SUBSTRATE AND ANTIBIOTIC BINDING SITES AT THE PEPTIDYL TRANSFERASE CENTRE OF *E. COLI* RIBOSOMES: BINDING OF UACCA-LEU TO 50 S SUBUNITS

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1. Introduction

In the past years we have been studying the peptidyl transferase centre of the larger ribosomal subunit [1–3 review]. This centre is responsible for the catalysis of peptide bond formation in protein synthesis. The peptidyl transfer reaction presumably takes place in several steps, amongst which are the binding of the peptidyl donor and acceptor substrates at the P- and the A-site on the peptidyl transfer centre. There is ample evidence that only the terminal moieties of tRNA interact with the centre, and we have been developing a system to resolve the binding steps using simple substrates containing the CpCpA oligonucleotides of tRNA. We have already reported a system for study of substrate-interaction at the P-site of the peptidyl transferase centre, based on the measurement of CACCA–Leu–Ac binding to 50 S ribosomal subunits in the presence of ethanol. Using this system, substrate-binding at the P-site was shown to be inhibited by a number of antibiotic inhibitors of peptidyl transfer but unaffected or even stimulated by others [4, 5]. The present work deals with a complementary assay for substrate-binding at the A-site. The assay is similar to that for substrate-binding at the P-site but uses UACCA–Leu (or CACCA–Leu) instead of CACCA–Leu–Ac. The present results, together with those of another study [6, 7], suggest that certain antibiotic inhibitors of peptidyl transfer act on both the A- and the P-site of the peptidyl transferase centre, others act only on one of the sites, and yet others act at neither site. A preliminary report of this work has already been published [8].

2. Materials and methods

2.1. *Materials*

Ribosomes and ribosomal subunits were prepared from log phase *E. coli* MRE 600 by the method of Staehelin [10]. Cross contamination of ribosome subunits was less than 5%. Sources of antibiotics were as previously indicated [11, 5]. CACCA–^{3}H-Leu was prepared essentially as described elsewhere [9].

2.2. *Assay of UACCA–^{3}H-Leu binding*

The standard incubation mixture contained (unless otherwise indicated) UACCA–^{3}H-Leu (*c* 1–2 nM; specific activity *c* 20 Ci/mmole), 3–5 mg/ml 50 S ribosomal subunits, 13 mM Mg acetate, 270 mM KCl, 33 mM tris-HCl (pH 7.4) and 50% (v/v) ethanol. 150 μl aliquots were incubated at 0° for 5–20 min and then centrifuged at 35,000 *g* for 5 min at 4°. 100 μl of the supernatant was mixed with 3 ml scintillation fluid (Bray's solution [12] with 4% CAB-O-Sil) and radioactivity estimated in a scintillation spectrometer. Parallel incubations without ribosomes were included for estimation of total radioactivity under identical quenching conditions. The amount of bound substrate was calculated by difference.

3. Results

3.1. *UACCA–Leu binding to ribosomes and their subunits*

In the experiments shown in table 1, up to 69%, 40% and 10% of the added UACCA–Leu was bound to 70 S ribosomes, 50 S subunits and 30 S subunits,

Table 1
Binding of UACCA–Leu to ribosomes and their subunits: effect of chloramphenicol.

Ribosomes or subunits	Percentage of added UACCA–Leu bound		
	No antibiotic	Plus 1 mM chloramphenicol	Difference
Exp. 1			
70 S	69	12	57
50 S	33	6	27
30 S	10	13	−3
Exp. 2			
50 S	40	0	40

3 mg/ml ribosomes or subunits were used. Time of incubation was 20 min. Other conditions and method of assay were as in text (2.3).

respectively, in presence of 50% (v/v) ethanol. The extent of binding varied with different ribosome preparations. Addition of 1 mM chloramphenicol lowered the binding to 0–12% with 70 S ribosomes or 50 S subunits but had no effect on binding with 30 S subunits. We conclude that the majority of the UACCA–Leu binding to 70 S ribosomes and 50 S subunits took place at a chloramphenicol-sensitive site. The remainder of the binding to these particles, as well as the binding to 30 S subunits, was presumably due to interaction at other sites. Since chloramphenicol acts on the peptidyl transferase centre [1, 3 review], it is reasonable to suppose that the chloramphenicol-sensitive binding represents interaction of UACCA–Leu at a site on the peptidyl transferase centre.

Table 2
Binding of UACCA–Leu to ribosomes and their subunits; effect of ethanol concentration.

Ribosomes or subunits	Percentage of added UACCA–Leu bound	
	33% (v/v) ethanol	50% (v/v) ethanol
70 S	44	57
50 S	16	27

3 mg/ml 70 S ribosomes or 50 S subunits were used. Time of incubation was 20 min. Other conditions and method of assay were as in text. Figures for percentage binding have been corrected for non-specific binding by subtraction of estimates obtained in parallel incubations with 1 mM chloramphenicol (for explanation see text).

