Binding of Chloramphenicol and a Fragment of Aminoacyl-Transfer Ribonucleic Acid to Ribosomes and a Ribosome Precursor from a Mutant of *Escherichia coli*

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During exponential growth, the mutant strain *Escherichia coli* 15-28 accumulates 47S particles, which are unusual precursors to 50S ribosomal subunits. The 47S particles have little ability to bind chloramphenicol, but binding of a fragment of aminoacyl-tRNA is about half that by completed subunits. The 70S (and 50S) ribosomes of strain 15-28 and its parent (strain 15TP) do not differ in chloramphenicol binding. Although ribosomes from the mutant are less able than those from the parent to bind the fragment, this difference is not as marked as was found previously [Sims & Wild (1976) *Biochem. J.* **160**, 721–726] for the binding of an analogue of peptidyl-tRNA and for peptidyltransferase activity. The altered activities may arise because strain 15-28 misassembles 50S subunits of altered conformation and because the few proteins that 47S particles lack have vital functions in some of the partial reactions of protein synthesis.

Escherichia coli strain 15-28 is a mutant with defects in ribosome assembly and function. Organisms in exponential growth accumulate ribonucleoprotein (47S) particles (MacDonald *et al.*, 1967). These precursors to 50S subunits contain mature rather than precursor 23S rRNA and lack only three of the proteins (L16, L28 and L33) found in the completed subunits. The 47S particles therefore differ from precursors to 50S subunits detected in the parent and other strains of *E. coli* (Markey & Wild, 1976; Markey *et al.*, 1976).

Although no differences are apparent between the protein and RNA complements of the 70S ribosomes of parent and mutant strains, 70S ribosomes from the latter are inefficient in cell-free protein synthesis (MacDonald *et al.*, 1967). By comparison with the parent organism, the 70S and 50S ribosomes of strain 15-28 have appreciably lower peptidyltransferase activities and show less binding of an analogue of peptidyl-tRNA (Sims & Wild, 1976). The 47S particles have very low activities in both these assays. This may be because their overall conformation is aberrant, or, more directly, because they lack protein L16, which is involved in these functions, albeit to an arguable extent (Brimacombe *et al.*, 1976).

The present paper extends these functional comparisons. There is appreciable binding of a fragment of aminoacyl-tRNA to 47S particles, presumably to the acceptor ('A') site of the transferase centre. However, little binding of chloramphenicol occurs. Completed ribosomes of parent and mutant strains show some differences in binding of the fragment, but not of chloramphenicol. The relevance of these

results to ribosome function in strain 15-28 is discussed.

Experimental

Growth and harvesting of bacteria

The organisms were *E. coli* 15 *thy*[−] *pro*[−] (15TP), and the mutant, 15-28, derived from it (MacDonald *et al.*, 1967). Cultures were grown, bacteria harvested and extracts prepared with an Aminco–French pressure cell as described previously (Sims & Wild, 1976).

Preparation of ribosomes and 47S particles

For studies of fragment binding, 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). Clarified extracts were layered on sucrose gradients that were centrifuged, analysed and collected as described previously (Sims & Wild, 1976). Pooled fractions of ribosomes or 47S particles were precipitated with ethanol and resuspended (concn. 20 mg/ml) in activation buffer (5 mM-Tris/HCl/5 mM-MgCl₂/50 mM-NH₄Cl, pH 7.5) in which there was no loss of activity after 1 month at −20°C.

Ribosomes and 47S particles used in the experiments on chloramphenicol binding were prepared similarly but in buffers (pH 7.4) containing (i) 10 mM-Tris/HCl/10 mM-magnesium acetate/100 mM-KCl (THMK buffer) or (ii) 10 mM-Tris/HCl/2 mM-

magnesium acetate/100mM-KCl (TLMK buffer). After precipitation with ethanol, ribosomes or 47S particles were dissolved (concn. 10mg/ml) in chloramphenicol-binding buffer (33mM-Tris/HCl/13mM-magnesium acetate/270mM-KCl, pH 7.4) and could be stored at -20°C for at least 14 days without loss of activity.

