ROLE OF 5S RIBOSOMAL RNA IN POLYPEPTIDE SYNTHESIS

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SUMMARY

Complete dissociation of 5S rRNA was obtained by mild treatment of 50S ribosomal subunits with EDTA. The 5S rRNA-free 50S subunits (50S E) could bind 30S subunits. The complex became attached to phenylalanyl-tRNA in the presence of poly U. The tRNA binding, however, was only 50% of that observed with normal ribosomes, and was resistant to tetracycline inhibition. Phenylalanyl-tRNA bound to 5S-rRNA-free ribosome-poly U complex was sensitive to pancreatic RNase. These results suggest that 5S rRNA may have a role in the specific binding of tRNA to ribosomes.

5S rRNA is a distinct species of RNA[1-3]. In *Escherichia coli* it is associated with 50S ribosomal subunits in 1:1 molar ratio per subunit[1,4,5]. The complete nucleotide sequence has been described for *E. coli* 5S rRNA[6], and several structural models were proposed[6-9]. The biological significance of 5S rRNA is unknown.

Our approach to studying the functional role of 5S rRNA was to dissociate it selectively from 50S ribosomal subunits. If 5S rRNA-free ribosomal particles can be recovered in a stable form, it should be possible to investigate the functional involvement of 5S rRNA in polypeptide synthesis. Morell and Marmur[10] obtained 5S rRNA-free ribosomal particles by treatment of 50S subunits with EDTA in the absence of Mg^{++}. The resulting particles sedimented close to 23S rRNA in a sucrose gradient centrifugation indicating an extensively unfolded structure[11,12] We have reported that dialysis of 50S ribosomal subunits against 0.5M ammonium

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Abbreviations: MAK column, methylated albumin kieselguhr column; TMA(-2) and TMA(-4), 10^{-2}M Tris-HCl pH 7.8 - 5 x 10^{-2}M NH_4Cl - 6 x 10^{-3}M mercaptoethanol and 10^{-2}M and 3 x 10^{-4}M Mg acetate respectively; TE, 10^{-2}M Tris HCl pH 7.5 - 10^{-3}M EDTA; TC, tetracycline.

chloride in absence of Mg^{++} produced partial dissociation of 5S rRNA[4]. By mild treatment of 50S ribosomal subunits with EDTA we obtained 5S rRNA-free particles which sedimented at 37S. The properties of these particles are described in this communication and the role of 5S rRNA is discussed.

Preparation of 5S rRNA-free ribosomal particles: Purified ribosomal subunits, prepared from *E. coli* Q13 as described earlier[23], were used throughout this work, unless stated otherwise. 100 A_{260} units of 50S subunits in 1 ml TMA(-4) were dialyzed vs. 1 liter of TE buffer at 4° for 1 hr. with a change. The sample was then placed on a biphasic sucrose solution in a Spinco rotor #50 tube. The lower phase was 1.75 ml 30% sucrose in TMA(-4) and the upper layer was 4.5 ml 10% sucrose in TE buffer. The tube was filled gently with TE buffer and centrifuged for 8 hrs. at 48,000 rpm. The pellet suspended in the lower 0.5 ml of solution was dialyzed overnight vs. TMA(-4). EDTA-treated subunits, referred to as 50S-E from hereon, have a sedimentation constant of approximately 37S (Fig. 1).

