STUDIES ON THE FORMATION OF TRANSFER RIBONUCLEIC ACID-RIBOSOME COMPLEXES

X. PHENYLALANYL-OLIGONUCLEOTIDE BINDING TO RIBOSOMES AND THE MECHANISM OF CHLORAMPHENICOL ACTION

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Received June 25, 1969

SUMMARY. The overall peptidyl transfer reaction can be subdivided into two distinct steps: a) binding of the aminoacyl-end of charged tRNA to ribosomes and b) the transfer of the nascent peptide from peptidyl-tRNA to aminoacyl-tRNA, the peptidyl transfer step. An assay is described which measures the binding of a phenylalanyl-oligonucleotide to ribosomes, a process which is considered to represent (a) in the absence of peptide bond formation (b). The binding of phenylalanyl-oligonucleotide to ribosomes requires Mg^{++} and K^+; it is stimulated by tRNA non-specifically. Chloramphenicol inhibits the binding of phenylalanyl-oligonucleotide to ribosomes at concentrations at which the antibiotic inhibits bacterial protein synthesis. The results are consistent with the hypothesis that chloramphenicol inhibits protein synthesis through interference with the binding of the aminoacyl-end of charged tRNA to ribosomes.

Chloramphenicol is a potent inhibitor of bacterial protein synthesis *in vivo* (1,2). *In vitro*, however, results have been variable depending on the assay used (3-6). Repeatedly, it has been shown that chloramphenicol inhibits the formation of peptidyl- and aminoacyl-puromycin (7-15). In fact, we recently showed that chloramphenicol is a competitive inhibitor of puromycin in the formation of N-acetyl-phenylalanyl-puromycin from N-acetyl-phenylalanyl-tRNA and puromycin on ribosomes (15). Since it appeared that chloramphenicol is a competitive inhibitor of puromycin, it was

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likely that it inhibits protein synthesis through competition with peptidyl- and/or aminoacyl-tRNA. Paradoxically, however, chloramphenicol interferes with net aminoacyl-tRNA binding to ribosomes little or not at all (16-18), although Wolfe and Hahn (19) reported a slight inhibition of lysyl-tRNA binding to ribosomes by the antibiotic.

Since chloramphenicol binds to and inhibits the overall peptidyl transfer function of the 50S subunit of E. coli ribosomes (13,20,21), possibly it interferes with the binding of the aminoacyl-end of aminoacyl-tRNA to 50S subunits. The peptidyl transfer reaction can be considered as two distinct steps: a) the binding of the aminoacyl-end of aminoacyl-tRNA (and peptidyl-tRNA) to the appropriate site; and b) the transfer of the nascent peptide from peptidyl-tRNA to the amino acid of aminoacyl-tRNA, the peptidyl transfer step. In this communication, a rapid assay for the first step (a) in the absence of peptide bond formation (b) is reported together with the effect of chloramphenicol on this reaction (a).

EXPERIMENTAL PROCEDURE

Ribosomes, tRNA and Phe-tRNA were prepared as previously reported (22). Aminoacyl-oligonucleotides (T1 ribonuclease digest) were prepared by minor modifications of the method described by Herbert and Smith (23). Chloramphenicol was generously supplied by Parke Davis. Radioactive phenylalanine was purchased from New England Nuclear (specific activities are recorded in the legend to the tables). Binding of Phe-oligonucleotide to ribosomes was determined by adsorbing the ribosomes on nitrocellulose filters (24). The details of each reaction are specified in each legend.

RESULTS AND DISCUSSION

The characteristics of Phe-oligonucleotide binding to 70S ribosomes are presented in Table I. Binding of the aminoacyl-oligonu-

TABLE I

REQUIREMENTS AND CHARACTERISTICS OF [^{3}H]PHE-OLIGONUCLEOTIDE BINDING TO 70S RIBOSOMES

ADDITION OR DELETION	pMOLES BOUND TO RIBOSOMES (cpm)	PERCENT OF CONTROL
Complete	0.09 (239)	100%
- Ribosomes	0.00 (11)	5%
- Mg^{++}	0.01 (23)	10%
+ poly U	0.10 (267)	112%
+ tRNA	0.20 (558)	233%
+ tRNA, + poly U	0.18 (501)	209%

Binding of [^{3}H]Phe-oligonucleotide to ribosomes was determined in 0.050-ml reaction mixtures containing the following components: 0.05 M Tris-acetate, pH 7.2; 0.05 M potassium acetate; 0.3 M ammonium chloride; 0.02 M magnesium acetate; 5159 cpm [^{3}H]Phe-oligonucleotide (sp. act. 4200) prepared from unfractionated [^{3}H]Phe-tRNA; 5.5 A_{260} units of ribosomes; 43 nmoles of base residues of poly U where indicated; and 5.1 A_{260} units of unfractionated E. coli B tRNA where designated. Incubations were performed at 24° for 10 min. and assayed as described under "Experimental Procedure". After the reaction, phenylalanine can be recovered as Phe-oligonucleotide and the amino acid is not transferred to a trichloroacetic acid precipitable state.