Fragment-binding assay

The 'fragment' is CpApCpCpA- $[\text{}^3\text{H}]$ leucine, an analogue of aminoacyl-tRNA, and was prepared as described by Monro *et al.* (1968). Binding of the fragment to ribosomes or 47S particles was measured by the method previously used for acetylated fragment (Sims & Wild, 1976). After incubation at 0°C , the assay mixtures (150 μl) were centrifuged (10000g for 5 min) and the radioactivity in samples (100 μl) of the supernatant was determined (Sims & Wild, 1976). The blanks used in the assays contained 30S subunits from strain 15TP.

Chloramphenicol-binding assay

The binding of $[\text{}^{14}\text{C}]$ chloramphenicol to ribosomes or 47S particles was measured by ultrafiltration at $1-2^{\circ}\text{C}$ essentially as described by Fernandez-Muñoz *et al.* (1971). An eight-channel apparatus, similar to that of Paulus (1969), was fitted with Diaflo XM50 membranes. Ribonucleoproteins were diluted to a concn. of 5mg/ml with chloramphenicol-binding buffer and portions (100 μl) mixed with 10 μl of $[\text{}^{14}\text{C}]$ chloramphenicol (in the same buffer) to give a range of antibiotic concentrations (typically 0.5–10 μM). Mixtures were incubated for 30 min at 0°C . Samples (100 μl) of each were then loaded into the apparatus, pressure (approx. 0.34 MPa) was applied and maintained for 30 min. About 60 μl of filtrate collected under each membrane; two 20 μl samples were counted for radioactivity in Bray's (1960) scintillant to measure unbound chloramphenicol. The total chloramphenicol concentration and, by difference, the chloramphenicol bound to the ribonucleoproteins, was determined from blanks (made from 100 μl of chloramphenicol-binding buffer plus 10 μl of chloramphenicol solution) that were incubated and filtered as described above. The equilibrium did not alter appreciably during filtration because the radioactivity (c.p.m./ μl) of the filtrate changed by less than 3% as its volume increased from 15 to 80 μl .

Chemicals

L-[4,5- ^3H]Leucine (55Ci/mmol) and D-threo-[di-chloroacetyl-1- ^{14}C]chloramphenicol (7.9mCi/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Ribonuclease T₁ was from Calbiochem, Hereford, U.K.; tRNA (from *E. coli* strain B) was from Gibco-Bio-Cult, Paisley, Renfrewshire, Scotland, U.K. Diaflo XM50 ultrafiltration

membranes were from Amicon, High Wycombe, Bucks., U.K.

Results

Chloramphenicol binding

The 70S ribosomes of both strains were obtained from extracts prepared in and centrifuged through sucrose gradients containing THMK buffer. These ribosomes have not been depleted of univalent cations and so should be active (Miskin *et al.*, 1968). To confirm this, portions of a sample of 70S ribosomes from the parent strain were dialysed at 4°C against (a) chloramphenicol-binding buffer and (b) against this buffer but without KCl. After dialysis, KCl was restored to sample (b), and a portion (c) incubated for 20 min at 37°C . The binding of chloramphenicol (3.6 μM) was then measured: that bound in samples (b) and (c) was 13 and 95% respectively of that in (a). Thus ribosomes made in THMK buffer are fully active.

Measurement of the binding of different concentrations of chloramphenicol to the 70S ribosomes of either strain gave hyperbolic curves when antibiotic bound was plotted against the concentration of free chloramphenicol. These curves could be converted into straight lines by the double-reciprocal procedure of Klotz *et al.* (1946). From the results of a binding experiment, the median value of the dissociation constant (K_d) and the number of binding sites per ribonucleoprotein particle (n) were computed by the method of Eisenthal & Cornish-Bowden (1974). Table 1 lists the results of several separate experiments and shows that there are no significant differences between the strains. The value of K_d is about 3 μM and is similar to those obtained by other workers, which range from 0.6 μM (Fernandez-Muñoz *et al.*, 1971) to 3 μM (Contreras *et al.*, 1974).

