes* is localized mainly in the ribosomal pellet, but in *P. aeruginosa* it is found almost quantitatively in the supernatant. The distribution of RNAase I and II among the cellular fractions prepared were rather similar in all three bacteria.

3. Discussion

In *E. coli* it is possible to localize enzymes to the periplasm or to the cytoplasm by a number of criteria (see Heppel [7]). Cytoplasmic enzymes can be further localized to whether they are in the supernatant or in

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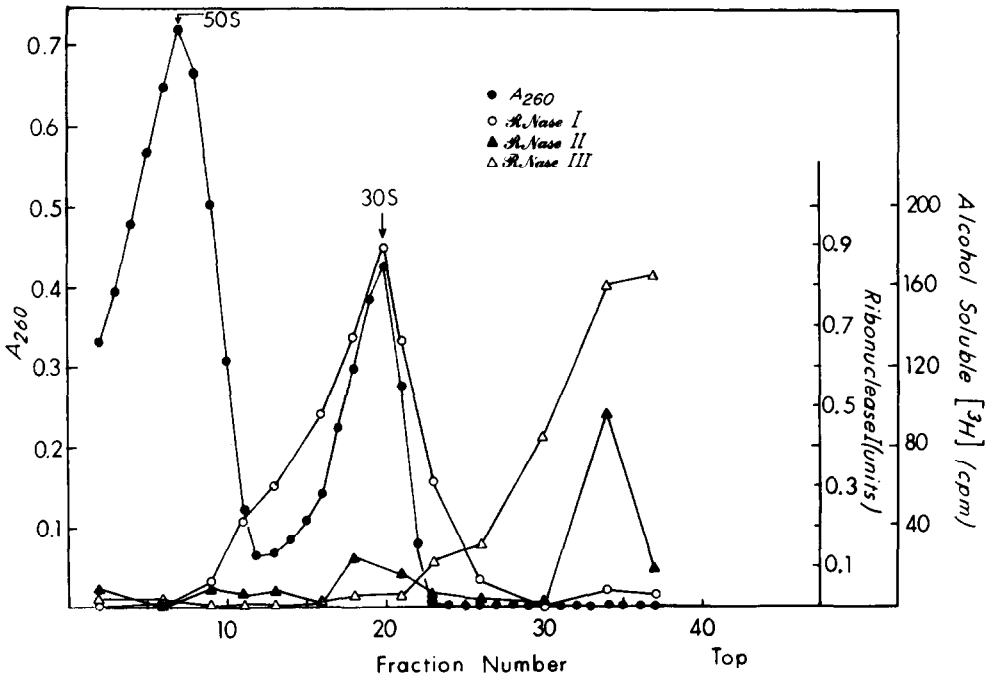

Fig. 1. Distribution of ribonuclease activities on ribosomal subunits. A 400 ml culture of strain AB301 (*E. coli* K12, requiring L-methionine) was grown to A_{560} of 0.8 at 30° in glycerol–casamino acids medium (minimal medium supplemented with 0.2% glycerol as carbon source and 0.5% Difco technical casamino acids), poured on ice and harvested. Cells were ground with alumina [14] and extracted in Tris-buffer (0.01 M Tris-HCl, pH 7.5; 0.01 M magnesium acetate; 1 mM dithioerythritol). The clarified extract (20,000 *g*) was centrifuged in a fixed angle rotor for 2 hr at 180,000 *g* (4°). The pellet was resuspended in the same buffer containing 0.1 M NH_4Cl in addition. This suspension was centrifuged as before. The ribosomal pellet was resuspended in TM4-buffer (0.01 M Tris-HCl pH 7.5; 0.1 mM Mg^{2+}) and dialysed against the same buffer for 16 hr. 76 A_{260} units of the dialysed ribosomes (about 0.6 ml) were layered on top of 28 ml sucrose gradient 5–20% in TM4-buffer) and centrifuged in a Spinco SW25/1 rotor at 40,000 *g* for 15 hr at 4°. Fractions of about 0.8 ml were collected from the bottoms of the tube. 0.1 ml of each fraction was diluted 1 to 10 in TM4 and the A_{260} determined. Ribonuclease I, II and III were assayed as described in the legend to table 1, with 80 μl samples of every third or fourth fraction, in a final volume of 0.1 ml. RNAase II assays were incubated for 20 min at 37°. Ribonuclease III was assayed for 60 min at 35°. The activities of RNAase II and III are expressed in counts per minute (cpm) released as alcohol soluble material.

the ribosomal pellet, after a high speed centrifugation, with the inference that enzymes that are found in the pellet are ribosomal and vice versa. The studies reported here suggest that such an inference is not always justified.

By release of enzymes from the *E. coli* cells after an osmotic shock, the enzyme RNAase I was shown to be periplasmic by Neu and Heppel [8, 9], while RNAase II [8] and III [5] were shown to be cytoplasmic. Moreover RNAase III is almost completely in the ribosomal pellet [5] while RNAase II is only partially there [4]. Therefore we thought that separation of the ribosomes to 50 S and 30 S subunits might reveal a specific localization on the ribosome for these two enzymes.

