IS RNase V A MANIFESTATION OF RNase II?

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SUMMARY

The relationship between RNase II and RNase V activities in Escherichia coli was investigated. The experiments suggest the possibility that the activity named RNase V was due to residual RNase II activity or a related enzyme on ribosomes.

Comparing the published data on RNase II (1-4) and RNase V (5) it became evident that both RNase II and V can function at a similar pH, both require K^+ (or NH_4^+) and Mg^{+2}, and both can degrade polyribonucleotides such as polyuridylic acid (poly U) to 5' mononucleotides. While these characterize the enzyme RNase II, there are further requirements for RNase V activity. These include ribosomes, sulfhydryl groups and all the elements required for ribosome translocation namely GTP, tRNA, G and T elongation factors (6). This realization together with the demonstration by Castles and Singer (7) that ribosomes and tRNA protect polyuridylic acid from degradation by the enzyme RNase II, suggested to us that there might be a relationship between RNase II and V activities. Our findings suggest that RNase V activity independent from RNase II may not exist. Similar findings were obtained by Holmes and Singer (8), see accompanying paper.

RESULTS

Since the reports on RNase V used strain N464 (5) in which RNase II activity is heat labile (9), we used this strain in our studies. We tried to find out if we could detect RNase activity under RNase V

assay conditions when RNase activity under RNase II conditions was inactivated.

Heat inactivation of RNase activity using a ribosome-free supernatant. We did a number of experiments when RNase activity was measured in a ribosome free supernatant, RNase II conditions, or in a supernatant supplemented with ribosomes, tRNA and GTP, RNase V conditions. In these experiments the supernatant was heated at 40^{0} (see Fig. 1) for various lengths of time and then added to the reaction mixture. It is evident that RNase activity under RNase V conditions was always lower than RNase activity under RNase II conditions. RNase activity under RNase V conditions became undetectable when the supernatant still demonstrated residual activity.

Since G and T elongation factors are required for RNase V activity (5), it was necessary to examine the possibility that these factors are inactivated by heating the supernatant. We used the same supernatant and ribosomes that were used for the RNase assays and performed a poly U directed polyphenylalanine synthesis. As can be seen in Fig. 1, the capacity of the supernatant to support protein synthesis is almost unchanged by the heat treatment. This indicates that the elongation factors G and T are functionally active, when RNase activities under II or V conditions are almost totally eliminated.

Since we used relatively low concentrations of supernatant proteins, ribosomes and poly U, we repeated a similar inactivation experiment (like the one reported in Fig. 1) using concentrations of reactants and experimental conditions similar to those published for RNase V (5). Again we could not find RNase activity which exceeded that found under RNase II conditions (Fig. 2).

RNase activity on ribosomes. About half of the RNase II activity found in crude extracts is in the ribosomal pellet (4). This activity was removed by successive washes in a buffer containing 1M NH_4Cl (see Figs. 1 & 3).

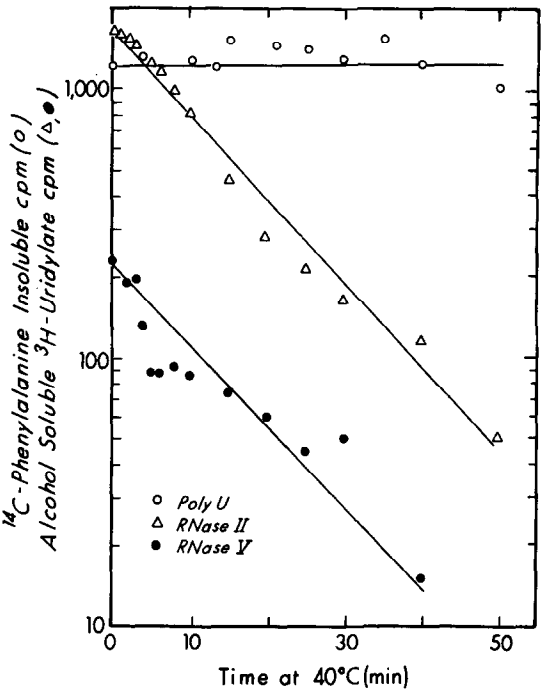

Figure 1. RNase activity after heating supernatant (low concentration).