Table 1. *Binding of chloramphenicol to 70S ribosomes*
The 70S ribosomes were prepared and assayed as described in the text. Median values of K_d and n were computed from the results of separate experiments.

Strain	Expt. no.	K_d (μM)	n
15TP	1	3.0	1.03
	2	3.3	0.84
	3	2.7	0.98
	4	3.5	0.88
	Mean ...	3.1	0.93
15-28	1	2.9	0.86
	2	2.6	0.84
	3	3.8	1.12
	4	2.6	0.77
	Mean ...	3.0	0.90

A comparison of chloramphenicol binding to the 50S subunits of both strains was also made. Results from the individual experiments again generated straight lines when plotted as double reciprocals. Computed values of K_d and n are listed in Table 2. The 50S subunits from the parent strain 15TP are most simply prepared by a single cycle of centrifugation through sucrose gradients containing TLMK buffer in which 70S ribosomes dissociate into their subunits. The 50S subunits thus obtained were fully active in an experiment similar to that described above for 70S ribosomes. Values of K_d and n were $4.1 \mu\text{M}$ and 0.96 respectively (Table 2a). An alternative procedure is to isolate 70S ribosomes from the parent strain in THMK buffer and then to dissociate these into subunits by a second centrifugation through TLMK buffer. The 50S subunits prepared in this way give (Table 2b) a not very different value of K_d ($3.4 \mu\text{M}$) for chloramphenicol binding, but the value of n (0.51) is appreciably less. The first procedure cannot be used to prepare 50S subunits from the mutant strain 15-28 (because of the presence of 47S particles in extracts): when made by the latter procedure, these 50S subunits bind chloramphenicol with values of K_d ($2.9 \mu\text{M}$) and n (0.49) not significantly different from those of the parent strain (Table 2c). The low values obtained for the number of binding sites after procedures involving two cycles of centrifuging can be ascribed to the 'extra' centrifuging rather than

exposure to THMK buffer. Table 2(d) shows that when 50S subunits of strain 15TP prepared in TLMK buffer are then re-centrifuged through this buffer, they bind chloramphenicol with an unaltered dissociation constant but a low value of n (0.60). Thus although the parameters for the binding of chloramphenicol to 50S subunits depend somewhat on experimental procedures, no significant differences between parent and mutant strains are apparent.

The binding of chloramphenicol to 47S particles was also examined. Fig. 1 shows that 47S particles bind much less chloramphenicol than do the 50S subunits from strain 15TP. This difference is even more marked when the non-specific binding to 30S subunits is taken into account. The accuracy of measurement of this low binding of antibiotic to 47S particles and 30S subunits was severely limited by the specific radioactivity of the chloramphenicol available. However, binding to 47S particles, when corrected for non-specific binding, was, over the concentration range tested, at most 15% of that to the 50S subunits.

Two possible explanations of the low binding activity of 47S particles were eliminated. The binding of $5.5 \mu\text{M}$ -chloramphenicol to either 47S particles or 70S ribosomes was unaffected when the time of

Table 2. *Binding of chloramphenicol to 50S ribosomal subunits*

The 50S subunits were prepared and assayed as described in the text. Median values of K_d and n were computed from the results of separate experiments.

Strain	Buffer used to prepare ribosomes (see the text)	Expt. no.	K_d (μM)	n
(a) 15TP	TLMK	1	3.9	1.14
		2	3.7	0.86
		3	4.6	0.87
		Mean ...	4.1	0.96
(b) 15TP	THMK+TLMK	1	3.4	0.50
		2	3.6	0.48
		3	3.9	0.57
		4	2.6	0.50
		Mean ...	3.4	0.51
(c) 15-28	THMK+TLMK	1	3.0	0.54
		2	2.9	0.46
		3	2.9	0.46
		Mean ...	2.9	0.49
(d) 15TP	TLMK+TLMK	1	4.2	0.69
		2	3.7	0.61
		3	2.4	0.51
		Mean ...	3.4	0.60

