ON THE SPECIFICITY OF THE TWO RIBOSOMAL BINDING SITES: STUDIES WITH TETRACYCLINE

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SUMMARY: The erroneous binding of isoleucyl-tRNA to ribosomes in response to poly U shows the same pattern of inhibition by tetracycline as the correct binding of phenylalanyl-tRNA, while the poly U directed binding of leucyl-tRNA is largely sensitive to tetracycline. It is concluded that the coding specificity of the peptidyl site on the ribosome is at least equivalent to that of the aminoacyl site.

The ribosome possesses two sites capable of binding acylated tRNA's, the aminoacyl site and the peptidyl site (1-3). During the course of protein synthesis, aminoacyl-tRNA's are bound enzymatically to the aminoacyl site (4,5). The initiator F-Met-tRNA is probably also bound initially at this site prior to translocation to the peptidyl site (6,7). This picture of protein synthesis places the entire burden of translation (i.e., the specific matching of codon and anticodon) on the aminoacyl site, and hence, in the simplest view, coding specificity in the peptidyl site should be unnecessary, and the general properties of the two sites should be quite different.

The nonenzymatic binding of aminoacyl-tRNA occurs directly to both sites (7-10). This binding is subject to considerable error (i.e., mismatching of codon and anticodon) (11). The antibiotic tetracycline (TET) has recently been shown to inhibit binding to the aminoacyl site without appreciably affecting direct binding to the peptidyl site (7,12-14). We have employed

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this antibiotic to test the specificity of the two sites toward nonenzymatic binding of various aminoacyl-tRNA's in response to poly U.

MATERIALS AND METHODS

Ribosomes washed 5 times with buffer containing 0.5 M NH_4Cl were prepared from E. coli Q13 (General Biochemicals, Inc.) as described by Nishizuka and Lipmann (15). tRNA was prepared by the method of von Ehrenstein and Lipmann (16) and further purified by chromatography on a DEAE-cellulose column (eluted between 0.3 and 0.7 M NaCl in 0.1 M Tris-Cl, pH 7.4). The tRNA was charged with amino acids as described by von Ehrenstein and Lipmann (16). The radioactive aminoacyl-tRNA's were as follows: H^3-phenylalanyl-tRNA, 32.9 μμmoles/A_{260} unit (6510 cpm/μμmole); H^3-leucyl-tRNA, 12.2 μμmoles/A_{260} unit (45,500 cpm/μμmole); H^3-isoleucyl-tRNA, 71 μμmoles/A_{260} unit (1560 cpm/μμmole). Labeled amino acids were obtained from New England Nuclear Corp., tetracycline from K and K Laboratories, poly U from Sigma and type HAWP filters from Millipore Corp.

Aminoacyl-tRNA-ribosome complexes were measured by Millipore filtration, following the method of Nirenberg and Leder (17). Binding mixtures (50 μl) contained 50 mM Tris-Cl, pH 7.4, 100 mM NH_4Cl, 10 mM 2-mercaptoethanol, 400 μg/ml poly U, and ribosomes, aminoacyl-tRNA, $MgAc_2$ and $6x10^{-4}$ M TET as indicated. All components of the binding mixture except aminoacyl-tRNA were preincubated 10 min. at 25°, and binding was initiated by the addition of aminoacyl-tRNA. After incubation for 30 min. at 25°, the reaction was terminated by the addition of a large volume of cold buffer containing 50 mM Tris-Cl, pH 7.4, 100 mM NH_4Cl and 15 mM $MgAc_2$, and the samples were immediately filtered and washed with this buffer. Washing of complexes formed at high Mg^{2+} with this low Mg^{2+} buffer was found to substantially reduce binding in the absence of poly U without appreciably affecting template directed binding. Liquid scintillation counting was performed in a toluene based scintillator at an efficiency of 47%.

RESULTS AND DISCUSSION

A typical pattern of TET inhibition of Phe-tRNA binding as a function of Mg^{2+} concentration is shown in Fig. 1a. Binding at low Mg^{2+} (5-10 mM) is completely resistant to TET inhibition, and in fact the binding of one preparation of Phe-tRNA was actually stimulated by TET under these conditions. This unexpected result may be related to the recent observations of Igarashi and Kaji (18), who found by a different method that the binding of Phe-tRNA to the 30s subunit was predominantly in the peptidyl site. TET-sensitive binding, by contrast, shows a maximum at approximately 30 mM Mg^{2+}.

The binding of Leu- and Ile-tRNA's in response to poly U represents mismatching of codon and anticodon by the ribosome. If the specificity of the peptidyl site were less stringent than that of the aminoacyl site, then this erroneous binding should be less sensitive to TET inhibition than the correct binding of Phe-tRNA. The binding of Ile-tRNA in response to poly U is shown in Fig. 1b. As can be seen, the pattern of TET inhibition of Ile-tRNA binding at various Mg^{2+} concentrations is virtually identical to that of Phe-tRNA binding, with a peak of TET-resistant binding at low Mg^{2+} and an inhibition of $\sim$ 50% at higher Mg^{2+} levels. Hence, the two sites are equally prone to make this particular translational error.