The assay mixture for RNase II contained: Tris-HCl (pH7.6), 0.04 M; NH_4Cl, 0.04 M; Mg^{2+}, 0.013 M; GSH, 0.01 M; ^{3}H-poly U (MW>50,000, Miles Laboratories), 2μg (80nC); and 25 μg supernatant protein (16); per ml. The RNase V assay mixture contained the RNase II components plus: GTP, 0.5mM; and E. coli tRNA, 100μg; and ribosomes, 0.60mg (10 A_{260} units); per ml. The ribosomes were obtained from an alumina ground extract (17), after it was clarified from alumina and cell debris, by centrifugation at 321,000g for 3 hrs in Tris-HCl (pH7.6), 0.01M; $Mg(CH_3COO)_2$, 0.1mM; mercapto-ethanol, 6mM. The ribosomal pellet was suspended in Tris-HCl, 0.01M; Mg^{2+}, 2mM; NH_4Cl, 1M(TMN) and centrifuged at 321,000g for 2 hrs. This procedure was repeated four times more with low speed washes following each high speed wash. After the final wash, the ribosomes were stored in TM buffer (Tris-HCl pH7.6, 0.01M; $Mg(CH_3COO)_2$, 0.01M). The ribosomes used in the experiments were washed four times in TMN and show no RNase activity under RNase II or RNase V conditions. The supernatant of the first high speed centrifugation was collected, Mg^{+2} concentration adjusted to 0.01M, and spun again for 2 hrs at 321,000g. The top 2/3 from tubes of this spin were the source of the supernatant used in our experiments. RNase assays were performed in 50μl and stopped with 50μl of Carrier RNA and 1ml of 66% ethanol. The Carrier RNA contained: Tris-HCl (pH7.5), 0.1M; NaCl, 0.6M; $CaCl_2$, 0.05M; and yeast RNA, 5mg per ml. The reaction tubes were spun at 8,000g for 20 min and 0.5ml from each tube was withdrawn and counted in 10ml Brays solution (18). Background counts were about 80-100 cpm in all RNase II and RNase V assays and have been subtracted.

The assay conditions for poly U directed protein synthesis contain: Tris-HCl (pH7.6), 0.04M; Mg^{2+}, 0.013M; $(NH_4)_2SO_4$ (pH7.6), 0.1M; and ATP, 0.4mg; GTP, 0.2mg; PEP, 1.66mg; poly U, 40μg; Pyruvate Kinase, 40μg; ^{14}C-Phenylalanine (pH7.6), 0.627μg (1u C); and ribosomes, 0.60mg; per ml. Reaction mixtures of 300μl were stopped by the addition of 3ml of 5% CCl_3COOH. The tubes were heated at 90° for 20 min, filtered on glass fiber filters,

dried and counted on a dry low background Nuclear Chicago Counter. Background was 1000 cpm and was subtracted.

In all three assays the supernatant in TM was heated at 40° for various times as indicated at a protein concentration of 0.25mg/ml and subsequently incubated at 37° for 10 min at a supernatant protein concentration of 25µg/ml in RNase II (this activity is directly proportional to the amount of supernatant protein added within this range) and RNase V assays and 150µg/ml in poly U assays. Assays performed without the supernatant being heated at 40°, were done in triplicates, other points are singlets. Heat inactivation of RNase II in the supernatant depends on the concentration of the supernatant (proteins?).

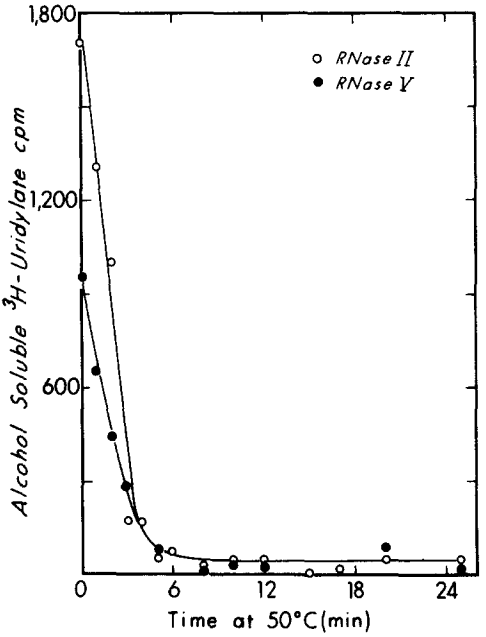

Figure 2. RNase activity after heating supernatant (high concentration). Assay conditions for RNase II and RNase V were the same as described in Figure 1 except: Ribosomes 2mg; supernatant, (protein concentration) 2mg; ^{3}H-Poly U, 14.6µg (80nC); per ml. The ribosomes added had no RNase activity under RNase II or RNase V conditions. The supernatant in TM, at a protein concentration of 3.35mg/ml, was heated at 50° and incubated for 30 min at 36°.

When ribosomes from the first and second washes were tested for RNase activities (II and V), after they had been heated at 40° for various time intervals, the activity under both conditions was identical (Fig. 3). The same was true for the remaining activity after further washes. It is interesting to note that the RNase activity of the once washed ribosomes is inactivated to approximately the same level as

that of the twice washed ribosomes and then inactivation stops (Fig. 3). This is in agreement with the idea of protection of a certain number of RNase II molecules by the ribosomes. (In both experiments the same quantities of ribosomes were used.)

