INTERACTION OF Ac-Phe-tRNA WITH *E. COLI* RIBOSOMAL SUBUNITS. 1. SPARSOMYCIN-INDUCED FORMATION OF A COMPLEX CONTAINING 50 S AND 30 S SUBUNITS BUT NOT mRNA

A.JIMENEZ, R.E.MONRO and D.VAZQUEZ
Instituto de Biologia Celular, Velazquez 144, Madrid-6, Spain

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1. Introduction

Sparsomycin inhibits protein synthesis by specifically blocking the ribosome-catalysed peptidyl transfer reaction [1–3]. In the conditions of the fragment reaction [4] the antibiotic stimulates binding of CCA-Leu-Ac to isolated 50 S subunits [5]. On the basis of experiments in this system [4–6] it was proposed [5] that sparsomycin induces the formation of an unreactive complex between the CCA-peptide moiety of the peptidyl donor substrate and the P-site on the peptidyl transferase centre. Since the presence of ethanol is obligatory in the fragment reaction system [4], confirmatory experiments were initiated in conditions closer to those of protein synthesis. We show here that sparsomycin induces complex formation in absence of ethanol, provided that intact donor substrate is employed. In such conditions both ribosomal subunits are required but mRNA is not necessary. These observations, while being fully consistent with the proposition that sparsomycin induces interaction between CCA-peptide and 50 S subunit, were unexpected in that they show that the 30 S subunit can act without mRNA. In this work we have used *N'*-acetyl-phenylalanyl-tRNA [7] as an analogue of peptidyl-tRNA [8]. The present paper gives a general characterization of sparsomycin-induced binding of Ac-Phe-tRNA to ribosomal subunits with and without mRNA, and shows in addition that amicetin and gougerotin also stimulate Ac-Phe-tRNA binding to ribosomes. The mRNA-independent action of 30 S subunits will be further considered in another paper [9]. A related study [10] reports the effect of complex formation on reactivity of Ac-Phe-tRNA towards hydroxylamine. Part of this work has already been briefly reported [11]. Herner, Goldberg and Cohen [12] have also observed the stimulation of Ac-Phe-tRNA binding to subunits by sparsomycin and gougerotin, but they did not examine the effect of omitting mRNA. Another difference is that their experiments were carried out in presence of initiation factors and GTP, whereas the present system contained only ribosomes and salts (with and without poly U).

2. Materials and methods

2.1. *Ribosomes and ribosomal subunits*

Ribosomes were prepared from log phase *E. coli* MRE600 and washed once by centrifugation through a layer of solution containing 20% (w/v) sucrose, 10 mM tris buffer (pH 7.4), 0.5 M NH_4Cl, and 10 mM Mg acetate [13]. 50 S and 30 S subunits were prepared from the washed ribosomes by a method similar to that of Staehelin [13] and of Moore et al. [14], based on dissociation into subunits and zonal centrifugation through a sucrose gradient containing 10 mM tris buffer (pH 7.4), 0.1 mM NH_4 acetate, and 1 mM Mg acetate. The ribosomes and subunits were stored in small batches at 0° in the same buffer, but with 10 mM Mg acetate. Cross contamination of 50 S and 30 S subunit preparations was always less than 10% as judged by analytical centrifugation and assay of poly U-directed polyphenylalanine synthesis.

2.2. *N'-acetyl-^{14}C-phenylalanyl-tRNA*

tRNA (from *E. coli* B) was charged with ^{14}C-phenylalanine (456 mCi/mmole) as described elsewhere [15], and the product was acetylated with acetic anhydride [7].

2.3. *Binding assay*

The assay for binding of Ac-^{14}C-Phe-tRNA to ribosomes was essentially as described by Lucas-Lenard and Lipmann [8]. The incubation mixture (0.2 ml/tube) contained 54 mM tris buffer (pH 7.4), 10 mM Mg acetate, 160 mM NH_4Cl, 10 mM dithiothreitol, Ac-^{14}C-Phe-tRNA (about 456 Ci/mole), and ribosomes or their subunits; 0.1 mM sparsomycin and 0.1 mg/ml poly U were added where indicated. The reaction was initiated by addition of Ac-Phe-tRNA and, after incubation for the indicated time at 30°, stopped by addition of 3 ml of "washing buffer" (at 0°) containing 54 mM tris buffer (pH 7.4), 10 mM Mg acetate and 160 mM NH_4Cl. The ribosome-bound radioactivity was determined by the method of Nirenberg and Leder [16].

