Peptidyltransferase Activity of Ribosomes and a Ribosome Precursor from a Mutant of *Escherichia coli*

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Escherichia coli strain 15–28 is a mutant with a defect in ribosome synthesis that causes the accumulation of ribonucleoprotein ('47S') particles during exponential growth. These particles are precursors to 50S ribosomes that lack three ribosomal proteins. Peptidyltransferase activity and binding at the peptidyl site of the peptidyltransferase centre are greatly decreased in 47S particles. Both these activities are lower in the 50S and 70S ribosomes of strain 15–28 than in its parent. Unusual assembly of the larger ribosomal subunit in strain 15–28 may produce completed ribosomes with diminished biological activity.

Escherichia coli strain 15–28, first described by MacDonald *et al.* (1967), is a mutant that accumulates unusual ribonucleoprotein ('47S') particles during exponential growth. We (Markey & Wild, 1976; Markey *et al.*, 1976) have shown that 47S particles are precursors to 50S ribosomes that, in common with other ribosome precursors, are more sensitive than completed ribosomes to degradation by ribonuclease. However, 47S particles differ not only from the precursors to 50S ribosomes detected by pulse-labelling of the parent strain but also from precursors with similar sedimentation coefficients described by other workers (for example, Blundell & Wild, 1969; Osawa *et al.*, 1969; Nierhaus *et al.*, 1973; Lindahl, 1975). By electrophoretic criteria, 47S particles contain 'mature' and not 'precursor' forms of 23S RNA. The particles lack three of the 34 proteins that are detected by two-dimensional electrophoresis of proteins from the 50S ribosomes of either strain 15–28 or its parent. The missing proteins are L16, L28 and L33 (Markey *et al.*, 1976).

In this paper we show that peptidyltransferase activity [as measured by the 'fragment reaction' (Monro, 1971)] and binding at the peptidyl site of the transferase centre are greatly decreased in 47S particles. Possible reasons for this are discussed. Moreover, both activities are lower in the 50S and 70S ribosomes of strain 15–28 than in its parent. It is likely that the unusual pathway of assembly of the larger ribosomal subunit in strain 15–28 generates ribosomes with an altered topography, which is reflected in decreased biological activity.

Experimental

Growth and harvesting of bacteria

Bacterial strains were *E. coli* 15 *thy*[−] *pro*[−] (15TP) and the mutant, 15–28, derived from it (MacDonald

et al., 1967). Cultures were grown in a shaking incubator at 37°C in glucose minimal medium (Blundell & Wild, 1969) supplemented with, per ml, 10 µg of thymine, 50 µg of L-proline and 0.1 mg of vitamin-free casamino acids (Difco Laboratories, West Molesey, Surrey, U.K.). Bacteria were harvested in the late exponential phase of growth ($E_{450} = 1.0$), and extracts prepared by using an Aminco–French pressure cell as described previously (Markey & Wild, 1976).

Preparation of ribosomes and 47S particles

The buffers (pH 7.5) used in the preparation of cell-free extracts and sucrose gradients were (i) 10 mM-Tris/HCl/10 mM-magnesium acetate/100 mM-NH₄Cl ('TAHM buffer') and (ii) 10 mM-Tris/HCl/1 mM-magnesium acetate/100 mM-NH₄Cl ('TAM buffer').

Portions (1.0 ml) of clarified extracts (E_{260} about 200) were centrifuged through 38 ml linear 15–30% (w/w) sucrose gradients for 16 h at 80000g (Spinco SW27 rotor). After centrifuging, the gradients were analysed and collected with an Isco model 185 density-gradient fractionator, by using E_{280} to locate ribosomes and 47S particles. Fig. 1 shows typical extinction profiles of these preparative gradients. Fig. 1(a) is of strain 15TP in TAM buffer: the 70S ribosomes have dissociated into their subunits. Fig. 1(b) is of strain 15–28 in TAHM buffer: 70S ribosomes are present together with appreciable quantities of 47S particles and 30S ribosomes. When 50S ribosomes of strain 15–28 were required, fractions containing 70S ribosomes were precipitated with ethanol (see below), dissolved in TAM buffer and the subunits obtained after further centrifuging in TAM buffer as for Fig. 1(a).