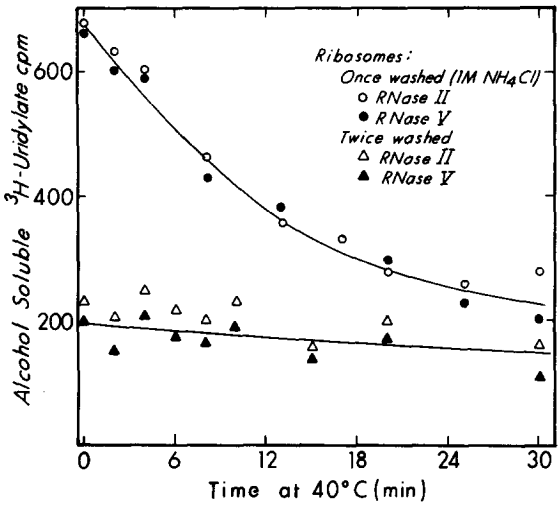

Figure 3. Heat inactivation of RNase activity on ribosomes. Ribosomes after one and two washes in 1M NH_4Cl buffer (see Fig. 1) were heated at 40° and assayed under RNase II and RNase V conditions (no supernatant added). At 50° the residual activity is inactivated much faster. Assay conditions were the same as described in Fig. 1.

Thus far RNase V activity was looked for as a combination of factors from the supernatant and washed ribosomes, and in partially washed ribosomes. Therefore, RNase activities were assayed after the supernatant and the ribosomes were heated together (V is II when tRNA and GTP are added). This was done in a number of combinations, varying the concentrations of the supernatant and ribosomes (using ribosomes with different levels of residual RNase activity). Again we could not find RNase activity which exceeded that found under RNase II conditions.

Protection of RNase activity in supernatant by ribosomes. In order to find out whether RNase II activity in the supernatant can be protected by ribosomes from heat inactivation, we performed two sets of experiments. In the first approach, ribosomes which did not have RNase II or V activities were mixed with excess supernatant, pelleted

and washed under the same conditions used to wash ribosomes isolated from the original extracts (see Fig. 3). The RNase activity of such pelleted ribosomes is very low (per ribosome unit), and sticks very poorly to them as compared with RNase activity on native ribosomes. However, as in the experiments with native ribosomes (Fig. 3), RNase activities under II and V conditions were identical and their heat inactivation was also identical. Moreover the reassociated RNase activity is relatively more resistant to heat inactivation than the activity of the supernatant itself.

In the second approach, small amounts of supernatant and an excess of ribosomes were mixed, heated and assayed. Under these conditions significant protection of RNase activity from heat inactivation was observed. For instance, after 10 min at 40°, almost no RNase II activity was detected when the supernatant was heated by itself, but about one-third of the original activity was preserved when the supernatant was heated in presence of ribosomes.

Relief of inhibition of RNase II activity by GTP. In general, the addition of ribosomes and tRNA to the supernatant reduced its RNase activity (see for example Fig. 1). Occasionally but not always the addition of GTP alleviated this inhibition to some extent. For instance, in one such experiment the RNase activity in the supernatant was reduced to about 40% by the addition of ribosomes. It was further reduced to about 20% by the addition of ribosomes and tRNA. However the addition of GTP restored RNase activity to the 40% level.

DISCUSSION

The various experiments presented here show that under no conditions RNase V-like activity was found when RNase II activity could not be demonstrated.

When extracts are prepared and separated to a ribosome pellet and supernatant, the enzyme RNase II can be purified either from the super-

natant (4) or from the ribosome pellet (2). Its distribution between supernatant and ribosomes is about equal (4). Castles and Singer (7) showed that the formation of a poly U-ribosome-tRNA complex inhibits degradation of poly U by RNase II. Since tRNA is present in supernatant, it is likely that the inhibition of degradation of poly U observed when ribosomes were added to the supernatant is due to the formation of such a complex. The inhibition is probably dependent on the concentrations of substrate, supernatant (tRNA) and ribosomes (see Figs. 1 and 2). However other factors besides tRNA and ribosomes might influence RNase II activity in extracts.

It is possible that when RNase V was originally assayed in strain N464, the free RNase II was inactivated while some of the RNase II bound to ribosomes remained active. Since RNase II activity could be inhibited by ribosomes (7), the requirement for tRNA, GTP, G and T for RNase V activity could have been the necessity for translocation of the ribosome in order to alleviate some of the inhibition of RNase II. Degradation of protected RNA after translocation could be simply due to the fact that after ribosome movement more polyribonucleotide becomes accessible to RNase II molecules, or that after translocation some of the inactive RNase II molecules can function again. Obviously a combination of both is also possible.

However, the idea of a unique RNase V like activity resulting from the combination of elements that participate in protein synthesis, each of which separately show no RNase activity, is not ruled out by these experiments. For instance, if such an activity is dependent on a supernatant factor, which is as heat labile as RNase II, such an activity would not have been detected in our experiments.

Since the experiments reported here cast some doubt on the existence of the postulated RNase V, we presently do not know of any exoribonuclease in E. coli that degrades single RNA chains from the 5' to the

3' end. Since the overall degradation of mRNA is from the 5' to the 3' end (10, 11) such an activity seems necessary. If RNase II, which degrades RNA from the 3' to the 5' end (12) is involved in mRNA degradation in E. coli (7,13,14), we propose that a combination of an endonuclease (15) and a 3' to 5' exonuclease are responsible for degradation of mRNA in bacteria.

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