AMINOACYL-tRNA BINDING SITES IN *E. COLI* AND RETICULOCYTE RIBOSOMES

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1. Introduction

The simplest and most commonly accepted ribosome model postulates the presence of only two distinct sites for tRNA binding, one donor (D) and one acceptor (A) site [1]. During chain elongation the new aminoacyl-tRNA enters the A-site near the D-site already carrying the growing peptide chain bonded to another tRNA molecule (peptidyl-tRNA). The growing peptide chain is then transferred to the entering aminoacyl-tRNA by the action of peptidyl-transferase, with the formation of a new peptide bond. By an energy-requiring enzymatic process, the new peptidyl-tRNA is translocated from the A-site to the D-site, and a new cycle starts again.

Experimental data supporting this model for *E. coli* ribosomes have been provided by Igarashi and Kaji [2], and by Roufa et al. [3].

Other authors, however, have obtained evidence indicating the presence of more than two aminoacyl-tRNA binding sites [4]. They observed that saturation curves of phenylalanyl-tRNA binding to *E. coli* ribosomes display three consecutive slopes of decreasing steepness. These and similar results led the authors to postulate the presence, beside the donor site, of three decoding sites, one of which functions as the acceptor site.

In the rabbit reticulocyte system data have been presented in favor both of the two-site model [5, 6], and of the three-site model [7]. The latter follows essentially the same chain elongation mechanism, but in addition to the D and A sites it postulates a third site E. This site would be the first that the new aminoacyl-tRNA enters before being enzymatically activated and then translocated to the A-site, as soon as the A-site has been freed from the previous aminoacyl-tRNA.

We tested the two alternative models by an experiment which takes advantage of sparsomycin's mechanism of action [8]. The results support the two-site model for both *E. coli* and rabbit reticulocyte ribosomes.

2. Materials and methods

2.1. Materials

[^{14}C]Phenylalanine was purchased from Schwarz Bioresearch. *E. coli* stripped tRNA (General Biochemicals) was charged with [^{14}C]phenylalanine as described by Conway [9]. GTP was obtained from Pabst, Sparsomycin from Upjohn, dithiothreitol (DTT) from Calbiochemicals, calcium phosphate gel from Sigma, DEAE-cellulose from Whatman and DEAE-Sephadex and Sephadex G-200 from Pharmacia.

2.2. Methods

2.2.1. Preparation of ribosomes from rabbit reticulocytes and from *E. coli*

Rabbit reticulocyte ribosomes were isolated and purified according to the procedure described elsewhere [10]. *E. coli* ribosomes were isolated from *E. coli* MRE 600 as described by Nishizuka and Lipmann [11].

2.2.2. Preparation of elongation factors from rat liver

The elongation factors T_1 and T_2 were purified from post-microsomal supernatant of male Wistar rat livers according to the procedure previously described [10].

2.2.3. Preparation of elongation factors from *E. coli*

The elongation factors were isolated from *E. coli* (MRE 600 strain) post-ribosomal supernatant. The fraction of a S150 preparation precipitating between 40 and 70% saturation with ammonium sulphate, after dialysis, was fractionated on DEAE-Sephadex column as described by Felicetti et al. [12].

2.2.4. Analysis of ribosome-bound phenylalanine polymerization products

The polymerization mixture, with bacterial ribosomes, was the following: 1.0 A_{260} units/ml of *E. coli* ribosomes, 50 μg/ml of T factor, 12 μg/ml of G factor, 200 μg/ml of poly-U, 0.4 mg/ml of [^{14}C]phenylalanyl-tRNA (420 counts/min/pmol), 0.25 mM sparsomycin, 50 mM Tris-HCl pH 7.5, 160 mM NH_4Cl, 10 mM Mg acetate, 4 mM dithiothreitol, 1 mM GTP.

The mixture, with reticulocyte ribosomes, contained: 0.8 A_{260} units/ml of reticulocyte ribosomes, 100 μg/ml of poly-U, 12 μg/ml of T_1 factor, 10 μg/ml of T_2 factor, 0.4 mg/ml of [^{14}C]phenylalanyl-tRNA (330 counts/min/pmol), 100 μg/ml of sparsomycin in 50 mM Tris-HCl pH 7.5, 80 mM KCl, 10 mM $MgCl_2$, 2 mM DTT, 0.05 mM GTP.

The mixture was incubated at 37°C for 10 min and then 2-ml samples were layered onto 3 ml of a solution containing 10% sucrose dissolved either in 50 mM Tris-HCl pH 7.5, 80 mM KCl, 10 mM Mg acetate for the reticulocyte system or in 50 mM Tris-HCl pH 7.5, 10 mM Mg acetate, 160 mM NH_4Cl for the *E. coli* system and centrifuged in a Spinco SW39 rotor at 38 000 rpm for 16 hr. The ribosomal pellets were hydrolyzed with 0.3 M KOH for 16 hr at 37°C and aliquots of the hydrolysate were analysed by descending chromatography in *n*-butanol NH_4OH as described by Felicetti and Lipmann [13]. At the end of the run, chromatograms were dried, cut into 1 cm sections and counted in a PPO–toluene scintillating system. Recovery of radioactivity in the paper sections was always above 90%.

