RIBOSOMAL PROTEINS S9 AND L6 PARTICIPATE IN THE BINDING OF [^{3}H]DIBEKACIN TO *E. COLI* RIBOSOMES

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(Received for publication January 16, 1981)

The degrees of binding of [^{3}H]dibekacin to LiCl-treated cores of *E. coli* ribosomes were reduced by increasing LiCl concentrations. The 1.15 M LiCl core lost 70~80% of the original binding capacity. The antibiotic attachment to the 1.15 M LiCl core was restored by reconstitution with the split proteins (SP), which were obtained by the treatment of 70S ribosomes with LiCl at concentrations of 0.8~1.15 M. The basic proteins, split off during the transition from 0.4 M LiCl core to 0.8~1.15 M LiCl core, seemed to be involved in the drug binding. $SP_{0.4\sim1.15}$, which was obtained by the treatment of the 0.4 M LiCl core with 1.15 M LiCl, was fractionated by CM-Sephadex C-25 column chromatography, and each fraction was assayed for protein composition and the capability of restoring the ability of the 1.15 M LiCl core to bind the drug. Of ribosomal proteins eliminated with 1.15 M LiCl, the addition of either S9 or L6 alone to the 1.15 M LiCl core was observed to restore approximately 50% of the binding as compared to the 70S ribosome alone, and both proteins restored about 70% of the binding. The results suggested that ribosomal proteins S9 and L6 were involved in the attachment of [^{3}H]dibekacin to the ribosome. The antibiotic binding to the 70S ribosome and 1.15 M LiCl core reconstituted with S9 or L6 was considerably inhibited by unlabelled dibekacin or kanamycin, and partially inhibited by gentamicin or neomycin, but was not significantly affected by streptomycin or viomycin.

The mechanism of action of the aminoglycoside group of antibiotics differ. Kasugamycin binds to the 30S ribosomal subunit and selectively inhibits initiation of protein synthesis[1,2]. Codon misreading is caused by streptomycin, kanamycin, neomycin, gentamicin and related aminoglycosides, but not by kasugamycin[3]. Translocation of peptidyl-tRNA from the acceptor site to the donor site on the ribosome is blocked by kanamycin, neomycin, gentamicin and related antibiotics but not significantly by streptomycin[4]. Kanamycin interferes with the translocation by fixing peptidyl-tRNA to the acceptor site but not to the donor site[5]. Streptomycin selectively binds to the 30S ribosomal subunit (*cf.* a review by WALLACE *et al.*[6]); but kanamycin, neomycin and gentamicin bind to both 30S and 50S ribosomal subunits[4]. In some kanamycin-resistant mutants, the resistance is attributed to mutational alterations of the 30S ribosomal subunit in some mutants, and to the 50S subunit in the others[7], but streptomycin resistance is due exclusively to changes of the 30S subunit[6].

For the purpose of elucidating what kind of ribosomal proteins are involved in the binding of kanamycin to the ribosome, we have studied the binding of [^{3}H]dibekacin to the reconstituted ribosomes, following the method of SCHREINER and NIERHAUS[8], and the results are presented in this publication. [^{3}H]Dibekacin is employed in the current experiment, because the highly radioactive compound is available.

Materials and Methods

[3′,4′-^{3}H]Dibekacin (66 μCi/mg) was kindly supplied by Dr. S. FUKATSU, Central Research Laboratories, Meiji Seika Kaisha, Ltd., Yokohama, Japan. Kanamycin, dibekacin, neomycin and streptomy-

cin are products of Meiji Seika Kaisha, Ltd., gentamicin of Schering Corporation, Bloomfield, New Jersey, and viomycin of Pfizer Taito Co., Ltd., Japan. All other chemicals were of the highest grade available commercially.