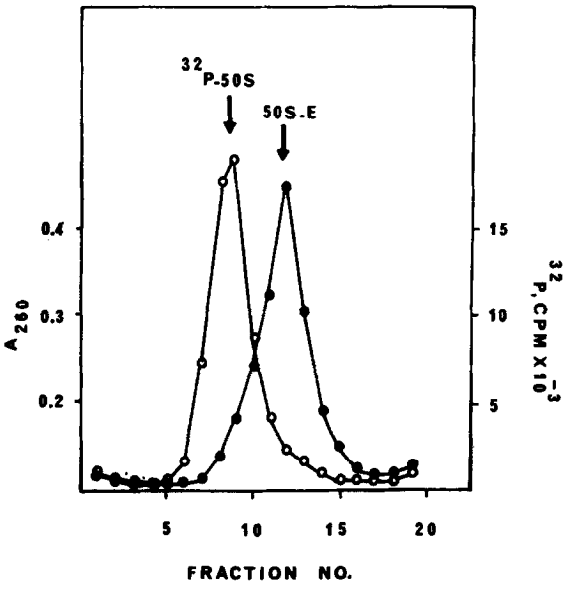

Figure 1. Sucrose gradient centrifugation of EDTA-treated 50S ribosomal subunits. 50S-E ribosomal subunits, 10 A_{260}, in 0.3 ml TMA(-4) mixed with 3.2×10^5 cpm (~0.002 A_{260} units) of ^{32}P-50S ribosomes were placed on 30-10% sucrose gradient made with TMA(-4). After 3 hr. centrifugation at 39,000 rpm in Spinco SW39 rotor, fractions of 3 drops were collected and analyzed for A_{260} and TCA-precipitable counts. Preparation and purification of ribosomes was described previously[23].

Absence of 5S rRNA from 50S-E was demonstrated by MAK column chromatography of phenol-extracted RNA (Fig. 2). An important step was to collect ribosomes in Mg^{++}-containing medium soon after the release of 5S rRNA. Prolonging the treatment with EDTA resulted in the appearance of slower sedimenting particles[10,11]. Gesteland[11] made similar observations and suggested that the change in the sedimentation constant was due to expansion or unfolding of the structure of ribosomes rather than to degradation. In fact, EDTA treatment of ribosomes caused no breakdown of rRNA or significant loss of protein[10,11]. The polyacrylamide gel electrophoresis pattern of proteins from 50S-E was identical to that from control 50S subunits.

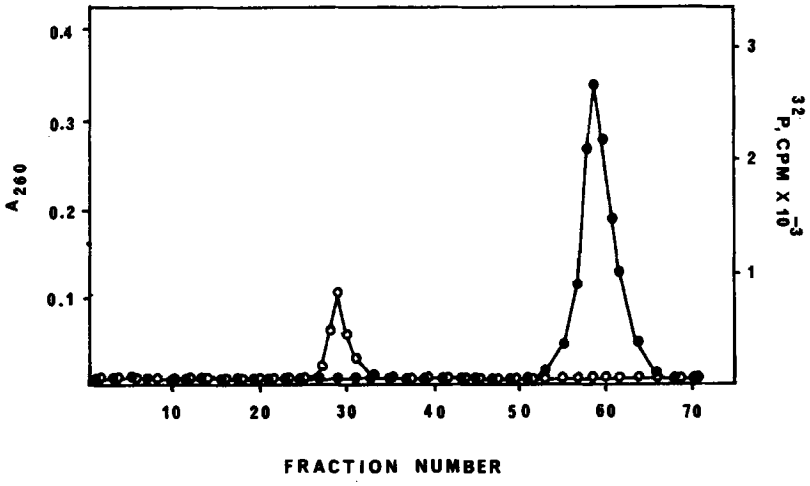

Figure 2. MAK column chromatography of RNA from 50S-E. 20 A_{260} units of RNA from 50S-E particles mixed with 10 µl of ^{32}P-5S rRNA (~ 2.5 x 10^3 cpm in 0.002 A_{260} units) were placed on MAK columns and eluted with 0.3 - 1.3 M NaCl gradient in 0.05M potassium phosphate buffer pH 6.7. ^{32}P-labeled and unlabeled RNA preparations and purification on Sephadex G-100 columns were described earlier[24]. MAK columns were made according to Mandell and Hershey[25] and chromatography was as described elsewhere[26]. Open circles, ^{32}P CPM; Closed circles, A_{260}.

Properties of 5S rRNA-free 50S ribosomal subunits: 50S-E was inactive for ^{14}C-phenylalanine incorporation carried out as described previously[23]. Gesteland[11] reported that re-dialysis of EDTA-treated ribosomes in Mg^{++}-containing-medium led to the recovery of activity for poly-phenylalanine synthesis. If this is

the case, absence of 5S rRNA from ribosomes in our system may have been detrimental to their biological activity. However, addition of purified 5S rRNA to the reaction mixture failed to restore the activity of 5S rRNA-free ribosomes. Sarkar and Comb[27] recently reported preparation of 5S rRNA-free particles which were inactive for poly U-directed ^{14}C-phenylalanine incorporation.

