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Properties of Purified Ribonuclease P from *Escherichia coli*†

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ABSTRACT: The purified protein moiety of ribonuclease P (EC 3.1.26.5) from *Escherichia coli*, a single polypeptide of molecular weight ~17 500, has no catalytic activity by itself on several RNA substrates. However, when it is mixed in vitro with an RNA species called M1 RNA, RNase P activity is reconstituted. The rate at which the purified RNase P cleaves any particular tRNA precursor molecule depends on the identity of that tRNA precursor.

RNase P (EC 3.1.26.5) is an enzyme which participates in the biosynthesis of all tRNAs in *Escherichia coli* (Altman, 1978; Altman et al., 1980). This enzyme can be reconstituted in vitro from separated, inactive protein and RNA components (Kole & Altman, 1979). An essential role in vivo for an RNA component in RNase P can be demonstrated with the use of temperature-sensitive mutant enzyme forms in complementation experiments in vitro (Kole et al., 1980). The enzyme behaves like a ribonucleoprotein in buoyant density gradients and can be inactivated by pretreatment with certain ribonucleases (Stark et al., 1978). Some of the earlier biochemical characterization of RNase P (Stark et al., 1978; Kole & Altman, 1979) was performed with enzyme preparations which were not homogeneous with respect to protein, thus complicating discussions of the true role of the RNA moiety of the enzyme. Furthermore, there are conflicting reports in the literature concerning the structure of the enzyme (Bikoff & Gefter, 1975; Shimura et al., 1978; Altman et al., 1980: Guthrie & Atchison, 1980). In this report we show that a single polypeptide species (called C5 protein), which has no demonstrable activity on several potential tRNA precursor substrates, manifests RNase P activity when mixed with the appropriate RNA species. In addition, neither native RNase P, reconstituted RNase P, nor crude C5 protein have any catalytic activity on mature tRNA or poly(A).

Experimental Procedure

Materials

Bacterial and Bacteriophage Strains. *E. coli* MRE600 used in the preparatin of RNase P was purchased as a frozen paste from Grain Processing Corp. (Muscatine, IA). Bacterial and bacteriophage strains used in the preparation of radioactive substrates have been described previously (Bothwell et al., 1976a,b).

Chemicals and Chromatographic Media. All column chromatographic media were purchased from Pharmacia (Piscataway, NJ). Materials (electrophoresis purity) used in making $NaDodSO_4$[1]-containing polyacrylamide gels were purchased from Bio-Rad Corp. (Rockville Centre, NY). Materials used in making polyacrylamide gels for routine RNase P assays were purchased from Eastman Kodak Corp. (Rochester, NY). PEI–cellulose thin-layer plates were purchased from Brinkmann Corp. (Westbury, NY) and cellulose acetate strips were purchased from Schleicher & Schuell (Keene, NH). Urea, ultrapure grade, was purchased from Schwarz/Mann (Orangeburg, NY). Bulk *E. coli* tRNA was a gift of Dr. B. F. C. Clark (Aarhus, Denmark). All other chemicals used were analytical grade.

Radioactive Polynucleotides. *E. coli* $tRNA^{Tyr}$, $tRNA^{Tyr}$ precursor, precursor to 4.5S RNA, and bacteriophage ϕ80 M3 RNA were made as previously described (Bothwell et al., 1976a,b). T4 tRNA dimeric precursors (Guthrie et al., 1973) were a gift of Dr. W. H. McClain (Madison, WI). Synthetic tRNA precursor (A_4 $tRNA_f^{Met}$) and [^{3}H]poly(A) (50.9 Ci/M phosphate; Miles Laboratories, Elkhart, IN) were gifts of Drs. S. Nishikawa and D. Soll (New Haven, CT). Cleavage of A_4 $tRNA_f^{Met}$ by purified RNase P was assayed by measuring the release of radioactive A_4 into acid-soluble material.