Fig. 1. *Binding of chloramphenicol to ribosomes and 47S particles*

The 50S ($\square$) and 30S (Δ) subunits from strain 15TP were prepared by a single cycle of centrifugation through TLMK buffer. The 47S particles from strain 15-28 ($\circ$) were prepared by a single cycle of centrifugation through THMK buffer. Chloramphenicol binding was assayed as described in the text. The final concentration of ribonucleoprotein was $1.8 \mu\text{M}$ (4.7 mg/ml for 50S subunits and 47S particles, 2.9 mg/ml for 30S subunits).

incubation at 0°C before the start of ultrafiltration was increased from 30min to 120min. Thus slow equilibration of chloramphenicol and 47S particles is not responsible for the poor binding. A second possibility was that 47S particles are particularly susceptible to inactivation, even in THMK buffer. Incubation at 37°C in chloramphenicol-binding buffer re-activates inactive 70S ribosomes. Samples of 47S particles (and 50S subunits from strain 15TP) were therefore incubated in this buffer for up to 40min without effect on their subsequent ability to bind the antibiotic.

Binding of fragment

The time course of binding of the CpApCpCpA-leucine fragment to ribosomes and 47S particles is shown in Fig. 2. Binding by the different ribonucleoproteins is 90% (or more) complete after 60min, so that this was subsequently taken as the standard incubation time in a series of measurements whose results are shown in Table 3. The following conclusions can be drawn. (i) The 47S particles from strain 15-28 consistently bind about 50% of the fragment bound by 50S subunits from strain 15TP. This result was rather surprising, in view of our earlier finding (Sims & Wild, 1976) that 47S particles bind acetylated P-site fragment poorly and have a peptidyltransferase activity only about 10% of that of 50S subunits from strain 15TP. Further experiments therefore measured binding at both A and P

Fig. 2. Binding of A-site fragment to ribosomes and 47S particles

The 70S (●) ribosomes and 50S (■) subunits from strain 15TP, 70S (○) ribosomes and 50S (□) subunits from strain 15-28 and 47S particles from the latter (△) were prepared and binding was assayed as described in the text. The final concentration of the ribonucleoproteins in all assays was 2.5mg/ml. The concentration of fragment was 10^4 c.p.m./assay (7.6nM). Radioactivity bound was measured on 100 µl samples from the assay mixtures.

Table 3. Binding of A-site fragment to 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. Binding of fragment was measured as c.p.m. bound/mg of ribonucleoprotein. In the Table, the binding to 50S subunits from strain 15TP is set at 100%; this corresponds to binding of about 1000 c.p.m./100 µl sample. The fragment concentration was 10^4 c.p.m./assay (7.6nM).

Expt. no.	Fragment bound (%)				
	15TP 50S	15-28 50S	15TP 70S	15-28 70S	15-28 47S
1	100	107	167	105	47
2	100	92	121	95	45
3	100	118	82	71	47
4	100	101	131	87	54
5	100	114	111	78	44
Mean ...	100	106	122	87	47

sites of the peptidyltransferase centre by using the same batch of 50S subunits or 47S particles. Equal concentrations of acetylated (P-site) or non-acetylated (A-site) fragment were added to separate portions of the same ribosome or 47S-particle preparation. In two experiments, 47S particles retained 44 and 47% of the amount of A-site fragment bound to 50S subunits and 17 and 15% of the acetylated (P-site) fragment. These measurements not only confirm our earlier observations (and those of Table 3), but emphasize the difference in binding of the two fragments to 47S particles. (ii) There is no clear difference in the binding of A-site fragment to the 50S subunits of either strain. However, the 50S subunits of the two strains were prepared rather differently. Extracts of strain 15TP were made in and centrifuged through TAM buffer to obtain 50S subunits directly, whereas, with strain 15-28, 70S ribosomes made in TAHM buffer were dissociated and centrifuged through TAM buffer to collect the subunits. When 50S subunits from strain 15TP were made by the procedure normally used for those of strain 15-28, their ability to bind fragment increased (in five experiments) to $119 \pm 8\%$ (s.d.) of that of 50S subunits prepared by the procedure used for Table 3. Thus the ability of 50S subunits from strain 15-28 to bind non-acetylated fragment may be slightly less than that of subunits from the parent strain, but even so the difference is much less marked than that noted in a comparison of binding of acetylated fragment to the peptidyl site of the 50S subunits of the two strains (Sims & Wild, 1976). (iii) The 70S ribosomes of the mutant bind appreciably less A-site fragment than do those from strain 15TP. (iv) The results in Table 3 are expressed per unit mass. When adjusted for the