2.4. *Sources of materials*

Frozen *E. coli* MRE600 cells: Microbiological Research Station, Porton, Wilts., England; tRNA: General Biochemicals; ^{14}C-phenylalanine and ^{32}P-tRNA: The Radiochemical Centre, Amersham, England; poly U: Miles Chemical Corporation; sparsomycin: Cancer Chemotherapy National Service, NC1, Bethesda, Maryland, U.S.A. other antibiotics: see ref. [17].

3. Results

3.1. *Time course of complex formation*

Effects of sparsomycin and poly U on the binding of Ac-Phe-tRNA to ribosomes are illustrated in fig. 1. Sparsomycin stimulated the binding both in presence and absence of poly U. With sparsomycin and poly U together binding reached equilibrium within 16 min at 30°, when about 80% of the added Ac-Phe-tRNA was bound. With sparsomycin alone binding was considerably slower but reached nearly the same extent after 90 min. With poly U alone binding was rapid but reached equilibrium when only about 50% of the added Ac-Phe-tRNA was bound. In the absence of both sparsomycin and poly U less than 5% of the added substrate was bound. The extents and rates of binding varied with different preparations of ribosomes and subunits, but in all experiments the responses were qualitatively the same as in fig. 1. Together with the evidence that sparsomycin action does not lead to any change in the covalent structure of the substrate [5], these results indicate that sparsomycin induces the formation of a stable complex between Ac-Phe-tRNA and the ribosome both in presence and absence of poly U. The relative extents of binding indicate that the complex induced by sparsomycin, both in presence and absence of poly U, is more stable than the complex induced by poly U alone. The difference in rate of Ac-Phe-tRNA binding in presence and absence of poly U can be explained on a kinetic basis (see Discussion). It might be thought that complex formation in absence of poly U involves natural mRNA. However, such a possibility is eliminated (i) by the observation (see below) that complex formation can take place with purified ribosomal subunits (which should contain very little mRNA) and (ii) by experiments with inhibitors which interfere with the interaction of mRNA with 30 S subunits and tRNA [9]. We conclude

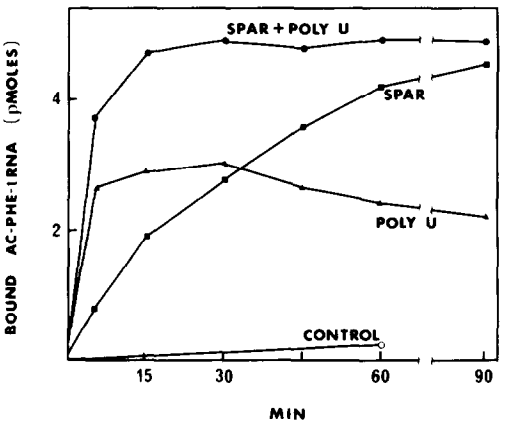

Fig. 1. Time course of Ac-Phe-tRNA binding to ribosomes: effects of sparsomycin and poly U. Incubation mixtures (0.1 ml) contained 6 pmoles Ac-^{14}C-Phe-tRNA, 3 mg/ml ribosomes, NH_4^+, Mg^{2+}, and 0.1 mM sparsomycin or 0.2 mg/ml poly U as indicated. Incubation was at 30°. Details of the assay conditions and procedure are given in the text (Section 2.3). Zero time blanks have been substracted: these were about 0.3 pmoles in samples with poly U and about 0.1 pmole in samples without poly U. •, sparsomycin plus poly U; ■, sparsomycin; ▲, poly U; ○, no addition.

that sparsomycin induces interaction of Ac-Phe-tRNA with ribosomes both in presence and absence of mRNA.

