MECHANISMS IN PROTEIN SYNTHESIS V. EVIDENCE FOR TWO RIBOSOMAL SITES FROM EQUILIBRIA IN BINDING OF PHENYLALANYL-tRNA *

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Saturation-curves for the poly-U-directed binding of phenylalanyl(Phe)-tRNA to E.coli-ribosomes display three subsequent slopes of decreasing steepness (Heller, 1966). From extrapolations of the third and second slopes towards a Phe-tRNA-concentration of zero, it appears that two sites, corresponding to the first and second slopes, are filled with nearly equal amounts of Phe-tRNA, even at very different stages of ribosome-purification. This equal filling of both hypothetical sites is seen at Mg^{++}-concentrations between 3 and 40 mM. The absolute amounts of binding are Mg^{++}-dependent, perhaps due to the sorption of poly-U. The first slope can be tentatively correlated with the filling of the "acceptor site", because 30S-subunits display it at its full length and nearly complete steepness. They show, however, the second slope only in the presence of 50S-subunits; the second slope therefore may correspond to the "donor site". In contrast, saturation of poly-A,U,G-programmed ribosomes with formylmethionyl(FMet)-tRNA is approached by a steady asymptotic FMet-atRNA concentration-curve.

METHODS AND RESULTS. Ribosomes from E.coli A19, ^{3}H-Phe-atRNA** (sp.act. = 3,000 c/mole), ^{14}C-F-^{3}H-Met-atRNA (sp.act. of F = 42,

*Reported at Frühjahrs-Tagung of the Ges.f.Biol.Chem., Tübingen, April 4, 1967; **the "a" indicates 19 non-labeled amino acids.

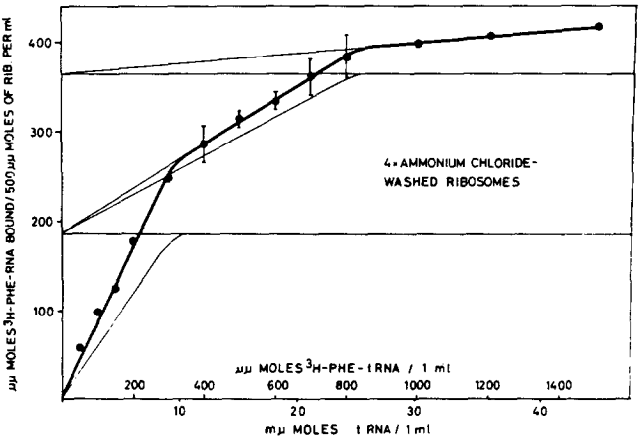

Fig. 1. Binding of Phe-tRNA to ribosomes: Three-sloped saturation-curve. 100 μl-reaction-mixtures with 50 μμmoles 4-times ammonium chloride-washed ribosomes*, 10 μg poly-U and the concentrations of ^{3}H-Phe-atRNA (3.4 % charged with Phe) given on the abscissa were incubated in 16 mM $MgCl_2$ - 10 mM Tris-HCl pH 7.2 - 13.5 mM KCl for 30 minutes at 33°C until equilibrium was reached at all tRNA-concentrations tested. Ribosomal complexes formed were adsorbed onto Millipore HAWP 0.45 μ, 25 mm diameter, washed with 10 mM Tris 7.2 and the Mg^{++}-concentration of the reaction mixture, and counted by liquid scintillation.