different particle weights of 70S ribosomes and 50S subunits, the binding of fragment to a 70S ribosome from strain 15TP becomes 195% of that to its 50S subunit. The value for strain 15-28 is 132%. These increases, also noted by Celma *et al.* (1971), perhaps result from a conformational change induced by association with 30S subunits. Ribosomes from the mutant strain 15-28 may be less able to undergo this change than those from the parent.

Discussion

Previous work (Sims & Wild, 1976) showed that the 50S and 70S ribosomes of strain 15-28 have a lower biological activity, as monitored by peptidyl-transferase activity and the binding of acetylated fragment to the peptidyl site, even though their composition is apparently no different from those of the parent strain 15TP. The results above show that non-acetylated (A-site) fragment also binds less well to the 70S ribosomes of the mutant, but that the difference between the 50S subunits from the two strains is slight. The aminoacyl-binding site therefore seems less affected by the conformational difference between the 50S subunits of the two strains that we proposed as the explanation for their different activities in the assays above and in cell-free protein synthesis. This view is supported by our finding that the binding of chloramphenicol to the 70S ribosome and 50S subunit of the mutant has parameters that cannot be distinguished from those for binding to the ribosomes of the parent strain. Chloramphenicol binds to a single site on the larger subunit (Fernandez-Muñoz *et al.*, 1971), at or near the aminoacyl-binding site; the antibiotic can completely inhibit binding of the fragment UpApCpCpA-leucine (Celma *et al.*, 1971), and its own binding is completely inhibited by puromycin (Nierhaus & Nierhaus, 1973). However, the chloramphenicol and aminoacyl-binding sites cannot be completely equated, because chloramphenicol has little effect on the binding of the intact aminoacyl-tRNA molecules (Suarez & Nathans, 1965; Vazquez & Monroe, 1967). The binding site for the small antibiotic would be expected to be less extensive than the site for aminoacyl-tRNA (and its derivatives), and so might well be less affected by conformational changes in mutant ribosomes.

There is little binding of chloramphenicol to 47S particles, although these ribosome precursors bind the CpApCpCpA-leucine fragment to about half the extent of completed ribosomes. This finding also shows that the antibiotic and fragment do not have identical binding sites. Different approaches have implicated protein L16 in the binding of chloramphenicol (Nierhaus & Nierhaus, 1973; Pongs *et al.*, 1973). This protein is one of three missing from 47S particles, so that our results are consistent with this role. There is strong evidence that protein L16 is also

involved in peptidyltransferase activity (Moore *et al.*, 1975; Tate *et al.*, 1975), although a vital function for this protein has been disputed (Brimacombe *et al.*, 1976), and other proteins, for example L11, are probably involved (Nierhaus & Montejó, 1973; Dietrich *et al.*, 1974; Hsuing *et al.*, 1974). We (Sims & Wild, 1976) noted that although the absence of protein L16 from 47S particles could explain the virtual absence of peptidyltransferase activity in these precursor particles, it is difficult to exclude the possibility that the overall conformation of the particles is so markedly different from that of completed ribosomes that activity, although latent in the components present, is not expressed. The appreciable ability of 47S particles to bind the A-site fragment shows that in this region, at least, the conformation of 47S particles is not grossly abnormal, and makes it rather more likely that their low peptidyltransferase activity can be ascribed to the absence of vital proteins, of which L16 is the obvious candidate.

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