Methods

Purification of RNase P. The first steps in the purification procedure were those in Scheme I as described by Stark et al. (1978). The S30 fraction was made from 400 g of *E. coli* MRE600 which had been ground with 800 g of alumina. The details of the various chromatographic columns are as follows: (a) DEAE-Sephadex A-50 (12 × 25 cm; 2.5-L bed volume) eluted stepwise with 2 L each of 0.2, 0.3, 0.4, and 0.5 M NH_4Cl in buffer A (5). (b) Sepharose 4B (2.5 × 90 cm; 4 mL/fraction collected at 16 mL/h). The fractions containing RNase P activity are shown by the dashed line in Figure 1A. The RNase P fractions which were most highly purified with respect to C5 protein, indicated by the solid bar in Figure 1A (and correspondingly for the other columns described in Figure 1), were pooled and precipitated with ammonium sulfate and subsequently layered on the G-200 column. (c) G-200 Sephadex (1.7 × 90 cm; 3 mL/fraction collected at 5 mL/h). The pooled material from this column (solid bars, Figure 1b) was made 2 M in ammonium sulfate and layered on the first *n*-octyl-Sepharose column. (d) *n*-Octyl-Sepharose (0.7 × 2 cm; 1 mL/fraction collected at 10 mL/h; total gradient volume of 50 mL). Active fractions from the *n*-octyl-Sepharose column (see Figure 1C) were pooled and rerun on a second, identical *n*-octyl-Sepharose column. Appropriate fractions from the second *n*-octyl-Sepharose column (see Figure 1D) were pooled, dialyzed against several changes of 0.05 M NaOAc, pH 7.2, and then made 7 M in urea by addition of solid urea. This material was applied to a CM-Sephadex column (1 × 3 cm) preequilibrated with the urea-containing acetate buffer. The column was eluted with a linear gradient from 0 to 0.5 M NaCl in the same buffer. Total volume of the gradient was 60 mL. Fractions of 1 mL were collected at a flow rate of 12 mL/h. The elution profile of a similar column has been published elsewhere (Kole & Altman, 1979). The fractions eluted from the various columns shown in Figure

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[1] Abbreviation used: $NaDodSO_4$, sodium dodecyl sulfate.

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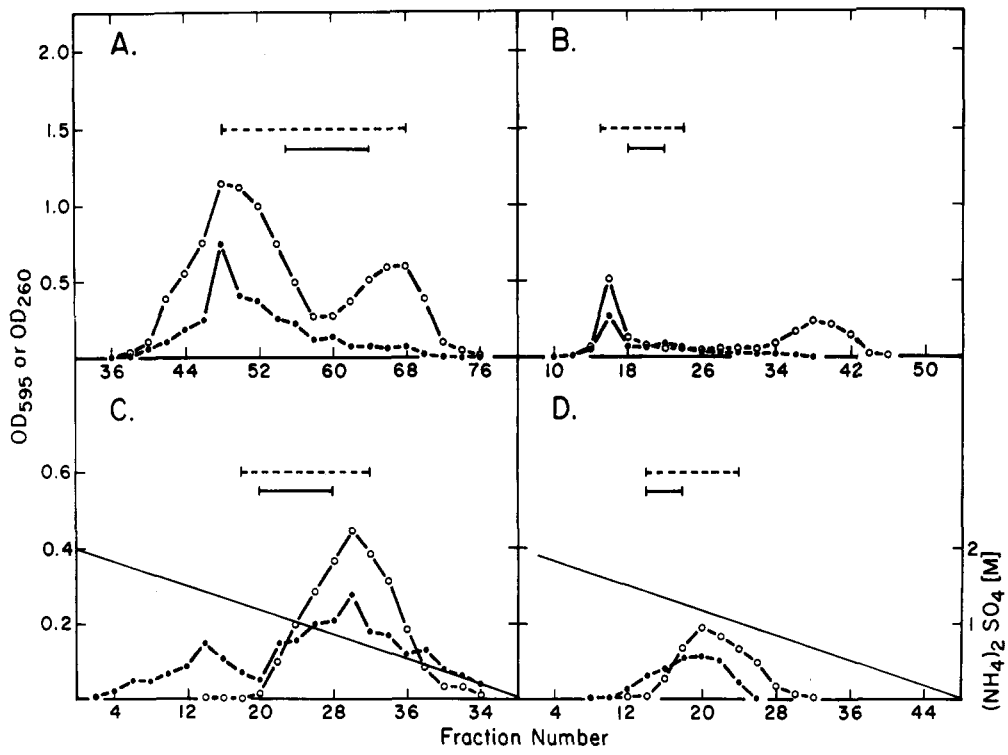

