Studies on the Action of Tetracycline and Puromycin*

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SUMMARY

By means of a convenient method for separating phenylalanyl-puromycin from diphenylalanyl-puromycin, it was found that, upon reaction with puromycin, phenylalanyl transfer RNA bound to ribosomes at 5 to 6 mM Mg++ yields phenylalanyl-puromycin exclusively while phenylalanyl-tRNA bound at 13 mM Mg++ yields diphenylalanyl-puromycin as well as phenylalanyl-puromycin. Tetracycline inhibited mostly the binding of phenylalanyl-tRNA to the acceptor site, but the binding to the donor site may be inhibited at 13 mM Mg++. Puromycin reaction of phenylalanyl-tRNA bound to the donor site was inhibited by the presence of N-acetylphenylalanyl-tRNA at the acceptor site.

It was found that tetracycline inhibits not only the acceptor site binding but also the donor site binding of phenylalanyl-tRNA in the presence of high Mg++. The reaction of puromycin with aminoacyl-tRNA on the ribosome has been used extensively for studying peptide bond formation. However, it has not been clear whether puromycin approaches the donor site from the side of the acceptor site or not. By use of the complex having N-acetylphenylalanyl-tRNA both on the acceptor and the donor site, we concluded that puromycin approaches the donor site from the side of the acceptor site.

MATERIALS AND METHODS

Escherichia coli Extract and Other Materials—Preparation of ribosomes, tRNA from E. coli B, and aminoacyl-tRNA have been described in the previous communications (1). The ribosomes were washed three times with a buffer containing 0.1 M Tris-HCl (pH 7.8), 0.01 M magnesium acetate, 0.06 M potassium chloride, 0.006 M β-mercaptoethanol, and 0.5 M ammonium chloride, and were free from aminoacyl-tRNA transfer factor (T factor) and initiation factors. Preparation of T factor and G factor was as described previously (13).

Binding of [14C]Phenylalanyl-tRNA to Ribosomes and Isolation of Complex of Ribosomes, poly(U), and [14C]Phenylalanyl-tRNA—A typical binding reaction mixture (0.5 ml) for the isolation of the complex, unless otherwise specified, contained 50 mM Tris-HCl (pH 7.2), 6 mM magnesium acetate, 40 mM ammonium chloride, 400 μg of poly(U), 5.2 μg of tRNA containing 8.5 × 10^6 cpm of [14C]phenylalanyl-tRNA, and 5.8 mg of ribosomes. This binding condition was called condition A. In some cases, the binding reaction was performed under identical conditions except that the Mg++ concentration was 13 mM. This was called condition B. When the enzymatic binding of aminoacyl-tRNA was carried out, 85 μg of T factor, 0.2 mM GTP, and 2 mM thioetherol were also added to the above binding mixture. The reaction mixture was incubated for 20 min at 22°C. To separate the complex of ribosomes, poly(U), and [14C]phenylalanyl-tRNA from unbound [14C]phenylalanyl-tRNA, the mixture was placed on 5 ml of a linear sucrose gradient solution (5 to 20%) containing 20 mM Tris-HCl (pH 7.2), 6 mM or 13 mM magnesium acetate, 40 mM NH₄Cl, and 6 mM β-mercaptoethanol. The gradients were centrifuged for 60 min at 48,000 rpm in a Beckman-Spinco rotor (SW 50.1), and the 70 S ribosome portions were collected as the ribosomal complex.

Reaction of Bound [14C]Phenylalanyl-tRNA with Puromycin—The reaction mixture (0.5 ml) for the puromycin reaction contained 20 mM Tris-HCl (pH 7.2), 13 mM magnesium acetate, 80 mM NH₄Cl, 0.2 mM GTP, 24 μg of G factor, 1 mM puromycin, and

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0.3 ml of the fraction containing the ribosomal complex isolated as described above. The reaction mixture was incubated for 60 min at 22°, mixed with 2 ml of 0.01 M Tris-HCl (pH 7.8), and shaken well with 6 ml of ethyl acetate. The ethyl acetate extract (0.5 ml) was mixed with 2 ml of Bray's solution (14), and then radioactivity was measured by liquid scintillation counter. The ethyl acetate extract (5 ml) was evaporated in a vacuum, redissolved in 0.6 ml of 0.5 M acetic acid, and applied on a Sephadex G-15 column as described below. Approximately 70 to 75% of the bound [14C]phenylalanyl-tRNA reacted with puromycin under these conditions.

