BINDING OF BLASTICIDIN S TO RIBOSOMES

TADATOSHI KINOSHITA, NOBUO TANAKA and HAMAO UMEZAWA

Institute of Applied Microbiology, University of Tokyo,
Bunkyo-ku, Tokyo, Japan

(Received for publication May 2, 1970)

The binding of blasticidin S to ribosomes is demonstrated using equilibrium dialysis. There is a single binding site per each ribosome and the association constant is $5 \times 10^5 \text{M}^{-1}$. The binding site is localized in the 50S ribosomal subunit. The binding of blasticidin S to ribosomes is inhibited by gougertotin, but not affected by chloramphenicol, lincomycin, erythromycin or puromycin.

Blasticidin S, an antibiotic containing a cytosine moiety, is a potent inhibitor of protein synthesis[1]. As reported previously[2], the antibiotic inhibits the reaction of puromycin with ribosome-bound peptidyl-tRNA, suggesting that it blocks peptidyl transfer which is catalyzed by a component integrated in the 50S ribosomal subunit[3]. Accordingly, it can be expected that blasticidin S acts on the 50S ribosomal subunit. To investigate this, binding of ^{14}C-blasticidin S to ribosomes and their subunits was studied by an equilibrium dialysis technique with the results presented in this paper.

Materials and Methods

^{14}C-Blasticidin S (labelled at 2-C of cytosine moiety; 4.6 μCi/μmole) was supplied by Dr. NOBORU OTAKE of our institute.

Ribosomes were isolated from *E. coli* B in the log phase of growth and washed with NH_4Cl-containing buffer as described by NISHIZUKA and LIPMANN[4]. T factor, G factor and tRNA were prepared as described elsewhere[5]. Ribosomal subunits were separated by centrifugation on a 5~25 % sucrose gradient in a Beckman SW-25.1 rotor at 22,500 r.p.m. (51,500 *g*) for 11 hours after dialysis of ribosomes against 10 mM Tris-HCl buffer, pH 7.4, containing 100 mM NH_4Cl, 0.1 mM magnesium acetate, and 0.5 mM dithiothreitol.

Equilibrium dialysis was carried out as follows: Ribosomes, or other components to be examined, in 0.25 ml of standard buffer (10 mM Tris-HCl, 100 mM KCl, 10 mM magnesium acetate, and 1 mM dithiothreitol, pH 7.4) were placed in a dialysis bag (Visking cellulose tubing), which was placed in a test tube containing ^{14}C-blasticidin S in 2.0 ml of standard buffer. The tubes were rotated at 4°C for 16~18 hours, a period determined to be long enough for equilibration under the conditions employed. The radioactivity of aliquots of materials inside and outside the dialysis bag was determined with a liquid scintillation counter. The difference in radioactivity between the two samples was taken to represent the amount of bound blasticidin S.

Results

By this equilibrium dialysis method ^{14}C-blasticidin S was shown to bind to ribosomes (Table 1). Little or undetectable ^{14}C-blasticidin S was found to bind to the proteins in S-100 fraction, T factor, G factor or tRNA.

To determine the number of binding sites on a ribosome and the association

Table 1. Binding of ^{14}C-blasticidin S to ribosomes

Contents in dialysis bag	mμmoles ^{14}C-blasticidin S bound
Ribosomes, 43 OD_{260} units	0.36
S-100, 3 mg protein	0.04
T factor, 0.5 mg	0
G factor, 0.5 mg	0
tRNA, 40 OD_{260} units	0
Ribosomes, 43 OD_{260} units + ^{12}C-blasticidin S, 70 mμmoles	0.07

Procedure of equilibrium dialysis is described in Materials and Methods. The outside buffer contained 7 mμmoles ^{14}C-blasticidin S.

Fig. 1. SCATCHARD plot of the equilibrium binding data.

r and A are defined in the text. To express the amount of ribosomes in terms of moles, it was assumed that 14.4 OD_{260} units are equivalent to 1 mg, and 1 mole weighs 2.8×10^6 g.

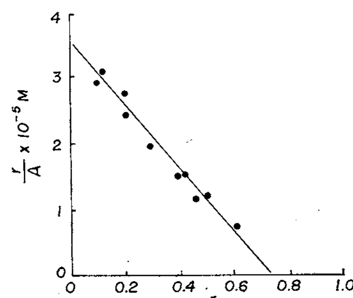

Table 2. Binding of ^{14}C-blasticidin S to 50S ribosomal subunits

	OD_{260} units	mμmoles ^{14}C-blasticidin S bound
30S	14.5	0.06
50S	29	0.35
30S + 50S	14.5 29	0.38
70S	43	0.33

The amount of ^{14}C-blasticidin S added to the outside buffer was the same as in Table 1.