FIGURE 1: Column chromatography of RNase P. In each case the broken horizontal bar represents the fractions containing RNase P activity and the solid horizontal bar represents those fractions pooled for further purification. (○) OD_{260}; (●) OD_{595}; protein concentration was as determined by the assay of Bradford (1976). Chromatography was carried out as described under Methods. (A) Sepharose 4B. (B) G-200 Sephadex. (C) First *n*-octyl-Sepharose column. (—) Ammonium sulfate concentration. (D) Second *n*-octyl-Sepharose column. (—) Ammonium sulfate concentration. In panels C and D the left-hand ordinate represents OD_{595} and OD_{260} and the right-hand ordinate represents $(NH_4)_2SO_4$ concentration. The left-hand ordinate in panel D has been expanded 2-fold in scale.

1 were analyzed in $NaDodSO_4$–polyacrylamide gels for their protein content. The fractions to be pooled (solid bars) were chosen in order to maximize purification of the C5 protein and not total activity of RNase P.

To scale up the preparation of RNase P, we ground 1300 g of *E. coli* MRE600 with alumina. The resulting S30 fraction (980 mL) was divided into three aliquots, and subsequently three DEAE-Sephadex A-50 columns [2.5-L bed volume; see above and Stark et al. (1978)] were used to process the material. The eluted RNase P activity from the three columns was pooled and chromatographed on a Sepharose 4B column (3.5 × 100 cm). Three such batches of RNase P from 1300 g of *E. coli* were processed in rapid succession, and the active fractions from the Sepharose 4B column of each preparation were combined and chromatographed on a Sepharose G-200 column (2.5 × 100 cm) and subsequently on two successive *n*-octyl-Sepharose columns as described above, except that the elution gradient in each case was 120 mL. Appropriate fractions from the leading edge of the RNase P activity peak from the second *n*-octyl-Sepharose columns were then chromatographed on an urea-containing CM-Sephadex column as described above. The purification of RNase P from the large preparations is not always as efficient as that for material derived from 400 g of cells. For example, rechromatography on the same columns of materials from the *n*-octyl-Sepharose or CM-sephadex columns is sometimes necessary to reach the degree of purity achieved with the procedure used for 400 g of starting material. The purified reconstituted RNase P, which as we have prepared it does not contain RNA and protein in equimolar amounts, has a specific activity somewhat higher than that of the material purified through the second *n*-octyl-Sepharose step after treatment with urea.

Preparation of ^{32}P-Labeled M1 and M2 RNA. M1 and M2 RNAs were prepared as described (Kole et al., 1980) from MRE600. As necessary, these species were further purified by electrophoresis[2] in 4.8% polyacrylamide gels (20 × 40 × 0.3 cm).

Preparation of Nonradioactive M1 and M2 RNA. RNase P from 400 g of *E. coli* MRE600 was purified through the Sepharose 4B step. The enzyme, in 1.5 mL containing ~30 mg of protein, was then dialyzed extensively against water and lyophilized. A mixture of ^{32}P-labeled M1 and M2 RNAs (60 000 cpm) was added as a tracer. The sample was divided into three aliquots and electrophoresed in three $NaDodSO_4$–polyacrylamide 7–15% linear gradient gels (20 × 20 × 0.3 cm). The material comigrating with the labeled RNA was isolated from the gel as described (Kole & Altman, 1979). The yield was ~0.6 mg of RNA. For separation of M1 and M2 RNA, this mixture of the two species was electrophoresed in a 4.8% acrylamide gel.

Assays for RNase P activity, quantitation of these assays, and reconstitution of RNase P from inactive RNA and protein were carried out as described previously (Kole & Altman, 1979; Stark et al., 1978; Bothwell et al., 1976a,b). The lower limit of sensitivity of our RNase P assay is ~3% cleavage of substrate.