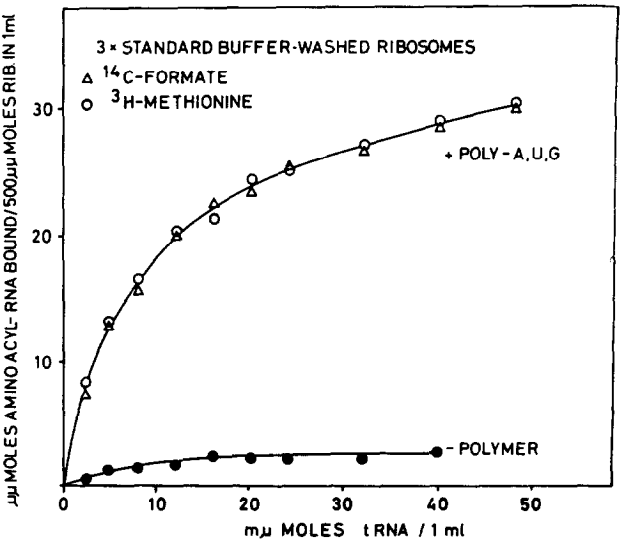

Fig. 2. Binding of FMet-tRNA to ribosomes: Substrate saturation-curve. 3-times standard buffer-washed ribosomes, ^{14}C-F-^{3}H-Met-atRNA 3 % charged with methionine, 1 % formylated; 3 mM $MgCl_2$, other conditions as in Fig.1.

*calculated at 45 (30,15) A_{260}-units/mμmole 70 (50,30)S-ribosomes.

of Met = 1,360 c:mole), and poly-A,U,G were prepared as described (Matthaei et al., 1967a; Matthaei and Voigt, 1967b). Uniformity of Millipore filters appears to be the accuracy-limiting factor in such experiments. To prove particularly the existence of the second slope, the mean deviations from five measurements per tRNA-concentration tested are given in Fig.1. Each assay was done at least in duplicate. Extrapolation of slopes to a substrate concentration of zero indicates an almost equal filling of two sites, regardless of quite different degrees of ribosomal purity. Table1 shows a few of these results. The ratio of filling of the two sites is also independent of Mg^{++}-concentrations between 3 and 40 mM (Fig.3 and Tab.1) at the rather low concentrations of monovalent

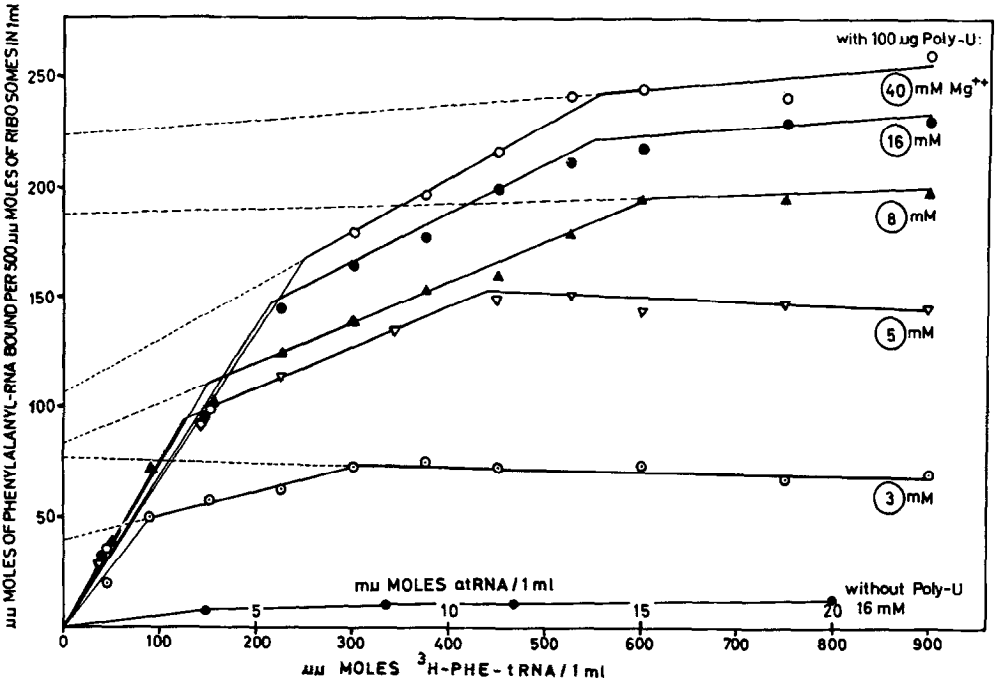

Fig.3. Binding of Phe-tRNA to ribosomes: Mg^{++}-dependence of substrate saturation-curves. 3-times ammonium chloride-washed ribosomes; Mg^{++}-concentrations indicated on curves, other conditions as in Fig.1.