The magnesium acetate concentration of pooled fractions was adjusted, if necessary, to 10 mM and

Fig. 1. Extinction profiles of sucrose density gradients

Extracts were prepared and gradients centrifuged and fractionated as described in the text. The direction of sedimentation is from left to right. (a) Strain 15TP in TAM buffer; (b) strain 15-28 in TAHM buffer.

ribosomes and/or 47S particles were precipitated by the addition of an equal volume of ethanol at -20°C . After standing at -20°C for 1 h, precipitates were collected by centrifuging at $35000g$ for 15 min. The pellets were dissolved in 'activation buffer' (5 mM-Tris/HCl/5 mM- MgCl_2 /50 mM- NH_4Cl , pH 7.5) at a concentration of 20 mg/ml ($E_{260} = 280$) and stored in small portions at -20°C . No loss of activity was observed after 1 month under these conditions.

Peptidyltransferase assay

Peptidyltransferase activity was measured by the 'fragment reaction'. The 'fragment' is CpApCpCpA-N-acetyl $^{[3]\text{H}}$ leucine, an analogue of peptidyl-tRNA, and was made from tRNA as described by Monroe (1971). Assay mixtures were prepared from a salts solution that contained 45% (v/v) methanol, 45 mM-Tris/HCl (pH 7.5), 363 mM-KCl, 18 mM-magnesium acetate and 0.9 mM-puromycin. Before use, ribosomes and 47S particles were diluted with activation buffer to $E_{260} = 50$, incubated at 37°C

for 20 min and then chilled to 0°C . Ribonucleoprotein ($10\mu\text{l}$) was mixed with $55\mu\text{l}$ of the salts solution and the reaction started by adding the fragment ($10\mu\text{l}$, containing about 3000 c.p.m.). Incubation was for 15 min at 0°C . The reaction was stopped with $50\mu\text{l}$ of 0.3 M-sodium acetate buffer (pH 5.5) saturated with MgSO_4 . The acetyl $^{[3]\text{H}}$ -leucyl-puromycin formed was extracted with $700\mu\text{l}$ of ethyl acetate. After centrifuging for 1 min in a Beckman Microfuge, $500\mu\text{l}$ of the upper organic phase was removed, added to 3 ml of Prockop & Ebert's scintillation fluid (described by Monroe, 1971), and radioactivity counted.

Assay of the binding of the fragment to ribosomes and 47S particles

Samples of ribosomes or 47S particles (20 mg/ml in activation buffer) were incubated for 20 min at 37°C and then chilled to 0°C . Their ability to bind the fragment was then measured essentially as described by Celma *et al.* (1970). Assay mixtures were prepared from $130\mu\text{l}$ of a solution that

contained 3000–12000 c.p.m. of the fragment, 38 mM-Tris/HCl (pH 7.5), 308 mM-KCl, 15 mM-magnesium acetate and 58% (v/v) ethanol. The reaction was started by adding 20 μ l of ribonucleoprotein. Incubation was for 30 min at 0°C. The particles were then removed by centrifuging for 5 min in a Beckman Microfuge and 100 μ l of the supernatant was taken for radioactivity counting in Bray's scintillant (Bray, 1960). The blanks used contained 30S subunits from strain 15TP.

Chemicals

L-[4,5-³H]Leucine (55 Ci/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Ribonuclease T₁ was from Calbiochem, Hereford, U.K. Puromycin was from Sigma (London) Ltd., Kingston-upon-Thames, Surrey, U.K. tRNA (from *E. coli* strain B) was from Gibco-Bio-Cult, Paisley, Renfrewshire, Scotland, U.K.