Preparation and Reconstitution of Ribosomes

The ribosomes, washed with 1 M NH_4Cl, were prepared from *E. coli* Q13, following the procedure described previously[4]. The preparation of LiCl cores and split proteins (SP), and partial reconstitution were carried out according to NIERHAUS and MONTEJO[9]. The 70S ribosomes (35 A_{260} units/ml) were incubated in TM-buffer (20 mM tris-HCl, pH 7.6, and 1 mM magnesium acetate), containing various concentrations of LiCl, for 5 hours at 0°C. The cores were sedimented at 143,000×g for 5 hours, suspended in core buffer (20 mM tris-HCl, pH 7.6, 20 mM magnesium acetate, 1 mM EDTA, 200 mM NH_4Cl and 2 mM 2-mercaptoethanol) and dialyzed against the same buffer. The supernatant containing SP was dialyzed against protein buffer (the same as core buffer with 400 mM NH_4Cl). The core particles 5 A_{260} units in 25 μl of core buffer, were added to 7 equivalent units of SP in 75 μl of protein buffer. The partial reconstitution was performed by incubation of the core and SP at 50°C for 60 minutes.

Molar concentrations of ribosomes, cores, and reconstituted particles were calculated from the optical density at 260 nm.

Fractionation of $SP_{0.4\sim1.15}$ by CM-Sephadex C-25 Chromatography

The core, prepared by the treatment of 70S ribosomes with 0.4 M LiCl, was further treated with 1.15 M LiCl, and the $SP_{0.4\sim1.15}$ was obtained. The acidic proteins and RNA in $SP_{0.4\sim1.15}$ were removed by DEAE-cellulose chromatography, using TM-buffer, pH 8.6. The basic protein fraction was dialyzed against HL-buffer (20 mM HEPES-NaOH, pH 7.0, and 0.15 M LiCl), and applied to CM-Sephadex C-25 column, packed in the same buffer. Adsorbed proteins were eluted by a 0.15~0.8 M LiCl gradient. A sample of each effluent fraction was tested for protein composition by SDS-polyacrylamide gel electrophoresis[10]. The gels were stained with amido black and scanned with a Toyo Digital Densitol DMU-33C, and the protein areas were computed. The remaining portion of each effluent fraction, after dialysis against protein buffer, was reconstituted with 1.15 M LiCl core, which was obtained by the treatment of 70S ribosomes with 1.15 M LiCl.

The protein composition of ribosomes, cores, and split proteins was analyzed by two-dimensional polyacrylamide gel electrophoresis by the method of KALTSCHMIDT and WITTMANN[11].

Binding of [^{3}H]Dibekacin

The binding of [^{3}H]dibekacin to ribosomes, cores and reconstituted particles was assayed by nitrocellulose (Millipore) filter method, as described previously[7]. The concentrations of the components of reaction mixture (100 μl) were: 1 μM ribosomes, cores or reconstituted particles, 10 μM [^{3}H]dibekacin, 20 mM tris-HCl, pH 7.6, 16 mM magnesium acetate, 280 mM NH_4Cl, and 2 mM 2-mercaptoethanol. Mixtures were incubated at 30°C for 5 minutes, and the radioactivity, collected on Millipore filters, was determined with correction for the values in a parallel mixture without ribosomal particles.

Results

Binding of [^{3}H]Dibekacin to LiCl-treated Cores of Ribosomes

By the treatment of 70S ribosomes with increasing concentrations of LiCl, a series of cores, which were increasingly devoid of proteins, was obtained, and the binding of [^{3}H]dibekacin to these cores was studied. As presented in Fig. 1, the extent of the antibiotic attachment to the LiCl-treated cores was reduced by increasing LiCl concentrations. The cores lost more than 70% of the original binding capability by the treatment with LiCl at concentrations above 1 M.

Binding of [^{3}H]Dibekacin to the Reconstituted Particles

The binding of [^{3}H]dibekacin to the 1.15 M LiCl core was effectively restored by reconstitution with split proteins (SP), which were obtained by the treatment of 70S ribosomes with 0.8~1.15 M LiCl

Fig. 1. Binding of [^{3}H]dibekacin to LiCl cores of ribosomes, treated with various LiCl concentrations.
100%=120 pmole of [^{3}H]dibekacin bound per 100 pmole of 70S ribosomes.

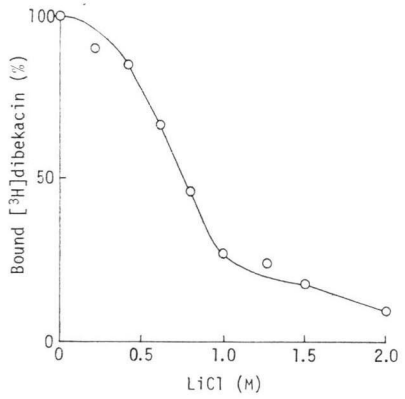

Table 1. Binding of [^{3}H]dibekacin to reconstituted particles (I).