cations used here. The absolute amounts of Phe-tRNA bound increase with purity of ribosomes and Mg^{++}-concentration. The highest value reached in the experiments reported here seemed to

Table 1: Sorption of Phe-tRNA depending on Mg^{++} and ribosomal purity

50 µµmoles ribosomes, stage of purification	Mg^{++}-conc. (mM)	Ratio of filling $2^{nd}/1^{st}$ site	µµmoles in "1^{st} site"	Fraction of offered Phe-tRNA bound in "1^{st} site"	Fraction of offered Phe-tRNA bound in "2^{nd} site"
unwashed	16	1.17	3.1	0.142	0.090
1 x stand.buffer washed	16	1.19	4.5	0.162	0.103
3 x NH_4Cl - washed	3	0.97	3.9	0.44	0.125
	5	1.19	7.1	0.57	0.195
	8	1.24	8.3	0.55	0.169
	16	1.08	9.3	0.50	0.190
	40	1.08	10.7	0.43	0.206
4x NH_4Cl-washed	16	1.00	18.0	0.60	0.160

be the sorption of one molecule of Phe-tRNA into the first site of 36 % of the 4-times ammonium chloride-washed ribosomes (Tab.1). At the low concentration of monovalent cations used here, the fractions of Phe-tRNA bound into the first and second sites appear to be essentially independent of Mg^{++}-concentrations between 5 - 40 mM for a certain stage of purification (see Tab.1); with three-times ammonium chloride-washed ribosomes, it averaged to 0.51 and 0.19 for the first and second slopes, respectively, corrected for the second and third slopes. FMet-tRNA, which should fill only one ribosomal site at 3 mM Mg^{++} (Matthaei and Voigt, 1967), saturates by a continuous asymptotic curve (Fig.2).

In an attempt to correlate the poly-U-dependent first and second slopes with ribosomal binding sites, the experiment shown in fig.4 was performed 3 times. The saturation of poly U-programmed 30S-subunits follows the first slope with almost the same steepness