Results

Properties of Purified RNase P. Purified RNase P is made by reconstituting C5 protein (M_r 17 500; see Figure 2) purified through the CM-Sephadex column chromatography step with a mixture of M1 and M2 RNA as described under Experimental Procedure. This complex is capable of cleaving a variety of tRNA precursor molecules at the 5′ termini of their tRNA sequences. We have incubated both the purified, reconstituted enzyme and the separated C5 protein with the precursor to *E. coli* $tRNA^{Tyr}$ and to two T4-encoded dimeric

[2] M. Baer, personal communication.

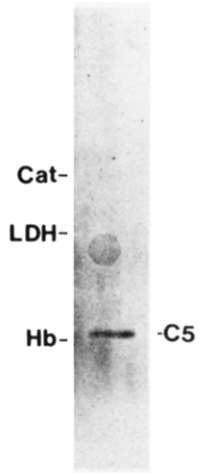

FIGURE 2: $NaDodSO_4$–polyacrylamide gel analysis of purified RNase P protein. Fractions 27–29 of a CM-Sephadex column (see Methods) were pooled, and ~4 μg of this material was electrophoresed in a 7–15% $NaDodSO_4$-containing gel as described in Kole & Altman (1979). The same material was used to prepare the pure, reconstituted enzyme described in the text. The positions of the molecular weight standards are shown in the figure. Standards: Cat, catalase (M_r 58 000); LDH, lactate dehydrogenase (M_r 36 000); Hb, hemoglobin (M_r 16 000); C5, C5 protein. The gel was stained with Coomassie brilliant blue (Fairbanks et al., 1971).

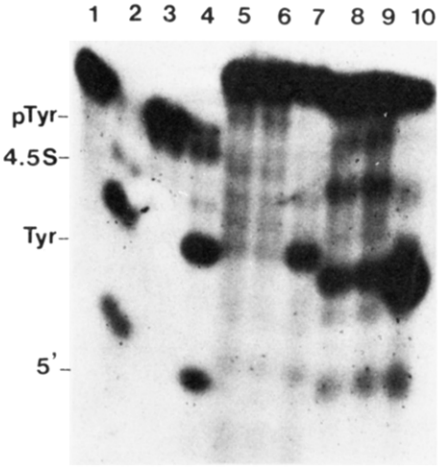

FIGURE 3: Substrate specificity of purified reconstituted RNase P. Reconstituted RNase P or C5 protein alone was incubated with various tRNA precursor molecules and assayed under standard conditions (Kole & Altman, 1979). Lane 1: $tRNA^{Tyr}$ precursor substrate; no added protein. Lane 2: $tRNA^{Tyr}$ precursor; RNase P purified through the G-200 Sephadex step, 0.05 μg of protein. Lane 3: $tRNA^{Tyr}$ precursor; C5 protein, 5 μg. Lane 4: $tRNA^{Tyr}$ precursor; reconstituted RNase P, 1 μg of protein. Lane 5: T4 Pro-Ser precursor; no enzyme added. Lane 6: T4 Pro-Ser precursor; C5 protein, 5 μg. Lane 7: T4 Pro-Ser precursor; reconstituted RNase P; 5 μg of protein. Lane 8: T4 Thr-Ile precursor; no added protein. Lane 9: T4 Thr-Ile precursor; C5 protein, 5 μg. Lane 10: T4 Thr-Ile precursor; reconstituted RNase P; 5 μg of protein. The cleavage products (Tyr and 5′) of $tRNA^{Tyr}$ precursor (pTyr) are marked on the left-hand side of the gel. The position of the product of cleavage of the precursor to 4.5S RNA is also marked as 4.5S RNA. The two cleavage products generated by RNase P cleavage of the T4 precursors are not resolved in this gel system and migrate as a single band as seen in the figure. Radioautolysis products of T4 precursors are apparent in the control and experimental lanes. The differences in results between, e.g., lanes 8 and 9, are more obvious in autoradiographs with shorter exposure times.

tRNA precursors (Guthrie et al., 1973). The results (Figure 3) indicate that the purified protein has *no* demonstrable activity against these substrates whereas the reconstituted enzyme produces cleavage fragments with the same mobility as those produced by the native RNase P. We have checked the exact site of cleavage of the $tRNA^{Tyr}$ precursor by isolating the cleavage fragments and performing the appropriate fingerprint analysis (Platt & Yanofsky, 1975; Sanger et al., 1965). The RNase A fingerprint of the RNase P cleavage product containing the tRNA sequence contains an oligonucleotide, pGpGpUp, which is the expected oligonucleotide from the 5′ terminus of the mature tRNA sequence (data not shown). Native, less pure RNase P makes the same cleavage in the $tRNA^{Tyr}$ precursor (Robertson et al., 1972).