There is no detectable binding of UACCA–Leu to 50 S subunits under normal ionic conditions in absence of alcohol. Table 2 shows that in presence of 33% ethanol (as used in the 'fragment reaction' for assay of the peptidyl transfer reaction [1] there is a weak but significant binding of UACCA–Leu to 50 S subunits at a chloramphenicol-sensitive site. Binding is more effective at 50% ethanol. 70 S ribosomes are more than twice as active as 50 S subunits in binding UACCA–Leu, both at 33% and 50% ethanol. The concentration of alcohol used in our standard assay is 50% (v/v).

3.2. *Characteristics of the complex*

The interaction between UACCA–Leu and 50 S subunits takes place rapidly, equilibrium being attained in less than 5 min at 0°. In contrast, binding of UACCA–Leu to 70 S ribosomes is relatively slow, and completion requires 20–60 min (fig. 1). With both 50 S subunits and 70 S ribosomes, the complex can be completely dissociated by resuspension of the ribosomes in buffer without ethanol. The binding is also reversed by chloramphenicol.

In order to characterize the products, UACCA–^{3}H-Leu was bound to 50 S subunits in the standard system. The complex was then isolated and dissociated by resuspension in buffer without ethanol, and the eluate characterized by paper ionphoresis at pH 6.5. About 90% of the radioactivity migrated as a single band at the same rate as a UACCA–Leu marker. The possibility remained that condensation takes place during the binding process with the formation of UACCA–$(Leu)_2$, which would migrate at about the same rate as UACCA–Leu. Such a possibility was eliminated by subjection of similar eluates to alkaline hydrolysis followed by chromatography on paper, using a butanol/acetic acid solvent. The product migrated at the same rate as Leu, and less than 10% of the radioactivity was coincident with a Leu–Leu marker. We conclude that the complex consists primarily of UACCA–Leu, bound loosely by non-covalent bonds to a chloramphenicol-sensitive site presumably on the peptidyl transferase centre.

3.3. *Effects of antibiotics*

Table 3 shows effects of a number of peptidyl

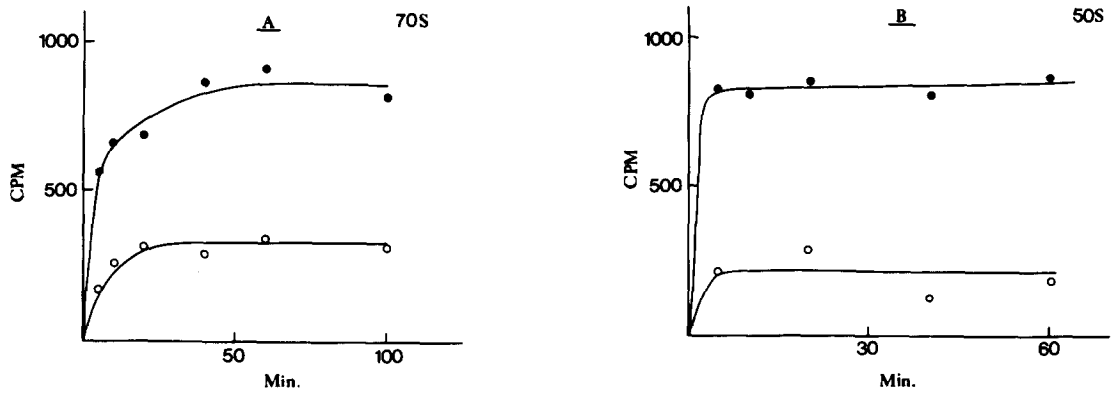

Fig. 1. Binding of UACCA–Leu to (A) 70 S ribosomes or (B) 50 S ribosomal subunits. 3 mg/ml of 70 S ribosomes or 50 S subunits were used and samples of incubation mixtures were taken at the indicated times. Other experimental conditions were as in Materials and methods. •: Binding of UACCA–Leu in the control; ○: binding of UACCA–Leu in the presence of chloramphenicol.

transferase inhibitors on the binding of UACCA–Leu to 50 S subunits. For comparison, effects of the same inhibitors on the binding of CACCA–Leu–Ac to 50 S subunits are reproduced from a previous publication [5]. Chloramphenicol strongly inhibited binding of the Leu substrate but stimulated binding of the Ac–Leu substrate. Lincomycin strongly inhibited binding of the Leu substrate and considerably less effectively inhibited binding of the Ac–Leu substrate. Streptogramin A, carbomycin and spiramycin III inhibited binding of both substrates. Oleandomycin weakly inhibited binding of the Leu substrate but weakly stimulated binding of the Ac–Leu substrate. Erythromycin, celesticetin and viridogrisein weakly stimulated the binding of both substrates. Other assays show that the binding of CACCA–Leu responds of these antibiotics in the same way as that of UACCA–Leu. The concentrations of antibiotics employed in these assays were sufficient to give nearly complete inhibition of the fragment reaction (except

Table 3
Binding of UACCA–Leu to 50 S ribosomal subunits: effects of antibiotics.