50S-E bound to 30S ribosomal subunits in equimolar ratio and the complex sedimented more slowly than 70S ribosomes (Fig. 3). Specific binding of aminoacyl-tRNA to this complex was tested next. It is known that there are at least two tRNA molecules bound per 70S ribosome[13,14]. A 30S ribosomal subunit alone can bind one aminoacyl-tRNA in response to the messenger RNA[15-17] on the amino acid site (site A), in which the 50S subunit may participate[18]. The 50S subunit

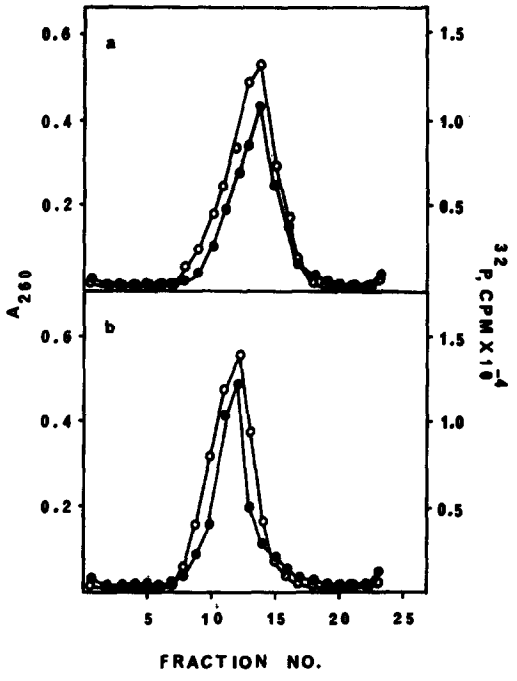

Figure 3. Binding of 50S-E and 30S ribosomal subunits. Equimolar quantities of ^{32}P-30S and 50S or 50S-E subunits were mixed and dialyzed vs. TMA(-2) for 4 hr. An aliquot of 0.3 ml (A_{260} 10) of the mixture was placed on 30-10% sucrose gradient in TMA(-2) in Spinco SW-65 rotor and centrifuged at 39,000 rpm for 1 hr. 50 min. Fractions of 3 drops each were collected and analyzed for A_{260} and TCA precipitable ^{32}P counts. Open circles, ^{32}P CPM; Closed circles, A_{260}. a) 50S-E + 30S. b) 50S + 30S.

alone cannot bind aminoacyl-tRNA, but when added to a 30S subunit-poly U-aminoacyl-tRNA complex it can generate a second binding site on the 70S ribosome[16]. It is believed that this second site (site P) is for peptidyl-tRNA[19,20]. The results summarized in Table 1 are consistent with this. The amount of ^{14}C-phenylalanyl-tRNA was constant and represents the saturation level for counts bound per unit of ribosomes. In the complete system, containing both 30S and 50S subunits, the counts were twice those with 30S subunits alone, but the binding capacity of the mixture containing 30S + 50S-E ribosomal complex was only 50% of that with normal ribosomes. The reduced binding capacity of 30S + 50S-E complex could be because:

Table 1

BINDING OF ^{14}C-PHENYLALANYL-tRNA TO RIBOSOMES

Reaction condition	CPM bound to ribosomes	
	30S + 50S	30S + 50S-E
complete system	1256	628
- poly U	129	116
-30S	83	45
-50S	569	569

Binding of ^{14}C-phenylalanyl-tRNA was assayed according to Nirenberg and Leder[28]. A typical reaction mixture contained, 0.5 A_{260} units of 30S and 1.0 A_{260} of 50S ribosomal subunits, 40 µg of poly U, 50 µg of ^{14}C-phenylalanyl tRNA, in 0.05 ml of buffer containing Tris-HCl, pH 7.4, 2.5 µmoles, Mg-acetate, 0.1 µmole and NH_4Cl, 5 µmoles. Incubation was at 35° for 15 min. unless otherwise stated. Samples were then diluted with 5.0 ml of same buffer (ice-cold) and filtered on a nitrocellulose membrane filter [28]. For preparing ^{14}C-phenylalanyl-tRNA, stripped *E. coli* B sRNA (General Biochemicals) was first freed of 5S rRNA by gel filtration through Sephadex G-100 columns[29]. For charging, 10mg of 5S rRNA-free tRNA was incubated in a total volume of 1.5 ml, with 0.03 µmoles of ^{14}C-phenylalanine (U.L.), specific activity 33.3 µC/µmole, 0.2 ml of nucleic acid-free enzyme preparations[30]; Tris-HCl, pH 7.4, 112.5 µmole; Mg acetate, 15 µmoles; mercaptoethanol, 6 µmoles and ATP, 2.25 µmoles. After 15 min. incubation at 36°, the mixture was shaken for 2 min. with equal volume of freshly-distilled phenol saturated with water. The aqueous phase was removed and phenol was re-extracted with 1.0 ml of H_2O. RNA was precipitated from the combined aqueous solution by adding 0.1 volume of 20% potassium acetate and 2 volumes of chilled 95% ethanol. Precipitation was for at least 6 hr. at -20° followed by centrifugation. The precipitate was washed once with 85% ethanol and dissolved in H_2O. The solution was lyophilized. The specific activity of the product was 5.2×10^4 cpm/mg.