Table 3. Effects of antibiotics on the binding of ^{14}C-blasticidin S to ribosomes

Antibiotic	mμmoles ^{14}C-blasticidin S bound
None	0.36
Gougerotin, 50 mμmoles	0.16
Chloramphenicol, 62 mμmoles	0.35
Lincomycin, 52 mμmoles	0.33
Erythromycin, 13 mμmoles	0.39
Mikamycin A, 16 mμmoles	0.51
Puromycin, 37 mμmoles	0.36

A dialysis bag contained 43 OD_{260} units of ribosomes and an antibiotic in an amount as indicated. Other conditions were the same as in Table 1.

constant, the equilibrium dialysis experiment was carried out over a range of ^{14}C-blasticidin S concentrations, and the data were plotted according to the SCATCHARD equation[6] for equilibrium binding, $r/A=Kn-Kr$, where r, A, n and K represent moles of bound ^{14}C-blasticidin S per mole of ribosomes, molar concentration of free ^{14}C-blasticidin S, number of binding sites on a ribosome and association constant, respectively. As illustrated in Fig. 1, the plots allowed us to draw a straight line. From its intercept on the abscissa the number of the binding sites was 0.7 and from its slope the association constant was estimated to be $5\times10^5\ M^{-1}$.

To localize the binding site with respect to ribosomal subunits, the binding experiment was carried out with separated 30S and 50S subunits. As shown in Table 2, 50S subunits alone were as active as native or reconstituted 70S ribosomes in the binding of ^{14}C-blasticidin S. In contrast, there was much less binding of ^{14}C-blasticidin S to 30S subunits. In this case the binding that did occur might be due to contamination with 50S subunits.

Effects of several other antibiotics on the binding of ^{14}C-blasticidin S to ribosomes were studied with the results presented in Table 3. In the presence of gougerotin the binding was remarkably reduced. On the other hand, chloramphenicol, lincomycin, erythromycin and puromycin did not substantially affect the binding. Mikamycin A was observed to increase the binding.

Discussion

In the present study it has been demonstrated that blasticidin S binds to ribosomes, but not to the polymerization factors or tRNA. An earlier experiment by Sephadex G-50 chromatography failed to show binding of the antibiotic to ribosomes, indicating that the binding is reversible. There is a single binding site on each ribosome and the association constant is $5\times10^{5}\ \mathrm{M}^{-1}$. The reciprocal of the association constant, $2\times10^{-6}\ \mathrm{M}$, is the concentration of free blasticidin S present when ribosomes are half-saturated with the antibiotic. This agrees fairly well with the concentration needed to give 50 % inhibition of polypeptide synthesis in the cell-free system of *E. coli* B[1,2]. This infers a close correlation between the binding of blasticidin S to ribosomes and the inhibition of polypeptide synthesis. The free energy change of the binding is calculated to be -7.3 kcal/mole, equivalent to approximately two hydrogen bonds. It is interesting to note that the N-methylguanidino group and the amino group in the cytosine moiety of blasticidin S have been reported to play an important role in inhibition of polypeptide synthesis[2].

From the study with separated ribosomal subunits, the binding site has been localized in the 50S subunit. Further localization of the binding site and correlation with peptidyl transferase is now under study.

The binding of blasticidin S to ribosomes is reduced in the presence of gougerotin. Since the two antibiotics have similar structures, the antagonism may be caused by competition for the binding site. On the other hand, chloramphenicol, lincomycin and erythromycin which binds to 50S ribosomal subunits[7,8,9] do not affect the binding of blasticidin S. This indicates that blasticidin S binds at a different site from these antibiotics. VAZQUEZ and MONRO have observed that the binding of ^{14}C-chloramphenicol to ribosomes is not inhibited by gougerotin[10]. The reason for stimulation of the binding of blasticidin S to ribosomes observed in the presence of mikamycin A is unknown at present. Puromycin has no effect on the binding, indicating that the mechanim by which blasticidin S inhibits the puromycin reaction is not competition for the identical site on the 50S ribosomal subunit.

Acknowledgement

The authors are grateful to Dr. N. OTAKE for kindly supplying ^{14}C-blasticidin S.

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