3. Results and discussion

Sparsomycin is an inhibitor of protein synthesis in both the 80 S and 70 S ribosome systems [14, 15]. It acts by blocking peptide-bond formation [16–20]. However, if the aminoacyl-tRNA in the donor site contains a free α-amino group, the inhibition cannot occur [8]. Accordingly, sparsomycin does not inhibit peptide bond formation between two aminoacyl-tRNA's, both carrying free α-amino groups; in a proper system of purified ribosomes, the first peptide bond between two aminoacyl-tRNA's can be formed, whereas the formation of the second and successive peptide bonds is inhibited.

Such a property of sparsomycin has been exploited in measuring the number of aminoacyl-tRNA's bound to each ribosome. Poly-U programmed ribosomes in the presence of phenylalanyl-tRNA and sparsomycin produce diphenylalanyl-tRNA molecules which occupy the D-site and leave any preceding site available to phenylalanyl-tRNA molecules. The molar ratio between ribosome-bound phenylalanyl-tRNA and diphenylalanyl-tRNA can then be easily determined. A one-to-one ratio is expected if only another site per ribosome is able to bind aminoacyl-tRNA besides the donor site. A molar ratio of two- (or more to-one is expected if more than one of such sites is present.

Fig. 1 shows the results of an experiment of this type in which *E. coli* ribosomes were incubated with labeled phenylalanyl-tRNA in the presence of sparsomycin. At the end of the incubation, the ribosome-bound radioactivity was analyzed by paper chromatography following alkaline hydrolysis. The phenyl-

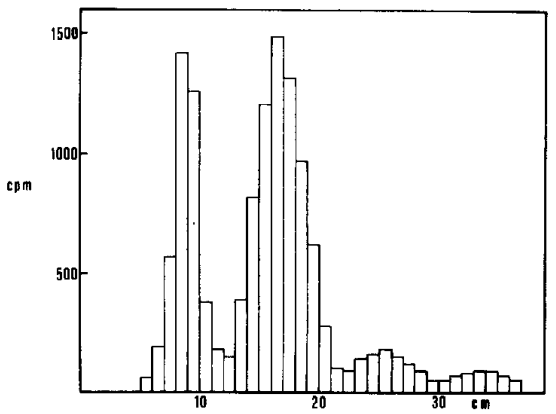

Fig. 1. Chromatography of ribosome-bound polymerization products obtained with *E. coli* ribosomes in the presence of sparsomycin. The technical details are described under *Methods*. The abscissa indicates the distance from the origin; the first peak co-migrated with phenylalanine and the second with diphenylalanine standards.

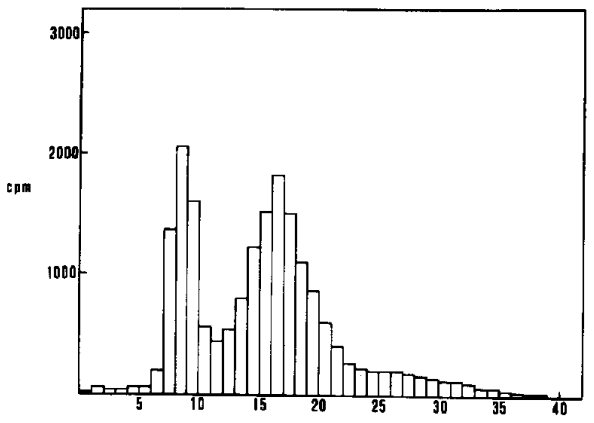

Fig. 2. Analysis of ribosome-bound polymerization products with reticulocyte ribosomes in the presence of sparsomycin. For details see *Methods* and the legend to fig. 1.

alanyl peak (first from the left) contains 3900 cpm while the other peak, diphenylalanine, contains 7100 cpm. The molar ratio monophenylalanine: diphenylalanine is thus 1, 1.

The results of a similar experiment carried out with rabbit reticulocyte ribosomes are illustrated in fig. 2. At the end of the single polymerization step allowed by sparsomycin, the chromatogram of ribosome-bound radioactivity showed 5800 cpm in the phenylalanine peak and 10 360 cpm in the diphenylalanine peak. The molar ratio between the two compounds was 1, 12.

These data indicate that only one molecule of phenylalanyl-tRNA could bind to each diphenylalanyl-tRNA carrying ribosome. Hence they support, for both ribosomal systems, the model that envisions the ribosome as having only two distinct sites for tRNA binding, one peptidyl and one aminoacyl site. Fairly strong evidence in favor of this notion had already been obtained by different approaches, with bacterial ribosomes [3], whereas, with 80 S ribosomes, the evidence supporting either one model was still somewhat inconclusive.

Since it has been shown that 80 S ribosomal hybrids (containing ribosomal subunits of different origin) can translate poly-U with the same efficiency as normal reticulocyte ribosomes [21, 22], the conclusion of the present experiment can be extended to 80 S ribosomes in general. Our data do not exclude, however, the possible existence of an exit site [23], accepting a discharged tRNA after the peptide transfer.

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