(a) phenylalanyl-tRNA did not bind on the 50S-E subunits and therefore the counts recorded were those bound to 30S subunits alone, or (b) of the two tRNA sites on the 30S + 50S-E complex, only one could accept tRNA while the other was inactive, or (c) that both sites became less efficient for the specific binding of aminoacyl tRNA. To distinguish between the alternatives we made use of the antibiotic tetracycline (TC). TC inhibits protein synthesis by blocking the binding of aminoacyl-tRNA on site A of ribosomes but has no affect upon binding on site P[19,21], since TC inhibits binding of lysyl-tRNA to 70S ribosomes but binding of polylysyl-tRNA remains unaffected[19]. Binding of aminoacyl-tRNA to 30S subunits is also sensitive to TC[16]. We found that the inhibition of TC on binding of ^{14}C-phenylalanyl-tRNA to 70S ribosomes in presence of poly U reached a plateau of 46% at 5×10^{-5}M of TC. A ten-fold increase in concentration of the antibiotic had no further inhibitory effect. The results are in agreement with previous reports which suggest that TC blocks only one of the two aminoacyl-tRNA binding sites on the ribosome. TC, 10^{-4}M, when added to the reaction

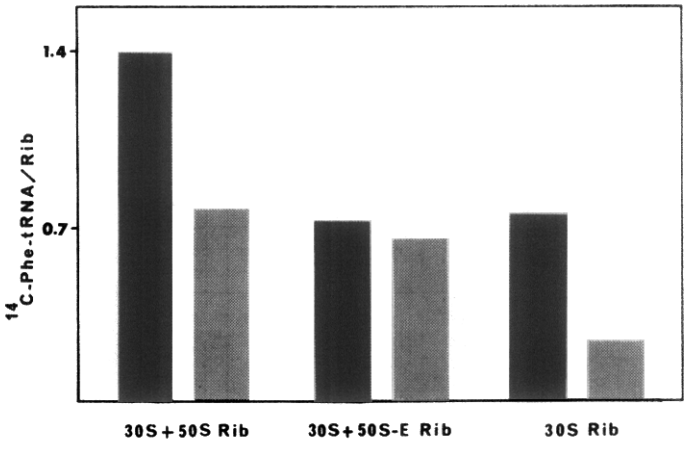

Figure 4. Effect of TC on non-enzymatic binding of ^{14}C-phenylalanyl-tRNA to ribosomes. TC, 10^{-4}M, was added to the binding mixture, 0.05 ml, before the addition of tRNA. Reaction conditions and concentrations of components were same as described in Table 1. The counts associated with normal ribosome, 30S and 50S, represent the saturation point for the number of tRNA molecules bound per 70S ribosomal subunit and calculations based on molecular weights of ribosomes and tRNA as 2.6×10^6 and 25×10^4 respectively indicated that the ratio of number of tRNAs bound per 70S ribosome was 1.4 assuming all ribosomes in the mixture were functional. Dark shadowed bar, no TC; Light shadowed bar, 10^{-4}M TC.

mixture containing 30S + 50S-E complex caused only 12% inhibition whereas in the absence of 50S-E, the binding capacity of 30S subunits was reduced by more than 65% (Fig. 4). Binding to normal 70S ribosomes was inhibited by about 50%, as expected. These results rule out the possibility that phenylalanyl-tRNA did not bind to 30S + 50S-E complex but only to 30S subunits, or one would expect the same inhibition of phenylalanyl-tRNA binding by TC whether 50S-E is present or not. The fact that binding of phenylalanyl-tRNA to the 30S + 50S-E complex was resistant to TC inhibition suggests that the binding was to a site other than site A, which is sensitive to TC inhibition.