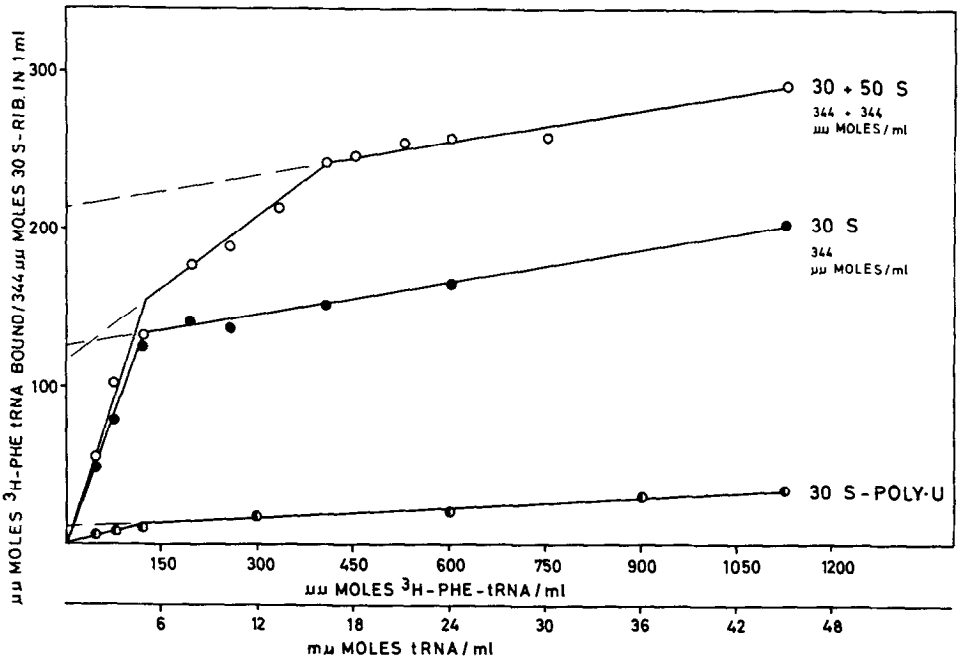

Fig.4. Binding of Phe-tRNA to ribosomal subunits: Only the first and third slopes are seen in the saturation-curve of 30S-particles. 3 times ammonium chloride-washed ribosomes were dialysed for 6 h against 0.7 mM $MgCl_2$ - 0.2 mM K-EDTA - 10 mM Tris-HCl pH 7.2 and diluted to 48 A_{260}/ml. 2 ml-samples were layered over 56 ml of linear gradients from 5 to 20 % sucrose in 0.5 mM $MgCl_2$ - 30 mM Tris-HCl pH 7.2, spun for 9 h at 2^0 in the Spinco-rotor SW-2 and harvested through a 1 mm flow-cuvette; 30S- and 50S-peak-fractions were dialyzed for 2 x 1 h against 1 and 10 mM $MgCl_2$ in 10 mM Tris-HCl pH 7.2, pelleted in 3 h at 50.000 rpm in the Spinco-rotor 50 Ti and resuspended to 55 and 110 A_{260}/ml respectively (Voigt and Matthaei, 1967). Assay conditions as given under fig.1, except 344 μμmoles particles/ml and 19 mM Mg^{++}. Assays in duplicate.

and length as obtained in the presence of both subunits. The second slope was seen only when both 30S and 50S-particles were present. The third slope appears to be essentially due to non-poly-U-dependent binding. The slightly lesser filling of the site represented by the second slope, may be due to sensitivity of 3 x ammonium chloride-washed ribosomes to the subunit fractionation procedure (Voigt and Matthaei, 1967).

DISCUSSION: Observations supporting one, two or three ribosomal binding sites for tRNA have been reported (Cannon et al., 1963; Warner and Rich, 1964; Wettstein and Noll, 1965; Matthaei and Voigt, 1967). Amino acyl-tRNA saturation curves may be a new source of evidence for the number of tRNA molecules bound per active ribosome; furthermore, they may allow us to measure the fraction of active ribosomes in a given ribosomal population. Because very different ribosome preparations seem to fill two sites to a nearly equal extent at the low salt concentrations used here, it appears most likely that in the binding system with poly-U, each active ribosome is programmed in both sites with a Phe-codon. An extension of the experiments reported in the high salt-environment of the polyphenylalanine synthetizing system (100 mM Tris-HCl - 80 mM KCl), has revealed the three slopes from 6 mM Mg^{++} upwards, whereas the synthesis of polyphenylalanine now requires 10 mM Mg^{++} at least (cf. Revel and Hiatt, 1965). Further investigation is therefore needed to explain the high Mg^{++}-requirement in polyphenylalanine-synthesis.

The first and steepest slope may correspond to the "acceptor site", because, (1) the site normally filled by the entering amino acid adaptor seems more likely to be the one which is easier to fill; (2) 30S-particles do not show the second slope (Fig.4). In the first communication of this series, these subunits were demonstrated and explained as having the site for binding both mRNA and amino acyl-tRNA in a specifically correlated manner; they have a "checking-site" (Matthaei et al., 1964) and may serve as the "acceptor-site" or part thereof in amino acid transfer. The second slope seems to require both the 30S- and 50S-subunits and is therefore tentatively correlated with the "donor site". It is not known whether this slope perhaps only

occurs in the binding system for Phe-tRNA coded by poly-U and by other synthetic mRNA containing Phe-codons. The third slope is, in part at least, due to non-specific binding, i.e. binding which is not stimulated by added poly-U. Further experiments to prove the possible meaning of the three slopes in relation to single steps in the synthesis of polypeptides on more natural mRNA-models are in progress.

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