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VEAL HEART RIBONUCLEASE P HAS AN ESSENTIAL RNA COMPONENT

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Summary: The activity of RNase P (EC 3.1.26.5) from veal heart can be abolished by pretreatment of the enzyme preparation with micrococcal nuclease, pancreatic RNase A, or RNase T_1. This indicates that veal heart RNase P contains an RNA component essential for function of the enzyme as has also been shown for E. coli RNase P (1-3). Additionally, veal heart RNase P has a buoyant density in Cs_2SO_4 of 1.33 g/cm^3, which is intermediate between that of protein and nucleic acid.

RNase P, which has been detected in procaryotic and eucaryotic organisms (4-6), is necessary for the biosynthesis of the 5' termini of tRNA molecules. This enzyme recognizes some aspect of structural conformation of its substrates, tRNA precursor molecules, rather than specific nucleotide sequences near its sites of cleavage. (4). Both functional and physical evidence have shown that E. coli RNase P consists of RNA and protein moieties (1-3). In this paper we show that an eucaryotic enzyme, veal heart RNase P, also requires an RNA component for function.

METHODS

Preparation of RNase P: 426 g of fresh veal hearts, defatted and sliced, were homogenized in 300 ml of ice cold buffer TM (20 mM Tris.HCl, pH 7.6/15 mM $Mg(OAc)_2$ / 6 mM 2-mercaptoethanol) containing 20 mM NH_4Cl and 20μM PMSF*. The buffer was then made 0.7% in Triton X-100 and 0.3% in sodium deoxycholate. The homogenate was left for 30 min on ice and then centrifuged at 16,300 x g for 30 min at 4°. The resulting supernatant (S16) was diluted two-fold with buffer TM containing 20 mM NH_4Cl and loaded onto a DEAE Sephadex A-50 column (2) (bed volume 1.5 l) equilibrated with the same buffer. The loaded material was eluted stepwise with successive two liter washes of buffer TM containing 0.06 M NH_4Cl, 0.2 M NH_4Cl and 0.35 M NH_4Cl. The RNase P activity was eluted in the last wash step. The pooled, active fractions were passed through a Biobeads (Biorad Corp.) column (18 ml bed volume) to remove the Triton X-100. The resulting active fractions were concentrated using $(NH_4)_2SO_4$. The 10-25% (0.1 g to 0.25 g/ ml original volume) $(NH_4)_2SO_4$ pellet was resuspended in buffer TM containing 0.2 M NH_4Cl and applied to a Sephadex G-100 column (1.3 l bed volume). The RNase P activity eluted just after the void volume.

Assay of RNase P activity: Radioactive substrate (E. coli $tRNA^{Tyr}$ precursor) was prepared and assayed as previously described (5). The standard reaction mixture (30 μl) contained 5 mM $MgCl_2$, 100 mM NH_4Cl, 1mM 2-mercaptoethanol, 0.1 mM Na_2EDTA, 50 mM Tris.HCl, pH 8 and 5,000-7,000 cpm of substrate.

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Ribonuclease inactivation of RNase P: Micrococcal nuclease: 10 µl of RNase P purified through the Sephadex G-100 step (about 20 µg of protein) was mixed with 1 µl of 50 mM Ca $(OAc)_2$, 3 µl of 50 µM PMSF and 1 µl of MN* (0.17 units or 1.7 units per 15 µl pretreatment mixture as desired) a kind gift of Dr. M. Laskowski, Sr., in 1 mM NaCl. The mixture was incubated at 37° for 40 min. 10 µl of 10 mM EGTA* was added to inactivate the MN by chelating Ca^{2+}. The RNase P activity was then assayed in standard reaction mixtures containing 1 mM EGTA and 10 µM PMSF.

RNase T_1 and RNase A: Pretreatment with these enzymes (separately) was carried out in buffer TM containing 0.2 M NH_4Cl and 10 µM PMSF. 1.5 units of RNase T_1 or 1 µl of RNase A (1 mg/ml) was added to 55 µl of RNase P (as above). The sample was incubated for 40 min at 37°. In some cases the amount of RNase A and RNase P were doubled to provide larger sample volumes. After incubation $(NH_4)_2SO_4$ (0.49 mg/ml) was added in the ratio of 90 µl of $(NH_4)_2SO_4$ solution to 110 µl of sample. After centrifugation the pellet was redissolved in buffer TM containing 0.2 M NH_4Cl. This procedure effectively removes most of the RNase T_1 and RNase A from mixtures containing these enzymes and RNase P. The resuspended pellet fraction was used directly for assay of RNase P or further purified by applying to a sucrose gradient. Gradients were formed by layering steps of buffer TM with 0.2 M NH_4Cl containing 20%, 15%, 10% or 7.5% sucrose. Each step was 150 µl. Centrifugation was carried out in tubes of 0.65 ml capacity in the Beckman SW65 rotor with special bucket adaptors for 4 hr at 59,000 rpm, or for 14 hr at 31,000 rpm at 4°. This gradient purification procedure was used because the molecular weight of RNase P is much larger than that of either RNase T_1 or RNase A. The tubes were punctured with a 27 gauge hypodermic needle and one drop fractions were collected.