Results

Peptidyltransferase activity of ribosomes and 47S particles

Fig. 2 shows the time-course of formation of acetyl-leucyl-puromycin in mixtures containing 50S ribosomes from strains 15TP and 15-28 and 47S particles from the latter. The rates of reaction of the 50S ribosomes are linear for the first 15–20 min, but then decline. This is probably due to substrate limitation, because, after 60 min, about 75% of the fragment added has been transferred to puromycin by the 50S subunits of strain 15TP. Therefore 15 min was chosen as the assay time for further

experiments. Fig. 2 also shows that 47S particles have a peptidyltransferase activity that is about 5% of that of the 50S ribosomes of the parent strain (15TP). In addition, 50S ribosomes from strain 15-28 have a somewhat lower (71%) transferase activity than those of the parent. This latter unexpected finding led to experiments in which the peptidyltransferase activities of 70S and 50S ribosomes of both parent (15TP) and mutant (15-28) strains were measured, together with that of 47S particles from the latter.

The results are in Table 1. Four different batches of ribosomes and 47S particles were used; two of the batches were also assayed on separate occasions. The following conclusions can be drawn. (i) 47S particles from strain 15-28 have a low activity that ranges from 5 to 13% of that of the 50S ribosomes from strain 15TP. It is difficult to exclude the possibility that some of this activity is due to contamination of 47S particles with ribosomes. However, after dialysis against buffer containing 10 mM-Tris/HCl (pH 7.5)/0.1 mM-magnesium acetate, 47S particles migrated as a single component through analytical sucrose gradients prepared in this buffer. Under these conditions the sedimentation coefficient of the particles is about 36S (Markey & Wild, 1976), and they are clearly resolved from 50S ribosomes. (ii) The transferase activity of 50S subunits from strain 15-28 is 20–40% lower than that of these subunits from strain 15TP, thus confirming the results of Fig. 2. (iii) There is a similar difference in the activity of the 70S ribosomes from the two strains. Thus the defect in the 50S ribosomes of strain 15-28 persists after association with 30S subunits. The activities for 70S ribosomes in Table 1 are lower

Fig. 2. Time-course of peptidyltransferase activity

The 50S subunits from strain 15TP (○) and strain 15-28 (△) and 47S particles from the latter strain (□) were assayed at 0°C as described in the text.

Table 1. Peptidyltransferase activity of ribosomes and 47S particles

The 70S, 50S and 30S ribosomes (strain 15TP), 70S and 50S ribosomes and 47S particles (strain 15-28) were prepared and assayed as described in the text. Fragment concentrations used ranged from 0.95×10^4 to 1.8×10^4 d.p.m./assay. Peptidyltransferase activities were measured as radioactivity (d.p.m./min per mg of ribonucleoprotein) extracted with ethyl acetate. The activity of 50S ribosomes from strain 15TP is set at 100%: in the different assays this corresponds to the transfer to puromycin of 49 ± 3 (s.d.)% of the added radioactivity.

Batch	Assay	Peptidyltransferase activity (%)					
		15TP 50S	15-28 50S	15TP 70S	15-28 70S	15-28 47S	15TP 30S
1	(i)	100	84	86	60	9.7	0.0
	(ii)	100	—	93	85	12.4	0.9
2	(i)	100	—	89	67	7.9	0.06
	(ii)	100	—	62	50	6.9	—
3	(i)	100	59	—	—	13.0	—
4	(i)	100	71	—	—	5.4	—

than those for the corresponding 50S subunits. This is because the values in Table 1 are per unit mass: when allowance is made for the different molecular weights of 70S and 50S ribosomes (2.65×10^6 and 1.65×10^6 respectively; Hill *et al.*, 1969), the activity of a 70S ribosome becomes about 140% that of the corresponding 50S subunit, suggesting that the combination of 50S with 30S ribosomes enhances the peptidyltransferase activity of the former.

Further experiments were carried out to substantiate these results. For Table 1, 50S ribosomes from strain 15TP were obtained directly from extracts made in and centrifuged through TAM buffer. Extracts of strain 15-28 were made in and centrifuged through TAHM buffer to obtain 70S ribosomes and 47S particles: some of the 70S ribosomes were then precipitated and dissolved in TAM buffer, and 50S ribosomes were collected after centrifuging through this buffer. Thus the 50S ribosomes of strain 15-28 were prepared after an extra cycle of centrifuging. When 50S ribosomes from strain 15TP were prepared by the method used for strain 15-28, their peptidyltransferase activity was slightly (10%) greater than that of 50S ribosomes of this strain prepared by the procedure used for Table 1. This increase is probably not significant, but, if it is, it enhances the differences between the strains.