Particle	[^{3}H]Dibekacin bound (%)
70S Ribosome	100
1.15 M LiCl core	23
+$SP_{0.4}$	39
+$SP_{0.8}$	70
+$SP_{1.0}$	93
+$SP_{1.15}$	95
+DEAE-$SP_{1.15}$	97
$SP_{1.15}$	4

The reconstitution was performed with 1.15 M LiCl core and split proteins. $SP_{0.4}$, $SP_{0.8}$, $SP_{1.0}$ and $SP_{1.15}$ represent split proteins, obtained by the treatment of 70S ribosomes with 0.4, 0.8, 1.0 and 1.15 M LiCl, respectively. DEAE-$SP_{1.15}$ stands for $SP_{1.15}$, from which the acidic proteins and RNA were removed by DEAE-cellulose chromatography.
100 %=120 pmole of [^{3}H]dibekacin bound per 100 pmole of 70S ribosomes.

(Table 1). Even when the acidic proteins and RNA were removed from $SP_{1.15}$ (split proteins with 1.15 M LiCl) by DEAE-cellulose chromatography, the remaining proteins restored the antibiotic binding to the same degree as with the untreated $SP_{1.15}$. The results suggested that the basic proteins, split off during the transition from 0.4 M LiCl core to 0.8~1.15 M LiCl core, play an important role in binding [^{3}H]dibekacin. Table 2 shows the protein composition (determined by two-dimensional gel electrophoresis) of the 0.4 M LiCl core, the 1.15 M LiCl core, and the $SP_{0.4\sim1.15}$ after removal of acidic proteins by DEAE-cellulose chromatography. The 1.15 M LiCl core contained: S4, S5, S6, S7, S8, S9, S13, S15, S17, S18, S19, S20, L1, L3, L4, L5, L9, L12, L13, L15, L17, L18, L19, L21, L22, L23, L24, L25, L26

Fig. 2. Elution profile of $SP_{0.4\sim1.15}$ in CM-Sephadex C-25 column chromatography.

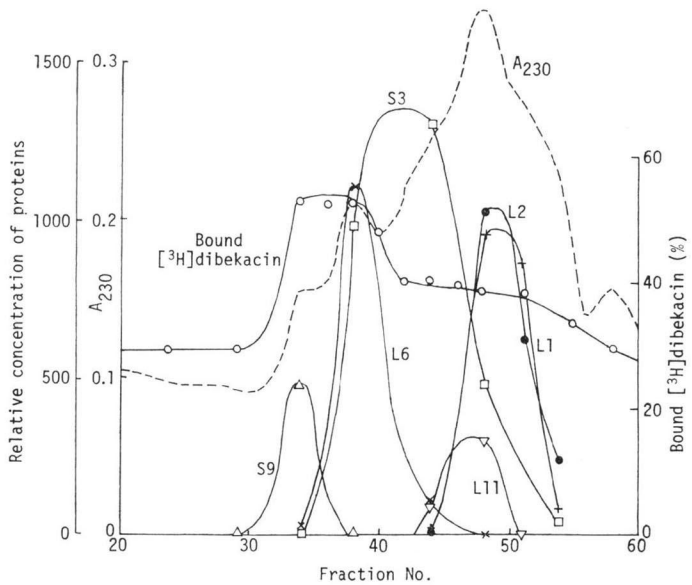

Table 2. Protein composition of LiCl cores and $SP_{0.4\sim1.15}$.