cleotide was dependent on the presence of ribosomes and magnesium. A poly U template was not required, a result expected since the Phe-oligonucleotide lacks an anticodon region. The addition of tRNA substantially stimulates the reaction; the presence of tRNA and poly U is approximately equivalent to addition of tRNA alone. Since purified $tRNA^{Phe}$ and $tRNA^{fMet}$ (15) could also stimulate the binding of Phe-oligonucleotide to ribosomes, the stimulation by tRNA may result from a relatively non-specific effect on ribosomes. The Phe-oligonucleotide contains the sequence CACCA (25). Thus, the stimu-

TABLE II

EFFECT OF MONOVALENT CATIONS ON

PHE-OLIGONUCLEOTIDE BINDING TO RIBOSOMES

MONOVALENT CATION	PMOLES [^{3}H]PHE-OLIGONUCLEOTIDE BOUND TO RIBOSOMES
NONE	0.00
0.1 M K^+	0.21
0.4 M K^+	0.43
0.1 M NH_4^+	0.24
0.4 M NH_4^+	0.51
0.1 M Na^+	0.00
0.4 M Na^+	0.02

Binding of [^{3}H]Phe-oligonucleotide to ribosomes was determined in 0.050-ml reactions containing the following components: 0.05 M Tris-acetate, pH 7.2; 0.03 M magnesium acetate; monovalent cation as indicated in the Table; 2.7 pmoles of [^{3}H]Phe-oligonucleotide (11,120 cpm); 4.7 A_{260} units of ribosomes washed additionally in 0.01 M Tris-acetate, pH 7.2, and 0.01 M magnesium acetate after four washes in 1 M NH_4Cl (22). Reactions were incubated at 37° for 10 min and assayed as described under "Experimental Procedure".

lation of Phe-oligonucleotide binding to ribosomes by $tRNA^{Phe}$ and $tRNA^{fMet}$ is not likely to represent attachment of the oligonucleotide to the template binding site.

The data of Table II show that K^+ or NH_4^+ ion are required for binding of the Phe-oligonucleotide; Na^+ cannot replace K^+ or NH_4^+. This requirement for K^+ or NH_4^+ for binding of the Phe-oligonucleotide to ribosomes resembles their requirement for the overall peptidyl transfer reaction (22,26,27). The binding of synthetic polyribonucleotide templates to ribosomes does not require K^+ or NH_4^+ (28). The K^+ or NH_4^+ requirement, therefore, also suggests

TABLE III

EFFECT OF CHLORAMPHENICOL ON BINDING OF [^{3}H]PHE-OLIGONUCLEOTIDE TO RIBOSOMES

CHLORAMPHENICOL MOLARITY	PERCENT OF CONTROL	pMOLES BOUND TO RIBOSOMES
0	100%	0.44
10^{-6}	79%	0.35
10^{-5}	51%	0.23
10^{-4}	26%	0.12
10^{-3}	15%	0.07

The binding of [^{3}H]Phe-oligonucleotide to ribosomes was determined as described in the legend to Table I. Chloramphenicol was added to the reactions prior to the addition of 4.3 pmoles of [^{3}H]Phe-oligonucleotide (5330 cpm), which was added last to begin the reaction. Each reaction contained 6 A_{260} units of 70S ribosomes.

that template binding is not being examined, but that binding of the Phe-oligonucleotide represents the binding of the aminoacyl-end of Phe-tRNA to ribosomes.

The effect of chloramphenicol on Phe-oligonucleotide binding to ribosomes was examined (Table III). At 10^{-6} M chloramphenicol, inhibition of Phe-oligonucleotide binding was detectable. At 10^{-4} and 10^{-3} M, there was 74 and 85% inhibition, respectively. This is comparable to the inhibition by chloramphenicol of peptidyl-puromycin formation (7-15) as well as protein synthesis *in vivo* (1,2). These results support the hypothesis that chloramphenicol inhibits protein synthesis by interfering with the binding of the aminoacyl-end of aminoacyl-tRNA to ribosomes. It is possible that the binding of peptidyl-tRNA also may be inhibited by chloram-

phenicol. Dunitz (29) and Jardetzky (30) have indicated that chloramphenicol structurally resembles a pyrimidine; and others (10,31) have previously suggested that chloramphenicol may compete with binding of the aminoacyl-end of tRNA to ribosomes. The present findings are consistent with and help to explain the varied effects of chloramphenicol on *in vitro* bacterial protein synthesis (3-6,32). In addition, the characteristics and requirements usually attributed to the peptidyl transfer reaction may reflect the requirements of binding of the aminoacyl-end of charged tRNA to ribosomes.

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