The binding of Leu-tRNA to the ribosome-poly U complex is rather poor (Fig. 1c), and there is considerable nonspecific binding in the absence of poly U. Both poly U-stimulated and nonspecific binding are dramatically inhibited by TET. At all Mg^{2+} concentrations, Leu-tRNA binding is significantly more sensitive to TET than Phe-tRNA binding. We therefore conclude that the peptidyl site possesses substantial specificity and, under these particular conditions, is more capable than the aminoacyl site of discriminating Phe- and Leu-tRNA's.

Once protein synthesis has been properly initiated, correct reading of the message depends not only on proper matching of codon and anticodon in the aminoacyl site, but also on maintenance of the proper reading frame. Correct

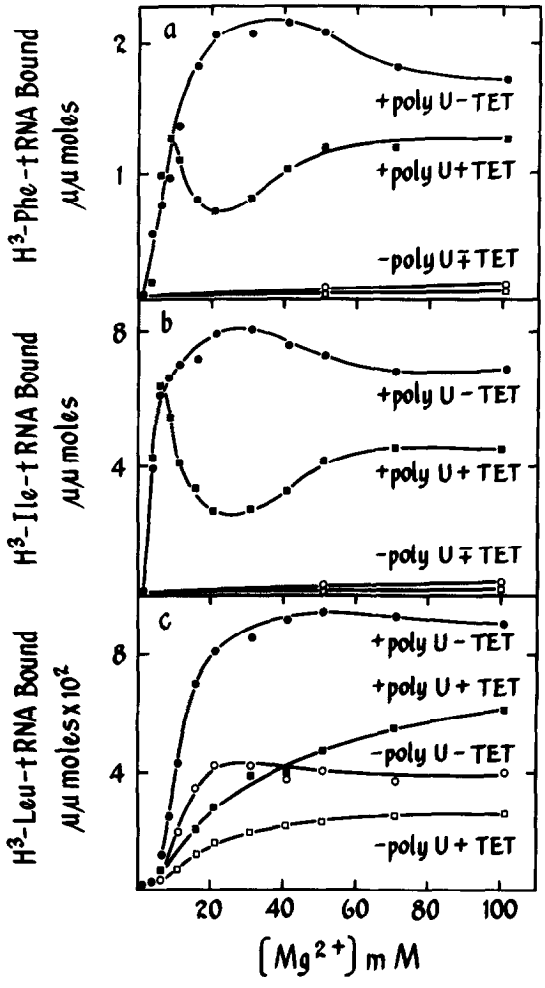

Figure 1. Inhibition by TET of poly U directed aminoacyl-tRNA binding. The reaction mixtures contained, in addition to the items listed in MATERIALS AND METHODS: a. 5.06 μμmoles H^3-Phe-tRNA, 9.25 μμmoles ribosomes (25 μg); b. 20.2 μμmoles H^3-Ile-tRNA, 92.5 μμmoles ribosomes; c. 0.63 μμmoles H^3-Leu-tRNA, 27.8 μμmoles ribosomes.

phasing could be a function of the peptidyl site on two occasions: during translocation of peptidyl-tRNA and while the peptidyl-tRNA-mRNA complex is bound to the peptidyl site prior to the binding of aminoacyl-tRNA. Bretscher recently showed that the triplet AUG was not released from the AUG-F-Met-tRNA complex bound in the peptidyl site unless F-Met was first released with puromycin, and he concluded that significant codon-anticodon interaction occurred in the peptidyl site (19). The present results indicate that the peptidyl

site is at least as demanding of correct codon-anticodon interaction as the aminoacyl site, and both of these observations may suggest a substantial role for the peptidyl site in maintaining the proper reading frame during peptide chain elongation.

In the presence of poly U, leucine is readily incorporated into peptides, while isoleucine is rather poorly incorporated (20). As reported previously (11), Ile-tRNA binds readily to poly U-ribosome complexes. Furthermore, the distribution of Ile-tRNA between sites, as determined by TET inhibition, is indistinguishable from that of Phe-tRNA. Although Leu-tRNA binds less well than Ile-tRNA, the bulk of the Leu-tRNA bound is in the aminoacyl site, which could account for its incorporation into peptides. We have previously shown that the product of this incorporation is a random copolypeptide of phenylalanine and leucine (21), which is presumably a reflection of random errors on the part of the aminoacyl site. The fact that Ile-tRNA binds well but is incorporated poorly suggests that some component of the incorporation system discriminates against Ile-tRNA. T factors offer themselves as the most likely agents for this discrimination. Experiments in progress deal with the role of the T factors in ensuring proper codon recognition during translation.

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