Pestka[22] recently reported that aminoacyl-tRNA bound to 70S ribosomes in the presence of template was resistant to pancreatic RNase; but that aminoacyl-tRNA bound to 30S subunits was highly susceptible to RNase digestion. The presence of 50S ribosomal subunits therefore afforded protection against RNase which suggests that aminoacyl-tRNA was tightly bound to 50S subunits[22]. To test whether 50S-E can afford similar protection, 0.025 µg of pancreatic RNase was added to the reaction mixture containing ribosomes, poly U and ^{14}C-phenylalanyl-tRNA as described in the legend of Table II. 0.025 µg of pancreatic RNase was found to digest more than 90% of phenylalanyl-tRNA in the absence of ribosomes. ^{14}C-phenylalanyl-tRNA bound to 30S ribosomal subunits alone in response to poly U was sensitive to RNase. When 50S ribosomal subunits were added to produce 70S ribosomes, the tRNA became RNase-resistant. Substituting 50S-E for normal 50S ribosomal subunits resulted in significant breakdown of ^{14}C-phenylalanyl-tRNA suggesting that 50S-E did not provide protection to the bound aminoacyl tRNA against RNase as did the 50S subunits. The susceptibility of ^{32}P-50S-E to 0.025 µg of RNase was tested in the presence of all the components present in the tRNA-ribosome binding reaction. It was found that 50S-E alone were much more sensitive to RNase digestion than 50S subunits but adding 30S subunits, poly U and phenylalanyl-tRNA produced resistance, as in the case of 50S ribosomal subunits.

The results suggest that removal of 5S rRNA from 50S ribosomal subunits, which is accompanied by a significant conformational change, caused an inactiva-

TABLE II

EFFECT OF PANCREATIC RNASE UPON ^{14}C-PHENYLALANYL-tRNA BOUND TO RIBOSOMES

Ribosomal subunits	TCA - insoluble CPM -RNase*	+RNase
30S	1104	68
30S + 50S	1206	734
30S + 50S-E	1192	97
- Ribosomes	1150	34

0.025 µg of pancreatic RNase was added to the reaction mixture, containing components for ^{14}C-phenylalanyl-tRNA binding to ribosomes described in the legend to Table I, after 10 min. of preincubation. The incubation was continued for an additional 10 min., and the reaction stopped by adding 2.0 ml of 10% TCA. The TCA-insoluble counts were recorded in a scintillation spectrometer.

* The counts in this column represent the total TCA-precipitable ^{14}C-phenylalanyl-tRNA added per tube, i.e., both bound and unbound to ribosomes.

tion of the aminoacyl-tRNA site (site A) on ribosomes. The fact that TC inhibited phe-tRNA binding to 30S subunits but not to the 30S + 50S-E complex indicated that tRNA binding to this complex in response to poly U was on a site other than site A. There was no direct evidence that P site was involved in the binding, but assuming that there are only two sites for tRNA available per 70S ribosome, one may conclude that one tRNA molecule binds to the 30S + 50S-E complex on the P site in response to poly U. 50S-E + 30S complex did not protect the bound ^{14}C-phe-tRNA against RNase as did normal ribosomes. It was suggested[22] that a single-stranded structural component on 50S ribosomes may interact with the single-stranded portions of aminoacyl-tRNA to make a double-stranded structure and thereby provide resistance to RNase digestion. That 5S rRNA has a role in providing such protection against RNase is highly speculative. It is not clear whether loss of 5S rRNA alone or the conformational change in 50S-E or both were responsible for the defective functioning of 50S-E ribosomal subunits. Gesteland[11] was able to restore amino-acid-incorporating activity by re-dialysis

of EDTA particles in Mg^{++}. Since re-dialysis did not restore the 50S conformation, it seems that lack of 5S rRNA, rather than conformational change, was responsible for the loss of amino-acid-incorporating activity in our experiments. Further work attempting to restore functional defects of 50S-E following specific binding of 5S rRNA is in process. Kirtikar and Kaji[31] reported that addition of 5S rRNA stimulated incorporation of amino acids directed by MS-2 RNA but not by poly U. Free 5S rRNA does not bind to 50S subunits under similar conditions[32], and further study will be necessary to explain the stimulatory activity of 5S rRNA reported by Kirtikar and Kaji.

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