The failure on one hand to find specific binding of either of these two enzymes (RNAase II and III) to any of the ribosomal subunits, and on the other hand the appearance of most RNAase III in *P. aeruginosa* in the supernatant, suggests that the binding of proteins to the ribosomal pellet could be fortuitous and does not necessarily reflect a cellular localization. This conclusion is further supported by the fact that RNAase I which is most likely a periplasmic enzyme [7–9] upon grinding cells with alumina attaches specifically to the 30 S ribosomal subunits [3], and fig. 1.

It is noteworthy that in *E. coli* while most of the RNAase III activity is found in the ribosomal pellet and only half of the RNAase II activity is there, never-

Table 1
Intracellular distribution of ribonucleases I, II and III in three bacteria.

Bacterial species	Enzymatic activity	S-30	S-150	Washed* ribosomes	Ribosome† washings
Escherichia coli	RNAase I	2.0	0.7	1.4	0.2
	RNAase II	478	497	313	421
	RNAase III	215	78	23	396
Enterobacter aerogenes	RNAase I	0.8	0.3	0.9	0.2
	RNAase II	591	518	230	488
	RNAase III	138	189	143	391
Pseudomonas aeruginosa	RNAase I	0.8	0.4	0.9	0.5
	RNAase II	189	393	207	244
	RNAase III	232	589	5	55

* After a wash in a TM2 buffer containing 0.2 M NH_4Cl.

† The supernatant after centrifugation of ribosomes that were resuspended in TM2 buffer containing 0.2 M NH_4Cl.

Bacteria were grown at 37° in Trypticase Soy Broth medium (BBL, Cockeysville, Maryland, U.S.A.) to A_{560} of approx. 1. The cells were harvested, washed in TM2 (Tris-HCl, 0.01 M, pH 7.6; magnesium acetate 0.01 M) buffer frozen and stored at −20°. The frozen cells were resuspended (1 g per 5 ml) in TM2 buffer and sonicated for 1 min. The sonicates were centrifuged at 30,000 *g* for 20 min to obtain cell-free extracts (S-30). All steps were carried out at 0° to 4°. S-30 supernatants were centrifuged at 150,000 *g* for 4 hr in a fixed angle rotor. The ribosomal pellets and the supernatants (the top 2/3 of the S-150) were separated. The ribosomal pellets were washed with TM2 containing 0.2 M NH_4Cl, since in *E. coli* almost all RNAase III can be found in this wash [5]. *Enzymatic assays: RNAase I.* The incubation mixture for RNAase I was essentially similar to that described by Neu and Heppel [8], but yeast RNA and Tris buffer pH 7.55 were used rather than *E. coli* tRNA and phosphate buffer. The amount of enzyme required to release 0.45 units (A_{260}) at 37° (260 nm) being defined as one unit of RNAase I activity. *RNAase II.* This activity was determined according to Singer and Tolbert [4] as described below. Reaction mixtures contained in a final volume of 0.1 ml, 0.01 M Tris-HCl pH 7.6; 0.1 M KCl; 5 mM magnesium acetate; and contained 2 μg of [^{3}H]polyadenylic acid (Schwarz/Mann, Orangeburg, New York, U.S.A). Assays were carried out at 35°. Reactions were stopped by adding 50 μl of carrier RNA (yeast RNA 5 mg/ml, 0.1 M Tris-HCl pH 7.6, 0.6 M NaCl) and 1 ml of cold 73% ethanol. The mixtures were allowed to stand in ice for 30 min and then centrifuged. 0.5 ml of the supernatant solutions were taken for counting radioactivity. *RNAase III.* RNAase III was assayed essentially as described by Robertson et al. [5]. The reaction mixtures contained in 0.1 ml, Tris-HCl, pH 7.5, 0.02 M; NaCl 0.1 M; magnesium acetate 0.01 M; and [^{3}H]poly (C)·poly (I) 3 μg of each (Schwarz/Mann). Reactions were stopped by addition of carrier RNA (500 μg yeast RNA) followed by 1.0 ml of 60% ethanol. 0.5 ml aliquots were used for determination of radioactivity. The RNAase II and RNAase III activities are expressed as nmoles of P_i released per mg protein per hr. RNAase I is expressed in units of activity. Protein concentrations were determined by the procedure of Lowry al. [5]. The amount of protein was similar in corresponding fractions from different bacteria. Each value is a result of a number of kinetic studies carried out at a number of protein concentrations. That each assay is specific is indicated by the results in fig. 1 where fractions with high levels of RNAase I show no RNAase II or III activity. Robertson et al. [5] showed that purified RNAase III has no activity against single stranded RNA molecules while Weatherford et al. [16] showed that purified RNAase II has no activity against poly (I)·poly (C).

theless the attachment of RNAase II is much firmer. While a single wash of the ribosome with a buffer containing 0.2 M NH_4Cl is sufficient to release most RNAase III [5 and table 1], it takes up to six or seven washes with a buffer containing 1 M NH_4Cl to release all RNAase II from ribosomes (see [10]). This firm attachment of RNAase II to the ribosome can also be seen in fig. 1, where centrifugation through a sucrose gradient removed completely RNAase III but not RNAase II from the ribosomes.

The studies reported and referred to here, point out also a real difficulty in the determination of what is a ribosomal protein and what is not. Since treatments like those necessary to remove ribonuclease II from the ribosomes would obviously remove quantitatively proteins like the initiation factors [11–13] that can be legitimately considered to be ribosomal proteins.

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