Protein	0.4M LiCl core	1.15M LiCl core	$SP_{0.4\sim1.15}$	Protein	0.4M LiCl core	1.15M LiCl core	$SP_{0.4\sim1.15}$
S1	(+)	−		L7	(±)	−	
S2	(+)	−		L8	+	−	
S3	(+)	−	+	L9	+	+	
S4	+	(+)	+	L10	+	−	
S5	+	+		L11	+	−	+
S6	+	+		L12	+	+	
S7	+	+		L13	+	+	+
S8	+	+		L14	(+)	−	
S9	+	(+)	+	L15	+	(+)	+
S10	+	−		L16	(±)	−	+
S11	−	−		L17	+	+	
S12	−	−		L18	+	+	+
S13	+	+		L19	+	+	+
S14	+	−		L20	−	−	
S15	+	+		L21	+	+	
S16	(±)	−	+	L22	+	+	
S17	(±)	+		L23	+	+	
S18	+	+		L24	+	+	
S19	+	+		L25	+	(+)	+
S20	+	+	+	L26 (S20)	+	+	+
S21	(±)	−	+	L27	(±)	−	+
L1	+	(+)	+	L28	−	−	
L2	+	(±)	+	L29	+	+	
L3	+	+		L30	+	(±)	
L4	+	+		L31	−	−	
L5	(+)	+		L32	+	+	
L6	+	−	+	L33	−	−	

+: Normal amount, (+): Reduced amount, (±): Trace, −: Not detectable.

(S20), L29, and L32: and $SP_{0.4\sim1.15}$: S3, S4, S9, S16, S20, S21, L1, L2, L6, L11, L13, L15, L16, L18, L19, L25, L26 (S20), and L27.

Ribosomal Proteins Participating in the Binding of [^{3}H]Dibekacin

For the purpose of determining the proteins necessary for the antibiotic binding, the basic proteins of $SP_{0.4\sim1.15}$ were fractionated by CM-Sephadex C-25 column chromatography. Each fraction was reconstituted with the 1.15 M LiCl core, and the binding of [^{3}H]dibekacin was assayed. A portion of each fraction was simultaneously analyzed by SDS-polyacrylamide gel electrophoresis; and each protein band on the gel was assayed as described in Materials and Methods. A part of the elution profile is illustrated in Fig. 2. Fraction 34, containing ribosomal protein S9, induced a significant increase of the drug binding to the 1.15 M LiCl core; and fraction 38, including L6 and S3, also enhanced the antibiotic attachment. In contrast, fraction 44, containing S3 but lacking in L6, showed less effect on stimulating the capability of the core to bind [^{3}H]dibekacin. Therefore, the data suggest that proteins S9 and L6 participated in the drug binding to the reconstituted particles. Either S9 or L6 alone was able to promote approximately 50% and the two proteins together about 70% of the original binding capacity of the

Table 3. Binding of [^{3}H]dibekacin to reconstituted particles (II).

Particle	[^{3}H] Dibekacin bound (%)
70S Ribosome	100
1.15M LiCl core	29
+fr. 34: S9	53
+fr. 38: L6 (S3)	53
+fr. 34 & 38: S6 & L6 (S3)	70

The reconstitution was carried out with 1.15M LiCl core and split protein fractions of CM-Sephadex C-25 column chromatography: Fraction 34 contained S9 protein, and fraction 38 L6 and S3 proteins.

100%=120 pmole of [^{3}H]dibekacin bound per 100 pmole of 70S ribosomes.

Table 4. Effects of antibiotics on the binding of [^{3}H]dibekacin to the 70S ribosome and reconstituted particles.

Antibiotic	Ribosomal particles			
	70S Ribosomes	Core+ S9	Core+ L6	Core*
None	100%	47	48	20
Dibekacin	43	20	18	11
Kanamycin	49	22	19	
Gentamicin	78	42	37	
Neomycin	63	40	39	
Streptomycin	92	46	43	
Viomycin	94	45	45	

* 1.15M LiCl core of ribosomes.
100% is the same as in Table 3.

70S ribosome (Table 3). The results suggested that ribosomal proteins S9 and L6 are involved in the attachment of [^{3}H]dibekacin to the ribosome.

Relationship of Binding Sites of Kanamycin, Dibekacin, Neomycin, Gentamicin and Viomycin on the Ribosome

Effects of 10-fold higher concentrations of some antibiotics on the binding of [^{3}H]dibekacin to the 70S ribosome and 1.15 M LiCl cores with S9 or L6 were examined, and the results are summarized in Table 4. The attachment of [^{3}H]dibekacin to the ribosome and reconstituted particles was reduced to 40~50% by the presence of unlabelled dibekacin, indicating that the observed binding was specific for the antibiotic. The 10 fold dilution by the unlabelled drug caused only *ca.* 60% reduction in binding, because increased antibiotic concentration resulted in enhanced binding of dibekacin.