Separation of Phenylalanyl Puromycin and Diphenylalanyl Puromycin by Sephadex G-15 Column Chromatography—The puromycin derivatives of phenylalanine formed as described in the preceding section were analyzed by Sephadex G-15 column chromatography (15). The puromycin derivative in 0.5 ml of 0.5 M acetic acid was applied to a Sephadex G-15 column (0.8 x 150 cm) which had been equilibrated with 0.5 M acetic acid. The elution was carried out by 0.5 M acetic acid with a flow rate of 1 ml per 12 to 15 min. Each fraction (1 ml) was collected and mixed with 5 ml of Bray's solution and the radioactivity of the [14C]phenylalanyl puromycin derivatives was measured. The elution profile is shown in Fig. 1.

Identification of Puromycin Derivatives of Phenylalanine—To identify the nature of two peaks of [14C]phenylalanyl puromycin derivatives (Fig. 1), NH2-terminal analysis of each peak was performed. Upon dinitrophenylation, [14C]phenylalanyl puromycin would give dinitrophenyl [14C]phenylalanyl puromycin which would be hydrolyzed by 6 N HCl into dinitrophenyl [14C]phenylalanine and puromycin. The dinitrophenyl phenylalanine is soluble in ether. Thus, under these procedures, 100% of radioactivity should become ether-soluble. On the other hand, under identical conditions [14C]diphenylalanyl puromycin would yield only 50% of its radioactivity as ether-soluble material because of insolubility of phenylalanine in ether. The materials of the first peak (Fractions 38 to 42 in Fig. 1) had been hydrolyzed by 6 N HCl in dinitrophenyl [14C]phenylalanine and puromycin. The dinitrophenyl phenylalanine is soluble in ether. Thus, under these procedures, 100% of radioactivity should become ether-soluble. On the other hand, under identical conditions [14C]diphenylalanyl puromycin would yield only 50% of its radioactivity as ether-soluble material because of insolubility of phenylalanine in ether. The materials of the first peak (Fractions 38 to 42 in Fig. 1, A and B) and the second peak (Fractions 48 to 58 in Fig. 1B) were separately pooled, dried in a vacuum, and dissolved in 0.2 ml of 1% triethylamine. Dinitrophenylation was carried out by adding the second peak (2 ml of 5% solution of dinitrofluorobenzene in alcohol) to the reaction mixture, and free dinitrophenol was removed by ether extraction. Under these acidic conditions, most of the dinitrophenyl [14C]phenylalanyl puromycin derivatives remained in the aqueous layer. The concentration of HCl of these aqueous layers was brought to 6 N by the addition of concentrated HCl, and acid hydrolysis was performed at 110° for 6 hours. The resulting hydrolysate was diluted to 0.6 N HCl with water, and ether-soluble materials extracted three times with 5-ml portions of ether. The radioactivities in the combined ether extracts and aqueous layer were compared. With material of the first peak, a major portion (about 90%) of its radioactivity was converted to ether-soluble material indicating that the first peak represents phenylalanyl puromycin. When the second peak was subjected to the identical NH2-terminal analysis, about 55% of its radioactivity was converted to the ether-soluble material showing that the second peak represented diphenylalanyl puromycin.

Materials—Preparation of N-acetylpheynylalanyl-tRNA was performed as described previously (16). Puromycin hydrochloride was purchased from Nutritional Biochemicals, and tetracycline hydrochloride was from California Biochemical Co. Specific activity of [14C]phenylalanine was 375 µCi per pmole and the counting efficiency was 10^6 cpm per µCi.