3.2. *Requirements for complex formation and maintenance*

Table 1 shows that binding of Ac-Phe-tRNA to ribosomes in absence of mRNA and alcohol was dependent upon sparsomycin, NH_4^+, Mg^{2+}, and both ribosomal subunits. Sparsomycin had little or no effect on Ac-Phe-tRNA interaction with isolated 50 S or 30 S subunits. Sparsomycin did not stimulate binding of non-acetylated Phe-tRNA or (in other experiments) of uncharged ^{32}P-tRNA to ribosomes either in presence or absence of poly U.

Table 1
Binding of Ac-Phe-tRNA to ribosomes without poly U: requirements.

Conditions	Bound Ac-Phe-tRNA (pmoles)	
	Minus SPAR	Plus SPAR
Expt 1 (ribosomes)		
Standard system	0.18	3.02
minus NH_4^+		0.12
minus Mg^{2+}		0.08
Expt 2 (subunits)		
50 S plus 30 S subunits	0.52	1.78
50 S	0.12	0.18
30 S	0.04	0.06
Expt 3 (subunits; Ac-Phe-tRNA replaced by Phe-tRNA)		
Standard system	0.04	0.12
plus poly U	1.62	2.08

Conditions were as in fig. 1 and text (Section 2.3). Poly U was not added unless indicated. Unfractionated ribosomes (3 mg/ml) were used in Expt 1, while purified 50 S (1 mg/ml) and 30 S (0.5 mg/ml) ribosomal subunits were used in Expts 2 and 3. Each tube contained a total of 9 pmoles of Ac-Phe-tRNA or, in Expt 3, of Phe-tRNA. Incubation was at 30° for 40 min in Expt 1 and for 30 min in Expts 2 and 3.

Table 2 shows that complex formation was inhibited by a number of peptidyl transferase-specific antibiotics. Their relative activities are similar to those observed in the fragment reaction system, both as regards reaction with puromycin [3] and interaction with 50 S subunits in presence of sparsomycin [5]. The incompleteness of the inhibition suggests that interaction at

Table 2
Sparsomycin-induced binding of Ac-Phe-tRNA to ribosomes: effects of peptidyl transferase inhibitors.

Addition	Concn. (mM)	Bound Ac-Phe-tRNA (% of control)
None	1	100
Chloramphenicol	1	62
Lincomycin	1	89
Carbomycin	0.1	22
Spiramycin III	0.1	33
Neospiramycin III	1	105
Streptogramin A	0.1	12
Amicetin	1	86
Gougerotin	1	95
Erythromycin	1	95

Conditions were as in text (Section 2.3) with 0.75 mg/ml 50 S subunits, 0.28 mg/ml 30 S subunits, 8 pmoles/tube Ac-Phe-tRNA, 0.1 mM sparsomycin, and the indicated additions. Poly U was not added. Incubation was for 30 min at 30°. 0.6 pmoles of Ac-Phe-tRNA were bound in the control. The data in the tube were not corrected for blanks without sparsomycin. Such blanks were 0.03 pmoles or less, except in samples with amicetin or gougerotin, which gave values in the range of 0.07 pmoles. Both the controls with sparsomycin and the blanks without sparsomycin gave less binding than in the other experiments in this paper owing to the use of a different preparation of ribosomal subunits.

Table 3
Stability of the sparsomycin-induced complex: effects of removing NH_4^+ or Mg^{2+} after formation of the complex.

Washing solution	Bound Ac-Phe-tRNA (pmoles)
Standard	4.15
minus NH_4^+	4.20
minus Mg^{2+}	0.02

Ac-^{14}C-Phe-tRNA was bound to ribosomes by incubation with sparsomycin in absence of poly U. The conditions were as in fig. 1, with a total of 9 pmoles Ac-Phe-tRNA per tube. Incubation was for 40 min at 30°. After incubation, 3 ml of cold, "washing buffer" (Section 2.3), or the same with omissions as indicated, were added and the samples were filtered. The filters were washed twice with the same buffers and the remaining radioactivity determined.

the peptidyl transferase centre was not the rate-limiting step of complex formation. In other conditions Herner et al. [12] also observed inhibition of Ac-Phe tRNA binding by chloramphenicol, and the extent of inhibition was greater than obtained here.