Of the three substrates shown in Figure 3, the precursor to $tRNA^{Tyr}$ is cleaved most rapidly. The T4 Pro-Ser precursor is cleaved at ~10% of this rate and the T4 Thr-Ile precursor is cleaved at ~20% the rate of the precursor to $tRNA^{Tyr}$. Interestingly, these T4 tRNA precursors have a longer half-life in vivo than does the *E. coli* precursor to $tRNA^{Tyr}$.

We have also used a synthetic tRNA precursor molecule[3] as a substrate for RNase P purified through the first *n*-octyl-Sepharose column. This molecule has a tetranucleotide, A_4, ligated to the 5′ terminus of *E. coli* $tRNA_f^{Met}$. The rate of cleavage of this substrate was ~10% of that of the $tRNA^{Tyr}$ precursor.

We previously reported that RNase P is capable of cleaving two other RNA molecules in addition to tRNA precursors, namely, the φ80-encoded M3 RNA (Bothwell et al., 1976b) and the precursor to 4.5S RNA of *E. coli* (Bothwell et al., 1976a). Some of our $tRNA^{Tyr}$ precursor preparations are contaminated with the precursor to 4.5S RNA since, fortuitously, these two species differ by only one nucleotide in size. As shown in Figure 3, the cleavage products of both these substrates may be evident in the same gel analysis of an RNase P reaction. The reconstituted RNase P is capable of cleaving the 4.5S RNA precursor to produce fragments with the expected mobilities. The rate of this reaction is 3%–10% of that with the $tRNA^{Tyr}$ precursor.

We have estimated that the rate of cleavage of M3 RNA is a few percent or less than that of the $tRNA^{Tyr}$ precursor (Bothwell et al., 1976b). We did not observe any cleavage of this substrate with purified, reconstituted RNase P. However, the specific activity of the purified enzyme was such that it limited the sensitivity of the reaction. Cleavage of M3 RNA would have been observed only if it occurred at a rate 5% or more than that of the $tRNA^{Tyr}$ precursor. With RNase P purified through the first *n*-octyl-Sepharose column, we calculated that the rate of cleavage of M3 RNA was ~0.5% of $tRNA^{Tyr}$ precursor. We previously suggested that the reactions of RNase P with M3 RNA and the precursor to 4.5S RNA were "secondary" reactions of the enzyme. We still believe this to be the case with the 4.5S RNA precursor, but because of the limited sensitivity of our assay we cannot say definitely if the purified, reconstituted RNase P retains the ability to cleave M3 RNA.

The purified, reconstituted RNase P requires Mg^{2+} and NH_4^+ simultaneously for maximum activity as do less pure preparations (Robertson et al., 1972; Stark, 1977). In addition, the purified enzyme is also inhibited completely, as are the less pure preparations, by 100 μg/mL bulk *E. coli* tRNA. Since the inhibitory tRNA is not radioactive, it could be postulated that the tRNA is a competitive substrate and that we are not detecting its cleavage in our assay. This suggestion implies that, if tRNA is a substrate for RNase P, then the K_m of the enzyme for it is several orders of magnitude higher than that for the tRNA precursor molecules [tRNA is not a sub-

[3] L. Kline, S. Nishikawa, and D. Söll, personal communication.