Cs_2SO_4 density gradient centrifugation: Preformed step density gradients were by layering 0.19 ml each of 45%, 37% and 31% Cs_2SO_4 solutions made in TM buffer containing 0.2 M NH_4Cl. The sample, 0.05 ml, was then layered on the gradient made in cellulose nitrate tubes of 0.65 ml capacity and the tube were centrifuged in the Beckman SW 65 rotor equipped with tube adaptors. Centrifugation was carried out for at least 6 hr at 3° and 59,000 rpm. Longer run times did not alter the results. Two drop fractions were collected after puncturing the bottom of the tubes with a 27 gauge hypodermic needle. Aliquots of each fraction were then assayed for RNase P activity in standard fashion.

RESULTS

MN pretreatment: When RNase P is pretreated with MN as described in the Methods, little or no residual RNase P activity is observed (Fig. 1A, lanes 1,2 and 7,8) as can be seen by the lack of RNase P cleavage products in the autoradiograph of the polyacrylamide gel analysis of the reaction mixture. By contrast, control incubations, carried out in the absence of MN or in the absence of Ca^{2+}, show RNase P is still active (Fig. 1A, lanes 3-6). The intactness of the precursor substrate as seen in Fig. 1A, shows that the MN is

*Abbreviations: PMSF - phenylmethylsulfonylfluoride
EGTA - ethylene glycol-bis-(β-aminoethyl ether) N - N'tetraacetic acid
MN - micrococcal nuclease

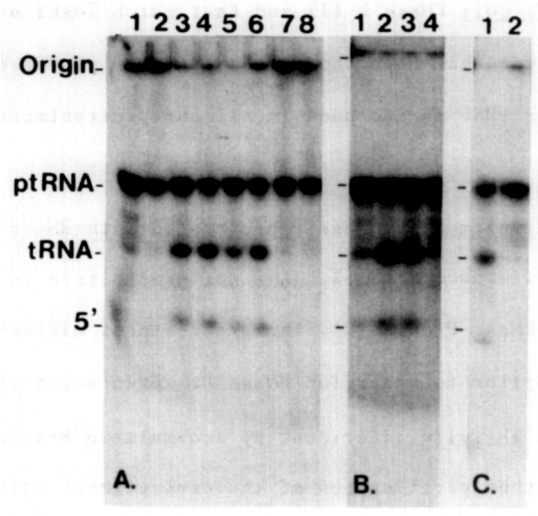

Figure 1: Inactivation of a veal heart RNase P by pretreatment with micrococcal nuclease or RNase T_1. A. Lanes 1 and 2: Pretreatment with 0.17 units of MN, 0.5 µl or 1 µl in assay mixture respectively. Lanes 3 and 4: Pretreatment with MN as in lanes 1 and 2 but no Ca^{2+} present, 0.5 µl and 1 µl assayed respectively. Lanes 5 and 6: Mock pretreatment, Ca^{2+} present but MN absent, 0.5 µl and 1 µl assayed respectively. Lanes 7 and 8: as in lanes 1 and 2 but 1.7 units of MN used. B. MN pretreatment followed by $(NH_4)_2SO_4$ fractionation as described in the methods. Experimental conditions as in Fig. 1A, lanes 2,4,6,8 respectively. C. Pretreatment with RNase T_1 followed by $(NH_4)_2SO_4$ fractionation as described in the Methods. Lane 1, Control pretreatment (no RNase T_1); 1 µl assayed. Lane 2: Pretreated with RNase T_1; 1 µl assayed. The position of the precursor of tRNA substrate and the RNase P cleavage fragments containing the mature tRNA sequence with the 'extra' 5' proximal fragment, are indicated in the figure.