Antibiotic	Concn. (mM)	Binding of:	
		UACCA–Leu (% of control)	*CACCA–Leu–Ac (% of control)
Chloramphenicol	1	0	126
Lincomycin	1	4	36
Streptogramin A	0.1	11	8
Carbomycin	1	10	12
Spiramycin III	1	24	12
Neospiramycin III	1	54	
Oleandomycin	1	64	125
Erythromycin	1	106	156
Celesticetin	1	120	123
Viridogrisein	1	120	160

Assays were carried out as indicated in the text. Edeine (2 μM), polydextran sulphate (5 μM), tetracycline (0.1 mM), anisomycin (0.1 mM) and siomycin (0.1 mM) were without significant effect.
* Data reproduced from ref. 5.

in the cases of erythromycin and oleandomycin) [13]. Similar effects of the antibiotics were observed using 70 S ribosome preparations. However, in this case celesticetin and viridogrisein had no significant effect on binding of UACCA–Leu at early times of incubation (at 50% ethanol).

4. Discussion

The existence of specific binding sites on the peptidyl transferase centre for the peptidyl donor and acceptor substrates is suggested not only by a priori considerations but also on experimental grounds. Thus, studies on the peptidyl transfer reaction with model compounds show that there is specificity towards the terminal C and A of the tRNA in aminoacyl-tRNA [14, 15] as well as a requirement for the correct linkage between the aminoacyl group and the A [16]. Studies on the fragment reaction show that for peptidyl donor substrates to be active there must be at least three nucleotides present, but that the presence of additional nucleotides does not affect activity [9]. The simplest explanation for such requirements is that there are specific binding sites for the termini of the peptidyl donor and acceptor substrates on the peptidyl transferase centre. Further evidence for a specific binding site in the case of the peptidyl donor substrate is provided by the demonstration that there is just one site available per 50 S subunit for binding of CACCA–Leu–Ac [1].

It is reasonable to suppose that CACCA–Leu–Ac would tend to bind at the P-site, while UACCA–Leu would tend to bind at the A-site. Thus, the former but not the latter is a good peptidyl donor substrate [9], while the latter but not the former is a peptidyl acceptor substrate. Our observations on the differential responses to antibiotics of the binding of these two substrates give us confidence that they do, in fact, bind at distinct sites in our assay systems, and that the two types of binding reflect interaction at the P-site and the A-site.

An assay for the study of fragment binding to ribosomes at the A-site has also been developed by Pestka [6, 7]. He reports that the binding of CACCA–Phe can occur in absence of alcohol if 70 S ribosomes (but not 50 S subunits) are employed. The system thus has the advantage over ours that it avoids the use of alcohol. On the other hand, the requirement for 70 S ribosomes, which has yet to be explained, lowers the resolution of the assay since binding might be affected not only by factors which directly influence the peptidyl transferase centre but also by factors which influence the interaction between 50 S and 30 S subunits. Moreover, the binding to 70 S ribosomes takes place slowly (and is relatively stable), in contrast to the binding to 50 S subunits (in presence of alcohol) which is rapid (and relatively weak) as is to be expected for a simple reversible binding reaction. It thus appears that the binding to 70 S ribosomes involves some secondary reaction, such as a conformational change of the ribosome. Although the present system and that of Pestka are both subject to limitations, the limitations are of different kinds, and the systems are thus complementary to one another. The responses of Pestka's system to antibiotics [7] are very similar to those reported here (for UACCA–Leu) and thus give confidence that the systems are not artefactual.

Inhibition of fragment-binding by an antibiotic does not necessarily imply that substrate and antibiotic compete directly for binding at overlapping sites. The substrate and antibiotic binding sites might be spatially-separated but allosterically-linked. We are inclined to think that the allostery model is closer to reality in cases where inhibition is only partial, such as the action of lincomycin on the P-site and of spiramycin III on the A-site. The allosteric model could also hold in cases of nearly complete inhibition. Thus, studies with puromycin suggest that lincomycin and chloramphenicol do not bind directly at the A-site [17] even though they effectively inhibit UACCA–Leu binding (table 3) and CACCA–Phe binding [7]. The failure of celesticetin to inhibit binding of either substrate is of particular interest, in view of the inhibitory action of celesticetin on the fragment reaction and the close relation of its structure to that of lincomycin (which is a good inhibitor of fragment binding). Celesticetin must clearly inhibit the peptidyl transfer reaction by some mechanism other than blockage of substrate binding.

Regardless of which models of antibiotic action are correct, there can be little doubt that the peptidyl transferase inhibitors in table 3 have a variety of effects on substrate-binding, including inhibition of substrate-binding at both, one, or neither site, and

stimulation of substrate-binding. Such a variety of effects is intriguing in view of the knowledge that these peptidyl transferase inhibitors all bind to the 50 S subunit at mutually exclusive sites [3: review; 8, 17].

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