In the absence of univalent cations, peptidyltransferase is reversibly inactivated (Miskin *et al.*, 1968). The methods by which the ribonucleoproteins were prepared used buffers that contain 100mM-NH_4^+ , so that activity should be retained; in addition, 20 min incubation at 37°C in activation buffer was included in the protocol. However, the lower transferase activities of 50S ribosomes and 47S particles from strain 15-28 could arise if the material was not fully active when assayed. The activity of 47S particles and 50S ribosomes from both strains was not increased when incubation at 37°C before assay was extended up to 160 min. Fig. 3 shows an experiment for which samples were prepared as usual and then inactivated by dialysis at 4°C against 5mM-Tris/HCl (pH 7.5)/ 5mM-MgCl_2 . NH_4Cl was then added (final concentration 50mM), and samples were incubated at 37°C for various times before assay at 0°C . The 50S ribosomes from both strains regained maximum activity after 10–20 min incubation; the subunits from strain 15-28 were then less active than those from strain 15TP. The activity of 47S particles remained low, even when incubation at 37°C was extended to 160 min.

Fragment binding

The CpApCpCpA-*N*-acetyl-leucine used in the assay of peptidyltransferase binds to the peptidyl ('P') site of the peptidyltransferase centre (Celma *et al.*, 1970). The lower peptidyltransferase activity of

Fig. 3. Activation of peptidyltransferase

The 50S subunits from strain 15TP (○) and strain 15-28 (△) and 47S particles from the latter strain (□) were dialysed against 5mM-Tris/HCl (pH 7.5)/ 5mM-MgCl_2 . NH_4Cl was then added to a final concentration of 50mM . Portions of the mixtures were incubated at 37°C for the times indicated and then chilled before assay at 0°C for 15 min.

Table 2. Binding of fragment by ribosomes and 47S particles

The 70S and 50S ribosomes from strains 15TP and 15-28 and 47S particles from the latter were prepared and binding was determined as described in the text. The binding of the fragment was measured as c.p.m. bound/mg of ribonucleoprotein. In the Table the binding to 50S ribosomes from strain 15TP is set at 100%. The fragment concentrations are the amount of fragment (in c.p.m.) added to each assay mixture (final volume $150\mu\text{l}$).

Batch	Fragment concn. (c.p.m./assay)	Binding of fragment (%)				
		15TP 50S	15-28 50S	15TP 70S	15-28 70S	15-28 47S
		50S	50S	70S	70S	47S
1	5681	100	78	97	69	20
	12066	100	76	88	73	40
2	4120	100	—	114	66	8.6
	13020	100	—	104	57	15
3	3480	100	77	—	—	0
	9957	100	55	—	—	0
	15072	100	66	—	—	22

ribosomes and 47S particles from strain 15-28 might be accompanied by, or be independent of, defective binding of this fragment to the P site.

To distinguish between these possibilities, the binding of fragment was measured, by using three different batches of the ribonucleoproteins, and in each case two or three different concentrations of the fragment. The results (Table 2) show a general similarity to those of the peptidyltransferase

assays. Binding to the 47S particles of strain 15-28 (as a percentage of that to the 50S ribosomes of strain 15TP) is low. The increased binding at higher fragment concentrations may be non-specific. Both 50S and 70S ribosomes from strain 15-28 bind less fragment than do the corresponding species from strain 15TP. The binding to a 70S ribosome is greater than that to a 50S subunit. This is probably a co-operative effect arising from association with 30S subunits. A similar and even more dramatic result was noted in a study of binding to the aminoacyl site (Celma *et al.*, 1971).

As was found for peptidyltransferase activity, 50S subunits prepared from strain 15TP by the protocol used for strain 15-28 showed somewhat increased binding of fragment. Two experiments gave values that were 15 and 22% greater than the binding to 50S ribosomes prepared as for the experiments of Table 2. Thus the lower binding of the 50S subunits of strain 15-28 is not due to their method of preparation.