completely inactivated after removal of Ca^{2+} and any residual ribonuclease activity must be due to active RNase P. The cleavage products generated by the active veal heart RNase P have been characterized by fingerprint analysis as the true products expected by accurate RNase P cleavage at the 5' terminus of the mature $tRNA^{Tyr}$ sequence (data not shown). Since some radioactivity was left at the origin of the gel (Fig. 1A, lanes 1,2,7,8), possibly complicating the interpretation of our results we decided to fractionate further the completed pretreatment mixture by precipitation of the RNase P with $(NH_4)_2SO_4$, as described in the Methods. Reassay of the pretreated RNase P after such fractionation is illustrated in Fig. 1B, lanes 1-4, in which little or no substrate is seen at the origin of the gel electrophoresis. The MN preparation we used in these experiments was the same as that used for

pretreatment of E. coli RNase P (1) and that which Koski used to inactivate KB cell RNase P (7), and has been shown to be free of proteases. In addition the protease inhibitor PMSF is included in all our pretreatment mixtures (see Methods).

RNase T_1 pretreatment: RNase P pretreated with RNase T_1 loses activity (Fig. 1C, lane 2). Since RNase T_1 does not precipitate in $(NH_4)_2SO_4$ under conditions where RNase P does (8), the pretreatment mixtures were fractionated by precipitation prior to assay for RNase P. Even so, a slight amount of residual RNase T_1 activity is evident by accumulated breakdown products in Fig. 1C, lane 2). Further purification of the pretreatment mixtures by sucrose gradient centrifugation (see Methods) removed the last residue of RNase T_1 from these mixtures (not shown). The end result observed with respect to the inactivation of RNase P is the same as shown in Figure 1C, lane 2.

RNase A pretreatment: RNase A pretreated mixtures were fractionated by $(NH_4)_2SO_4$ and then further purified by velocity sedimentation as described in the Methods. Even after these steps some residual RNase A was evident in the assays of the sucrose gradient fractions (Fig. 2B). In particular, there is some degradative activity in the bottom fraction of the gradient, presumably due to unresuspended RNase A which precipitated in the $(NH_4)_2SO_4$ fractionation, and at the top of the gradient where this enzyme is expected to sediment because of its small size. Nevertheless, the presence of RNase P in the relevant fractions from the gradient containing the control treated sample (Fig. 2A, lanes 2-4) is apparent whereas no such activity is seen in the same fractions from the gradient containing the RNase A pretreated sample (Fig. 2B, lanes 2-4). These experiments were carried out with various concentrations of soluble RNase A and with RNase A bound to polyacrylamide beads and yielded the same result. To exclude the possibility that RNase P cleavage products might be digested by residual RNase A in reaction mixtures containing the pretreated samples, we assayed reconstruction mixtures, containing both control treated and RNase A pretreated samples, for RNase P activity. We detected both RNase