RESULTS

Characterization of Complex Formed under Conditions A and B—It has been postulated that in the presence of 5 to 6 mM Mg2+ concentration, phenylalanyl-tRNA binds to the donor site alone, and in the presence of 13 mM Mg2+ concentration, both donor and acceptor sites are occupied. Our previous studies, on the mode of action of antibiotics (9), were dependent on this assumption. It was therefore desirable to determine the evidence which would indicate that this is indeed the case. From this assumption it follows that we should have mostly phenylalanyl puromycin when the binding was carried out at low Mg2+ (condition A) while diphenylalanyl puromycin as well as phenylalanyl puromycin would be formed when the binding was carried out at high Mg2+ (condition B).

Fig. 1A shows that the puromycin derivative formed under condition A gives one peak corresponding to a phenylalanyl puromycin upon passage through the Sephadex G-15 column. As expected from the function of the G factor, the addition of the factor did not influence the nature or the quantity of the product as shown in this figure. On the other hand, when the complex was made under condition B, the addition of the G factor and GTP caused a large increase of formation of phenylalanyl puromycin and diphenylalanyl puromycin as shown in Fig. 1B.

Effect of Tetracycline on Binding of Phenylalanyl-tRNA—It has been proposed that tetracycline has an exclusive inhibitory effect on the binding of aminoacyl-tRNA to the acceptor site (10-12). On the other hand, during the studies on the relationship between the acceptor site and the donor site, it was observed that, under certain conditions, tetracycline may also inhibit the binding of phenylalanyl-tRNA to the donor site (7). It was therefore desirable to determine exactly the percentage of inhibition of the binding of aminoacyl-tRNA to each site.

In the experiment shown in Table I the ribosomal complex was prepared under various conditions in the presence or absence of tetracycline. The puromycin derivatives of [14C]phenylalanine were formed from these complexes and analyzed with Sephadex G-15 column. It should be pointed out that the inhibitory effect of tetracycline on the binding reaction was much more pronounced when the binding reaction was carried out in the presence of high Mg2+ or T factor.

In order to determine which site was more susceptible to tetracycline, the formation of puromycin derivatives of the bound phenylalanyl-tRNA was studied with or without G factor. As shown in Table I the addition of G factor stimulated the formation of phenylalanyl puromycin derivatives when the ribosomal complex was prepared at high Mg2+ (13 mM) or in the presence of T factor was used. Table I also shows the results of the analysis of the puromycin derivatives formed from these complexes. The relative amounts of phenylalanyl puromycin and diphenylalanyl puromycin were calculated. With these values, it was possible to estimate the distribution of phenylalanyl-tRNA reaction with puromycin between the donor and the acceptor site. The principles used in calculating the distribution of phenylalanyl-tRNA between the two sites are (a) phenylalanyl puromycin formed in the absence of G factor was assumed to be located at the donor site; (b) diphenylalanyl puromycin was derived from phenylalanyl-tRNA bound to two sites, thus the
FIG. 1 (left and center). Sephadex G-15 column chromatography of puromycin derivatives of [14C]phenylalanine. The puromycin derivatives of [14C]phenylalanine prepared under Conditions A (5,520 cpm) or B (20,292 cpm) were extracted in ethylacetate, the ethylacetate was evaporated and dissolved in 0.6 ml of 0.5 M acetic acid. The solution was then poured onto a column (0.8 x 150 cm) of Sephadex G-15 which had been equilibrated with 0.5 M acetic acid. The sample was eluted with 0.5 M acetic acid with flow rate of 1 ml every 12 to 15 min. Fractions (1 ml) were collected and 1 ml aliquot was mixed with 5 ml of Bray's solution (14) and the radioactivity was counted. O- - O, puromycin derivatives formed in the presence of G factor; , puromycin derivatives formed in the absence of G factor. A and B represent puromycin derivatives formed under Conditions A and B, respectively.