Table 3 shows that maintenance of the complex, after its formation, required Mg^{2+} but not NH_4^+. This contrasts with formation of the complex, which requires NH_4^+ in addition to Mg^{2+}.

3.3. *Centrifugal analysis of complex*

Ac-^{14}C-Phe-tRNA was incubated with ribosomal subunits (without poly U) in presence and absence of sparsomycin, and the resultant mixtures analysed by zonal centrifugation through sucrose gradients. In the ionic conditions employed the subunits partially reassociate, so that the OD_{260} profiles had peaks corresponding to 50 S – 30 S couples as well as to free 50 S and 30 S subunits. Fig. 2 shows that all of the radioactivity remained at the top of the gradient when the sample incubated without sparsomycin was centrifuged. The sample with sparsomycin contained a new radioactive component which migrated slightly faster than the 50 S – 30 S couples. (There was little increase in OD_{260} corresponding to formation of the radioactive peak, because on a molar basis the bound Ac-Phe-tRNA corresponded to less than 2% of the total ribosome population.) There were no radioactive peaks in the regions of the 50 S or 30 S subunits, although there was a trail of radioactivity near the top of the tube, which presumably resulted from the breakdown of less stable complexes. These results together with the evidence that both ribosomal subunits are required, indicate that the sparsomycin-induced complex contains Ac-Phe-tRNA plus one 50 S subunit and one 30 S subunit. The fact that the complex has a slightly greater mobility than the simple 50 S – 30 S couples suggests

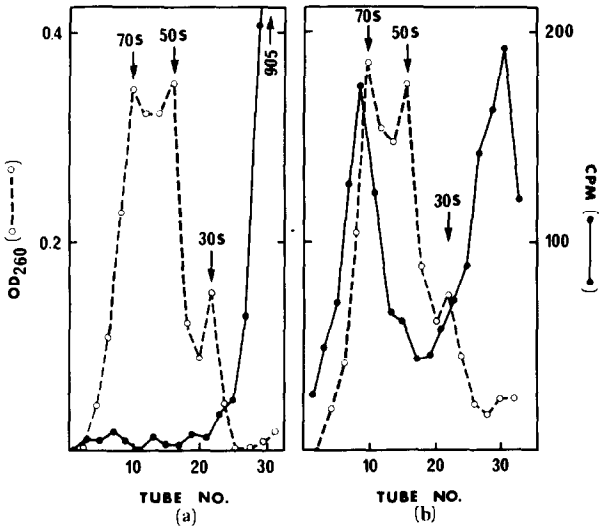

Fig. 2. Zonal centrifugation of ribosomal subunits incubated with Ac-^{14}C-Phe-tRNA (a) alone and (b) with 0.1 mM sparsomycin. In neither case was poly U added. Conditions of incubation were as in Section 2.3 with 3 mg/ml of 50 S and 1.5 mg/ml of 30 S subunits. The samples were incubated for 40 min at 30°, cooled, and then 80 μl were layered onto a 5 ml 20–5% linear sucrose gradient with an ionic composition identical to that of the incubation mixture. The gradient in which the sample containing sparsomycin was to be centrifuged had a final concentration of 10 μM sparsomycin, to avoid possible dissociated of the complex. Centrifugation was in a Beckman SW-50 rotor at 49,000 rpm for 70 min. Alternate fractions (150 μl) were collected (i) in 3 ml water for OD_{260} determination and (ii) in vials for determination of radioactivity. The latter samples were mixed with 3 ml Bray's solution [18] containing 4% CAB-O-Sil (Nuclear Chicago), and the radioactivity determined in a scintillation spectrometer.