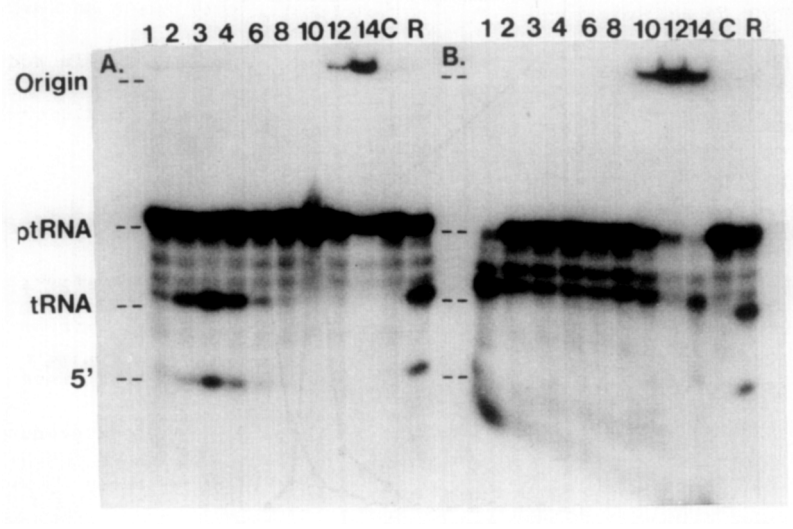

Figure 2: Sucrose gradient fractionation of RNase P after pretreatment with RNase A. RNase P was pretreated with RNase A as described in the Methods. The mixtures (with RNase A and control lacking RNase A) were then fractionated by ammonium sulfate precipitation and the relevant resuspended pellet layered on sucrose gradient as described in Methods. Gradient fractions (1 µl aliquots) were then assayed for RNase P activity. The figure shows an autoradiogram of the polyacrylamide gel analysis of the RNase P assay. The numbered lanes indicate relevant gradient fractions with number 1 being the bottom of the gradient. Fractions not shown in the autoradiogram had no activity. A. Control pretreatment (no RNase A). The lane labeled 'C' was a control incubation with no added material from the sucrose gradient. The lane labeled 'R' was a reconstruction (see Text) with equal amounts of material from Fig. 2A lane 4 and Fig. 2B lane 4. The position of the RNase P cleavage products are indicated in the figure. Small amounts of spontaneous breakdown products or RNase A break down products (Fig. 2B) are visible in the autoradiogram. They do not have the same mobility as the RNase P cleavage fragments (see Fig. 2B lane 1 especially).

P and RNase A activities at the same level as seen in the samples alone before mixing for the reconstruction experiment (compare Fig. 2A, lane R with Fig. 2A, lane 4 and Fig. 2B, lane 4). Thus, this experiment demonstrates that RNase P activity is abolished by the pretreatment with RNase A and is not lost in the subsequent assay for RNase P activity under the conditions we used.

Buoyant density of crude veal heart RNase P: The S16 fraction from an RNase P preparation was further fractionated by $(NH_4)_2SO_4$ precipitaion (see Methods) and dialysed versus buffer TM with 0.2M NH_4Cl and centrifuged in a preformed Cs_2SO_4 gradient (see Methods). The results, shown in Figure 3, indicate that the buoyant density of this material is about 1.33 g/cm^3. The recovery of activity, 3-5% is very poor. E. coli RNase P has a buoyant

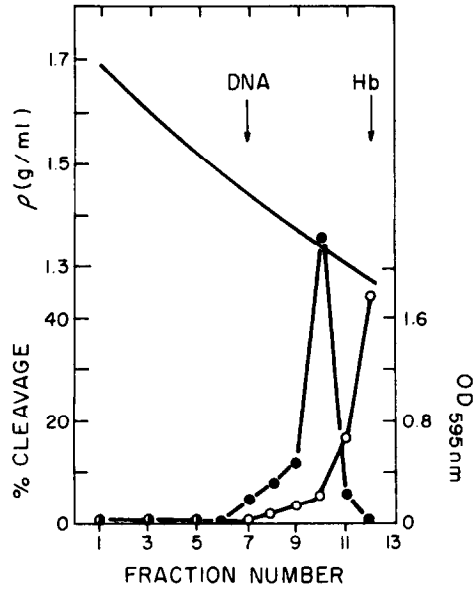

Figure 3: Cs_2SO_4 buoyant density determination of veal heart RNase P. Centrifugation was carried out as described in the Methods. (●) RNase P activity quantitated by measuring radioactivity in gel slices from analyses of assay mixtures for RNase P activity in gradient fractions. (X) Protein concentration as determined by the method of Bradford (10). The two arrows indicate the position of DNA and hemoglobin markers as determined in separate experiments (see Text).

density of 1.55 g/cm^3 under similar conditions (unpublished experiments). The buoyant densities of various markers (hemoglobin: 1.25 g/cm^3; calf thymus DNA 1.43 g/cm^3; and *E. coli* $tRNA^{Tyr}$: 1.38 g/cm^3) show that the veal heart RNase P complex is denser than protein but less dense than RNA or DNA (9), presumably due to the presence of a nucleic acid component.

DISCUSSION

We have shown that veal heart RNase P can be inactivated by pretreatment with any of the three ribonucleases, micrococcal nuclease, RNase T_1 or pancreatic RNase A. Consistent with the functional inactivation studies is the observation that the buoyant density of veal heart RNase P in Cs_2SO_4 is intermediate between that of RNA and protein. *E. coli* RNase P is also inactivated by pretreatment with MN and RNase A and also has a buoyant density characteristic of an RNA-protein complex (1). In this latter case further evidence has been presented to show that the enzyme requires an RNA component

in vivo. The reason for the discrepancy between the buoyant density of the veal heart and E. coli RNase P complexes is not apparent but several explanations can be put forward. The mammalian enzyme may have a smaller RNA associated with it than does the procaryotic RNase P, or it could be more highly modified or have more secondary and tertiary structure under our centrifugation conditions. Lastly, it could be complexed with membrane fragments. All these factors could contribute to making the buoyant density somewhat smaller than that observed for E. coli RNase P. Our results, taken together with data regarding E. coli and KB cell RNase P, suggest strongly that RNase P from any source will consist of an RNA-protein complex.

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