strate at concentrations similar to those at which precursor tRNAs are tested (Stark et al., 1978)]. Because of the limited availability of our purest, reconstituted RNase P, it was necessary to use less pure preparations of enzyme to test adequately the hypothesis regarding substrate potential of tRNA. We reasoned further that less pure preparations of RNase P and C5 protein should be as likely as the purest preparations to manifest the activity against tRNA and a second test substrate, poly(A). Accordingly, we tested crude RNase P and C5 protein (prepared from RNase P purified through the G-200 step) for activity with uniformly ^{32}P-labeled tRNA^{Tyr} and [^{3}H]poly(A) as substrates. An aliquot of this crude C5 protein preparation was reconstituted with purified M1 RNA (the same material as was used in the experiment shown in Table I, line 1) and represents the partially purified RNase P we used. The amounts of reconstituted, partially purified RNase P (and equivalent amounts of C5 protein) used in our reactions were in a 4-fold excess compared to that normally used to cleave the tRNA^{Tyr} precursor completely in our standard reactions. We also tested native RNase P purified through the Sepharose 4B step in 1000-fold excess. The concentrations of labeled poly(A) or tRNA^{Tyr} used as substrates were also ~3 orders of magnitude greater than that normally used for tRNA precursors in our reactions. No endonucleolytic cleavage of the tRNA substrate was observed (in our gel electrophoresis assay) with either RNase P or C5 protein nor did either show any ability to acid solubilize the poly(A) substrate. We conclude that there is no low level of activity present in either RNase P or C5 protein which can hydrolyze either poly(A) or tRNA. We have also found that native, partially purified RNase P does not cleave several bacteriophage-encoded mRNAs or HeLa cell hnRNA.[4]

RNA Component of RNase P. The RNA component extracted from RNase P purified through the G-200 step contains a mixture of M1 and M2 RNA (Kole et al., 1980) found in various proportions in different preparations ranging from 40 to 90% M1. The fingerprints of these RNAs, when analyzed by homochromatography on PEI plates (Kole et al., 1980), show that they are distinct species but they are very close to each other in size (~360 nucleotides). We have purified these species further by an additional electrophoresis step and have used the enriched, separated species in reconstitution experiments to determine if one or both are active in reconstitution with C5 protein to make RNase P. The results, shown in Table I, indicate that as the proportion of M1 RNA is increased in the mixture, the specific activity of the reconstituted enzyme is enhanced considerably. These data show that M1 RNA is the active species in reconstituting the catalytic activity of C5 protein. A preparation of M1 RNA which was 94% pure was used in the reconstitution experiments with pure C5 protein described above.

Table I: Effectiveness of M1 RNA in Reconstitution of RNase P[a]

expt	M1/(M1 + M2) in the RNA prepn	rel sp act. of reconstituted enzyme
1	0.94	300
	0.40	100
	0.13	10
2	1.0	45
	0.0	<1

[a] (Experiment 1) A fixed amount (3 μg) of various mixtures of M1 and M2 RNA was used in the reconstitution reaction with 7 μg of protein isolated by CM-Sephadex chromatography (Kole & Altman, 1979) from RNase P purified through the G-200 Sephadex step. The relative amounts of M1 and M2 RNA were judged by two methods as follows. (1) The RNA mixtures were terminally labeled with RNA ligase and [^{32}P]pCp as described by Bruce & Uhlenbeck (1978). The labeled RNA species were then analyzed on a 4.8% acrylamide gel with in vivo labeled M1 and M2 RNA present as standard mobility markers (Kole et al., 1980). The relative proportion of M1 and M2 was determined by cutting out appropriate slices from the analytical gel and determining the amount of radioactivity in each. (2) The RNA mixtures were electrophoresed in a 7–15% linear gradient NaDodSO$_4$-polyacrylamide gel which was then stained with methylene blue (Peacock & Dingman, 1967). The gel was photographed and the negative scanned with a Joyce-Loebl densitometer to determine the relative amount of material in the bands corresponding to M1 and M2 RNA. The values for M1 RNA purity in the table are the averages of the numbers determined by the two methods, each of which give values in good agreement with the other. (Experiment 2) In this experiment 1.96 μg of protein and 0.84 μg of RNA were used in the reconstitution mixtures. The relative amounts of M1 and M2 RNA were judged as follows. Uniformly labeled RNA of each type (Kole et al., 1980) was added to an unlabeled mixture of M1 and M2 RNA (Kole & Altman, 1979). The mixture was electrophoresed in an 8% polyacrylamide gel containing 7 M urea, and the RNA comigrating with each separated, labeled species was cut out of the gel and eluted electrophoretically. The eluted RNAs were then reelectrophoresed separately in a 5% polyacrylamide gel (no urea), and the amount of radioactive RNA appearing in the positions corresponding again to those expected for M1 or M2 RNA was observed in each case. In the final preparations used in the experiment reported here, there was no cross contamination of M1 RNA with M2 RNA or vice versa.