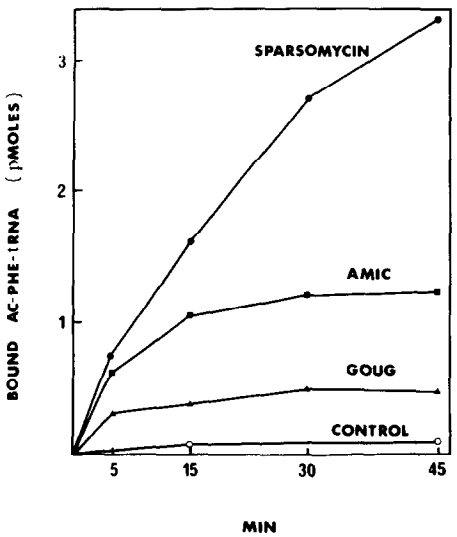

Fig. 3. Time course of Ac-Phe-tRNA binding to ribosomes without poly U, in presence of: •, 0.1 mM, sparsomycin; ■, 1 mM amicetin; ▲, 1 mM gougerotin; ○, no addition. The assay was carried out as in fig. 1. Each tube contained a total of 9 pmoles of Ac-Phe-tRNA.

that it is in a relatively compact form and that there is a strong and specific interaction of 30 S subunits with the 50 S...Ac-Phe-tRNA...sparsomycin complex even in absence of mRNA.

3.4. *Amicetin and gougerotin*

These two antibiotics are similar to sparsomycin in that they inhibit peptidyl transfer and are active against both 70 S and 80 S ribosomes. Fig. 3 shows that amicetin and gougerotin stimulate the binding of Ac-Phe-tRNA to ribosomes in the present conditions. They are active in the order: sparsomycin > amicetin > gougerotin. Gougerotin is only weakly active. Other results indicate that amicetin and gougerotin stimulate the ethanol-dependent binding of CACCA-Leu-Ac to silated 50 S subunits: the stimulation is weak at 33% (v/v) ethanol [5] but strong at 50% (v/v) ethanol [6]. At 33% ethanol they partially reverse the sparsomycin-induced binding of CACCA-Leu-Ac to ribosomes [5]. Herner et al. [12] have observed a partial inhibition by gougerotin of sparsomycin-induced Ac-Phe-tRNA. binding. These various lines of evidence suggest that the sites of action of sparsomycin, amicetin and gougerotin are all in the same region on the peptidyl transferase centre, and that their modes of action are closely related to one another. However, a possible difference in action is indicated by the observation that amicetin, but not sparsomycin or gougerotin, weakly stimulated the mRNA-directed binding of non-acylated aminoacyl-tRNA to ribosomes [17].

4. Discussion

The effects of sparsomycin on Ac-Phe-tRNA binding to the ribosome are similar to those on CCA-Leu-Ac binding [5], and support the proposition that the antibiotic induces formation of an inert complex between the CCA-peptide moiety of the peptidyl donor substrate and the peptidyl transferase centre on the 50 S subunit. In particular the activity of Ac-Phe-tRNA but not of Phe-tRNA suggests that sparsomycin affects the peptidyl donor rather than peptidyl acceptor substrate, while the inhibition by peptidyl transferase-specific antibiotics indicates involvement of the peptidyl transferase centre on the 50 S subunit.

Sparsomycin-induced binding of CCA-Leu-Ac requires ethanol and can take place with isolated 50 S subunits [5]. In contrast sparsomycin-induced binding of Ac-Phe-tRNA does not require alcohol but does require ribosomal subunits (when ethanol is absent). The difference between the two systems is in accord with the concept that interaction of the peptidyl donor substrate with the peptidyl transferase centre normally requires 30 S subunits and mRNA and the anticodon loop of the substrate, but that alcohol promotes the interaction in absence of these components [4, 6]. The observation that 30 S subunits can act without mRNA was unexpected. This phenomenon and its bearing on the structure and function of 30 S subunits is further considered in another paper [9].

The observation that Ac-Phe-tRNA binding to ribosomes is much slower when induced by sparsomycin alone than when poly U is present (with or without sparsomycin) is presumably a reflection of the stability of the intermediates involved. It is reasonable to suppose that in presence of poly U, a 50 S... poly U complex is first formed and that to it are added successively Ac-Phe-tRNA, 50 S subunits and sparsomycin (50 S subunits might be added at an earlier stage). Each of the intermediates is reasonably stable and has been characterized. In contrast, none of the intermediates formed in absence of poly U is stable enough to have been characterized, and their concentrations must be so low as to be severely rate-limiting.

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