Discussion

The 50S and 70S ribosomes of strain 15-28 have appreciably lower peptidyltransferase activities than the equivalent ribosomes from their parent strain, 15TP. This finding was rather unexpected because the 50S ribosomes of both strains have, qualitatively at least, the same normal complement of ribosomal proteins (Markey *et al.*, 1976). However, MacDonald *et al.* (1967), using polyuridylic acid as the messenger RNA, found that the 70S ribosomes of strain 15-28 were only 50-70% as efficient as those of strain 15TP in protein synthesis *in vitro*. Our results suggest that this defect could be largely explained as a lower peptidyltransferase activity of the 50S subunits, although the other reactions in chain elongation have not yet been investigated. There is at present no information about the ability of ribosomes of strain 15-28 to initiate or terminate protein synthesis.

Interpretation of the results for binding of the fragment used as an analogue of peptidyl-tRNA is complicated by several factors. Peptidyltransferase assays are kinetic measurements, whereas fragment binding measures an equilibrium process. The relationship between the two requires a more detailed knowledge than is at present available of the rate-limiting steps in peptidyltransferase as assayed in the fragment reaction. Functional overlap of the peptidyl-binding site with that of the enzyme centre is expected, but could be associated with different ribosomal proteins. However, the decreased binding of the fragment to 50S and 70S ribosomes of strain 15-28, together with their lowered peptidyltransferase activity, provides evidence for our view (Markey *et al.*, 1976) that the genetic lesion

in strain 15-28, besides giving rise to an unusual pathway of ribosome assembly, generates 50S subunits of altered topography and biological activity.

There are alternative explanations for the low peptidyltransferase activity of the 47S particles from strain 15-28 and their greatly decreased capacity to bind the fragment. Although these precursors to 50S ribosomes lack only three of the proteins found in completed 50S subunits, they do not associate with 30S subunits, their sedimentation coefficient differs from that of 50S ribosomes, they unfold in buffers in which 50S ribosomes do not, and their sensitivity to ribonuclease is greater than that of the completed subunits (Markey & Wild, 1976; Markey *et al.*, 1976). These general properties argue that the overall conformation of 47S particles may be such that, although the components of peptidyltransferase are present, little activity is generated. On the other hand, ribosome 'cores', prepared by the removal of up to 30% of the protein from ribosomes by treatment with salt solutions, can retain peptidyltransferase activity until crucial proteins are subsequently lost (Staehelin *et al.*, 1969; Monro, 1971). Moreover, the peptidyltransferase activity of the ribosomal cores used by Moore *et al.* (1975) was restored by the addition of one ribosomal protein (L16) in the absence of at least nine others. It may be more than a coincidence that 47S particles do not contain protein L16. This protein, which binds specifically to 23S RNA (Stöffler *et al.*, 1971), has been implicated in the binding of both chloramphenicol and aminoacyl-tRNA (Nierhaus & Nierhaus, 1973; Pongs *et al.*, 1974; Traut *et al.*, 1974) and in peptidyltransferase activity measured by the ability to form peptide bonds (Moore *et al.*, 1975) and by the hydrolysis of peptidyl-tRNA in the termination stage of protein synthesis (Tate *et al.*, 1975). Although other proteins, for example protein L11 (Nierhaus & Montejó, 1973; Dietrich *et al.*, 1974; Hsiung *et al.*, 1974), may also be involved in the peptidyltransferase reaction centre, the lack of protein L16 would by itself provide an explanation for the low peptidyltransferase activity of 47S particles and possibly for their inability to combine with 30S ribosomes (Kazemie, 1975).

Further work is necessary to decide between these alternatives, which in any case need not be completely separate. The interplay between structural elements of ribosomes is well known (see, for example, Apirion *et al.*, 1969). Changes in one subunit of a 70S ribosome may even be reflected in the properties of the other (Saltzman & Apirion, 1976). An altered partial activity in protein synthesis after the removal or addition of ribosomal protein(s) can be accompanied by conformational changes, which may be subtle and extensive (see, for example, Michalski & Sells, 1975). Thus it is at pre-

sent often difficult to separate the structural role of ribosomal proteins from the functional activities to which they may contribute.

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