Discussion

The protein component of RNase P has a molecular weight of ~17 500 and shows no hydrolytic activity on any known RNase P substrates nor, in fact, on any of the RNAs we have tested. However, when reconstituted with the appropriate RNA species, the C5 protein–M1 RNA complex manifests the enzymatic activity previously characterized as RNase P. In reconstituting RNase P from C5 protein and RNA, we have found that the RNA species we call M1 RNA is the effective agent. We do not know why the species called M2 RNA copurifies with M1 RNA at least through the *n*-octyl-Sepharose columns of our purification scheme. M1 and M2 RNAs are very similar in size, and their fingerprints differ only in the positions of a few oligonucleotides (Kole et al., 1980). M2 RNA appears to be more highly modified than M1 RNA.[2] Whether or not these two species differ only by posttranscriptional modification of nucleotides or endonucleolytic cleavages or whether they are totally unrelated species remains to be determined.

Reconstituted, purified RNase P has the same cleavage specificity and general properties as less pure enzyme preparations. Most importantly, it generates the 5′ termini of mature tRNA sequences from tRNA precursors. We may thus consider that the basic RNase P complex is made up of C5 protein and M1 RNA. Thus, arguments invoking contaminating molecular constituents as explanations for the requirement for M1 RNA in RNase P function in vitro (Guthrie & Atchison, 1980) can be eliminated with a consideration of the data reported here. We do not know if other molecules, like M2 RNA, are part of a larger RNase P complex with catalytic or regulatory functions we have not yet identified. It is noteworthy that the rates of reaction of RNase P with different tRNA precursors are not identical. The tRNA^{Tyr} precursor was cleaved faster than the T4 tRNA precursors or the synthetic tRNA precursor. Nucleotide modification is less complete in the tRNA^{Tyr} precursor (Guthrie et al., 1973; Schaefer et al., 1973) than in other precursors. Small differences in precursor structure, to which

[4] Unpublished experiments.

RNase P may be sensitive, could be determined by nucleotide modifications. Another factor governing the rate of RNase P cleavage, at least in T4 tRNA precursors, is the nature of the extra sequences on precursor molecules. T4 tRNA precursors with the CCA_{OH} termini of the mature tRNA species are cleaved faster than those with the extra precursor sequence at the 3′ terminus still intact (Schmidt et al., 1976). We do not know if the differences in rates of cleavage in vitro by RNase P are relevant to the half-lives in vivo of tRNA precursors. It is true, however, that the half-lives of T4 tRNA precursors are longer than that of *E. coli* $tRNA^{Tyr}$ precursor and that these T4 RNA precursors are not cleaved in vivo at all by RNase P until they have CCA_{OH} at their 3′ termini (Guthrie et al., 1973; Schmidt et al., 1976).

The absence of any demonstrable hydrolytic activity of the C5 protein is striking and implies that the M1 RNA must be involved in activating the catalytic mechanism of the RNase P complex. We cannot yet say if this RNA is also directly involved in substrate recognition. The possibility that an RNA molecule participates in the formation of an active site of an enzyme appears novel. However, the formation of active sites in ribosomes offers an analogy to the case of RNase P. For example, dissociated ribosomal proteins and rRNA exhibit no ability to carry out the peptidyltransferase reaction (Krayevsky & Kukhanova, 1979). This activity, however, is manifest in reconstituted ribosomes which are complexes of noncovalently linked RNA and protein species, as is RNase P. Another possible example of this kind of phenomenon may be the amylose isomerase isolated from rabbit muscle (Petrova & Shvedova, 1974) which contains an essential RNA component as well as protein.

Acknowledgments

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