Kanamycin was found to reverse the binding of [^{3}H]dibekacin at the same level as cold dibekacin. The radioactive binding seemed to be somewhat decreased by gentamicin or neomycin but was not significantly affected by streptomycin or viomycin. The competition profile of dibekacin binding to the core with S9 or L6 appeared to be identical with that to the 70S ribosome, also suggesting that S9 and L6 are involved in the binding site of dibekacin as well as kanamycin.

Discussion

The current experiments suggest that ribosomal proteins S9 and L6 are involved in the attachment of dibekacin to *E. coli* ribosomes, or are the binding site of the drug. S9 is contained in the 30S subunit and L6 in the 50S subunit. Therefore, the results are in accord with the previous findings that kanamycin, neomycin and gentamicin bind to both 30S and 50S ribosomal subunits[4] and that kanamycin resistance is linked to the 30S subunit in some of resistant mutants and to the 50S subunit in others[7]. It is worthy of note that ribosomal protein L6 is altered in some mutants resistant to gentamicin[12].

Of the aminoglycoside group of antibiotics, the binding site on the ribosome has been most extensively investigated with streptomycin. OZAKI *et al.*[13] and BIRGE and KURLAND[14] have shown by reconstitution experiments that streptomycin resistance and dependency are due to alteration of ribosomal protein S12. S12 controls the binding of the drug to the ribosome, but neither itself binds the antibiotic nor forms a portion of the attachment site. None of the separated ribosomal proteins binds streptomycin, and the conformation of the 30S ribosomal subunit seems to be required for the drug bind-

ing. CHANG and FLAKS[15] have demonstrated by successive treatment of the 30S ribosomal subunit with trypsin that the removal of S9 and S14 results in a loss of dihydrostreptomycin binding. SCHREINER and NIERHAUS[8] have found that the 30S ribosomal subunit loses the capacity to bind dihydrostreptomycin by washing in 1.15~2.0 M LiCl. Of proteins eliminated, S3 and S5 can restore the attaching ability by the nonbinding core, and the capacity of S3+S5 is enhanced by addition of S9, S10 and S14; but the binding is independent of the presence of S12. By treating ribosomes with graded concentrations of N-ethylmaleimide, which reacts with sulfhydryl groups, GINZBERG *et al.*[16] have assigned S1, S14 and S21 to the streptomycin binding site. By affinity labelling of ribosomes with different photoactive analogs of streptomycin, PONGS and ERDMANN[17] have labelled S3 and S4, and GIRSHOVICH *et al.*[18] S7, S14 and S16/S17. LELONG *et al.*[19] have investigated the binding site of streptomycin, using specific antibody fragments (Fab) for 30S ribosomal proteins, and observed that antibodies for S1, S10, S11, S18, S19, S20 and S21 interfere with the binding of dihydrostreptomycin to 30S or 70S ribosomes. BISWAS and GORINI[20], and GARVIN *et al.*[21] have presented evidence that 16S RNA is the binding site of streptomycin and that it binds 2 molecules per RNA molecule. As described above, the diverse results obtained by a number of investigators using different methods make it impossible to specify any ribosomal component(s) for the attachment site of streptomycin. Likewise, the precise binding site of dibekacin remains to be determined, although the current experiments suggest participation of ribosomal proteins S9 and L6 in the binding of dibekacin to the ribosome. Moreover, the 1.15 M LiCl core shows approximately 20% of the capability of intact ribosomes to bind dibekacin, suggesting that ribosomal proteins remaining in the core are also involved in the drug binding.

The competition experiments reveal that the binding site of dibekacin may be identical with or closely related to that of kanamycin. The site may be somewhat related to but different from those of gentamicin and neomycin, but it may differ considerably from those of streptomycin and viomycin. The experiments also suggest that ribosomal proteins S9 and L6 are involved in the binding site of kanamycin on the ribosome.

Acknowledgement

The authors express their deep thanks to Dr. HAMAO UMEZAWA, Institute of Microbial Chemistry, Tokyo, for his generous advice and cooperation during the current study.

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