FIG. 2. (right). Effect of additional binding of aminoacyl-tRNA on the puromycin reaction of the donor site-bound N-acetyl[14C]phenylalanyl-tRNA. For the preparation of the ribosomal complex having N-acetyl[14C]phenylalanyl-tRNA mostly at the donor site, the binding reaction mixture (0.4 ml) contained 40 mm Tris-HCl (pH 7.2), 40 mm NH₄Cl, 5 mm magnesium acetate, 80 µg of poly(U), 2.5 mg of ribosomes, and 42 µg of tRNA mixture containing 2 x 10⁶ cpm of N-acetyl[14C]phenylalanyl-tRNA. After incubation for 15 min at 22°, 2.3 mg of tRNA mixture containing 1.2 µmoles of N-acetyl[14C]phenylalanyl-tRNA or 3.0 µmoles of [14C]phenylalanyl tRNA were added to the above binding reaction mixture and Mg²⁺ was adjusted to 13 mM. The mixtures were incubated again for 15 min at 22° to assure the additional binding to the acceptor site. The ribosomal complex thus prepared was isolated as described in the text. The reaction mixture for the puromycin reaction (0.5 ml) of the bound N-acetyl[14C]phenylalanyl-tRNA was as described in the text and it contained approximately 2,200 cpm of bound N-acetyl[14C]phenylalanyl-tRNA, but no G factor. N-Acetyl[14C]phenylalanyl-pyromycin formed is expressed as percentage of the total bound N-acetyl[14C]phenylalanyl-tRNA. O---O, complex with no additional [14C]aminoacyl-tRNA at the acceptor site; A---A, complex with N-acetyl[14C]phenylalanyl-tRNA at the acceptor site; , complex with additional [14C]phenylalanyl-tRNA at the acceptor site.

TABLE I

<table>
<thead>
<tr>
<th>Binding condition of [14C]phenylalanyl-tRNA</th>
<th>Bound [14C]phenylalanyl-tRNA</th>
<th>Total phenylalanyl puromycin formed</th>
<th>Puromycin derivatives formed (percentage of total)</th>
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<tr>
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<td>Total cpm</td>
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<td>Diphenylalanyl puromycin</td>
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<td>13</td>
<td>—</td>
<td>5 x 10⁻⁴</td>
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The calculations of the amount of the puromycin-reactive [34C]-phenylalanyl-tRNA at the acceptor and the donor site were carried out as follows. For example, from the ribosomal complex formed in the presence of 6 mM Mg++, 5190 cpm of the puromycin derivatives of [34C]phenylalanine were formed in the absence of the G factor (line 1 in Table I). Of this, 92% (5095 cpm) were phenylalanyl puromycin and 8% (495 cpm) were diphenylalanyl puromycin. Since diphenylalanyl puromycin must come from the phenylalanyl-tRNA at the donor and the acceptor site, 248 cpm (5905 - 5695 = 215) of phenylalanyl-tRNA derivatives of [34C]phenylalanine were formed (line 2 in Table I). 89% (5910 cpm) and 11% (730 cpm) were phenylalanyl-puromycin at the donor and the acceptor site, respectively. Thus, 215 cpm (5910 - 5695 = 215) of phenylalanyl puromycin were formed due to the addition of G factor. Of this, 92% (5695 cpm) were phenylalanyl-tRNA bound at the donor site and translocated to the acceptor site. The diphenylalanyl puromycin (5095 cpm) must come entirely from the donor site. In the presence of the G factor, 6640 cpm of puromycin derivatives of [34C]phenylalanine were formed (line 2 in Table I). Of this, 89% (5910 cpm) and 11% (730 cpm) were [34C]phenylalanyl-puromycin and diphenylalanyl puromycin, respectively. Thus, 215 cpm (5910 - 5695 = 215) of phenylalanyl puromycin were formed due to the addition of G factor. We regard that this was bound at the acceptor site and translocated to the donor site by the G factor. The diphenylalanyl puromycin formed due to the addition of G factor was 235 cpm (730 - 495 = 235). We assign 117 cpm (235 x 0.495 = 117) to each site. The total [34C]phenylalanyl-puromycin bound to the donor site is therefore 5095 + 248 + 117 = 580 cpm. The sum of these values (5095 + 5695 = 1170 cpm) represent total puromycin-reactive [34C]phenylalanyl-tRNA bound in the presence of 6 mM Mg++. The action of Tetracycline and Puromycin Vol. 247, No. 1

It is clear from Table II that the effect of tetracycline was most exclusively at the donor site, G factor did not appreciably stimulate the puromycin reaction of this diphenylalanyl-tRNA at the acceptor site. It can also be seen in this table that the stimulation of the binding of [34C]phenylalanyl-tRNA by T factor was mainly on the acceptor site in confirmation of our previous conclusion with the experiment where NH₂-terminal analysis of polyphenylalanine was employed to determine the amount of bound phenylalanyl-tRNA at these sites (2).

Studies on Puromycin Action and Influence of Aminoacyl-tRNA at Acceptor Site on Reactivity of Aminoacyl-tRNA Bound at Donor Site—Since the puromycin reaction with the donor site-bound aminoacyl-tRNA is analogous to the peptide bond formation between aminoacyl-tRNAs bound at the donor and acceptor sites (18–20), it is reasonable to assume that puromycin would attack the aminoacyl-tRNA at the donor site from the side of the acceptor site. If this assumption is correct, one would expect that the presence of nonreactive aminoacyl-tRNA at the acceptor site would hinder the reactivity of the aminoacyl-tRNA at the donor site. The report contrary to this expectation (21) prompted us to examine this possibility with the present system. In the experiment described in Table III, the complex of [34C]phenylalanyl-tRNA, poly(U), and ribosomes was prepared at 5 mM Mg++. This complex was then incubated with or without N-acetylphenylalanyl-tRNA or phenylalanyl-tRNA at 13 mM Mg++. Under these conditions, [34C]phenylalanyl-tRNA or N-acetyl[34C]phenylalanyl-tRNA and [34C]phenylalanyl-tRNA were at the acceptor and the donor site, respectively. Using these complexes, the formation of puromycin derivative was examined. It is clear from this table that the amount of puromycin derivative of [34C]phenylalanine formed in the absence of G factor was greatly reduced when [34C]phenylalanyl-tRNA or N-acetyl[34C]phenylalanyl-tRNA were added to the reaction mixture for the additional binding of aminoacyl-tRNA at the acceptor site. Since the [34C]phenylalanyl-tRNA was bound almost exclusively at the donor site, G factor did not appreciably stimulate the puromycin reaction of this [34C]phenylalanyl-tRNA when no additional aminoacyl-tRNA was bound at the acceptor site. On the other hand, in the presence of [34C]phenylalanyl-tRNA at the acceptor site, [34C]phenylalanyl-[34C]phenylalanyl-tRNA was formed and located at the acceptor site. Consequently, the puromycin reaction of this diphenylalanyl-tRNA was dependent on G factor. When N-acetyl[34C]phenylalanyl-tRNA was bound at the acceptor site, no diphenylalanyl-tRNA could be formed and, consequently, G factor had no appreciable stimulation on the formation of puromycin derivative of [34C]phenylalanine. Thus, the inhibitory effect of N-acetyl[34C]-phenylalanyl-tRNA at the acceptor site could be attributed solely to the steric hindrance.

The data presented in Fig. 2 shows similar effects of the [34C]-phenylalanyl-tRNA and N-acetylphenylalanyl-tRNA present at the acceptor site on the puromycin reaction of N-acetyl[34C]-phenylalanyl-tRNA bound at the donor site. Since the peptide bond formation takes place much more rapidly with N-acetyl-
phenylalanyl-tRNA than with phenylalanyl-tRNA, the rate of formation of puromycin derivative was studied after a short incubation period. As can be seen from the figure, at the initial period of the reaction a marked inhibition of the puromycin reaction of N-acetyl[14C]phenylalanyl-tRNA at the donor site was observed. It should be pointed out that no G factor was added in this experiment. Thus, the inhibitory effect of [14C]phenylalanyl-tRNA because the former inhibited the puromycin reaction for two possible reasons, i.e. steric hindrance, as well as formation of N-acetyldiphenylalanyl-tRNA, while the latter inhibited only because of the possible steric hindrance. These results are consistent with the concept that puromycin attacks the donor site from the direction of the acceptor site.

The experiments described above were based on the assumption that the binding sites for N-acetylphenylalanyl-tRNA were the same as those for phenylalanyl-tRNA, and N-acetylphenylalanyl-tRNA behaved similarly to phenylalanyl-tRNA with respect to the binding to ribosomes. To ascertain that this assumption was correct, the binding sites for N-acetylphenylalanyl-tRNA were compared with those for phenylalanyl-tRNA. As shown in Table IV, the amount of bound N-acetyl[14C]phenylalanyl-tRNA at the donor site was mostly to the donor site while both donor and acceptor sites were occupied with phenylalanyl-tRNA in the presence of high Mg++. Binding of phenylalanyl-tRNA to ribosomes took place more efficiently at three different Mg++ concentrations. Thus, one can conclude that in the presence of low Mg++, N-acetylphenylalanyl-tRNA binds mostly to the donor site while it binds to both donor and acceptor sites at high Mg++.
small, if any, stimulation of the formation of diphenylalanyl puromycin. On the other hand, complex B yielded diphenylalanyl puromycin as well as phenylalanyl puromycin in the presence of G factor. These results give further support of the previous notion (2) that in the presence of low Mg"+ (condition A) only the donor sites are occupied with phenylalanyl-tRNA, whereas both acceptor and donor sites are occupied with phenylalanyl-tRNA in the presence of high Mg"+ (condition B).

With the analysis of the puromycin derivatives formed from the bound phenylalanyl-tRNA, it became clear that, in confirmation of our previous results with NH_{4}-terminal analysis of polyphenylalanine (7), tetracycline inhibited significantly the binding of phenylalanyl-tRNA at the donor site as well as the acceptor site in the presence of high Mg"+ (condition B). In the presence of low Mg"+ and T factor the action of tetracycline was relatively specific to the acceptor site. It should be pointed out, however, that in the normal situation, the donor site is occupied with either unesterified tRNA or peptidyl-tRNA; thus the possibility that the aminoacyl-tRNA directly binds to the donor site does not exist in the normal protein synthesis. Only when the empty ribosomes are presented to an aminoacyl-tRNA with synthetic messenger RNA, such a situation takes place.

The presence of N-acetylphenylalanyl-tRNA or phenylalanyl-tRNA at the acceptor site has a strong inhibitory effect on the puromycin reaction of the donor site-bound [14C]phenylalanyl-tRNA. The inhibition by the presence of [14C]phenylalanyl-tRNA at the acceptor site is due to two reasons. The first is formation of [14C]phenylalanyl-[14C]phenylalanyl-tRNA bound to the acceptor site. In support of this, a marked increase of the puromycin reaction was observed by the addition of G factor. The second reason for the inhibition is a possible steric hindrance caused by the presence of aminoacyl-tRNA at the acceptor site. This notion was supported by the observation that the presence of N-acetyl[14C]phenylalanyl-tRNA at the acceptor site had strong inhibitory effect on the puromycin reaction of the donor site-bound [14C]phenylalanyl-tRNA. The inhibitory effect of the acceptor site-bound N-acetyl[14C]phenylalanyl-tRNA could be due to its effect on the conformation of the ribosome. At any rate, these observations indicate that puromycin attacks the donor site peptidyl-tRNA from the side of the acceptor site. Although there is no direct evidence that puromycin is actually at the acceptor site when it reacts with the donor site peptidyl-tRNA, our present observation is consistent with the current notion that puromycin will react with peptidyl transferase in a way similar to the reaction of acceptor site aminoacyl-tRNA.

REFERENCES