THE ROLE OF RIBOSOMAL PROTEIN FOR THE BINDING OF DIHYDROSTREPTOMYCIN TO RIBOSOMES

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Although the lethal action of streptomycin (SM) may not be related to its effect on the protein biosynthesis, it appears clear that SM influences ribosomal function through inhibition or miscoding (Davies et al., 1964). The target of SM action is apparently 30S ribosomal subunits (Cox et al., 1962). Thus, SM inhibits the specific binding of phenylalanyl tRNA to the complex of poly U-30S ribosomal subunits (Kaji et al., 1966). On the other hand, no miscoding effect was observed with the binding of aminoacyl tRNA to 30S ribosomal subunits while miscoding takes place with 70S ribosomes (Kaji, 1967) suggesting that 50S ribosomal subunits may play some role for the miscoding by SM. In our preceding communication we reported that one molecule of dihydrostreptomycin (DHSM) binds to a 30S ribosomal subunit. In this paper we report that proteins of core particle, but not RNA, isolated from SM-sensitive 30S ribosomal subunits are responsible for the binding of ^{3}H-DHSM.

MATERIALS AND METHODS

Escherichia coli B and SM-resistant mutant of the same strain were grown as described (Kaji and Kaji, 1964). Binding of ^{3}H-DHSM to ribosomes and specific binding of ^{14}C-phenylalanyl tRNA to the complex of poly U and ribosomes were performed as described (Kaji and Kaji, 1964; Kaji and Tanaka, 1968). For the preparation of core particles and split proteins (Meselson et al., 1964) 2.5 mg of 30S ribosomal subunits were suspended in 1 ml of buffer containing 0.04 M magnesium acetate, 0.02 M Tris-HCl buffer (pH 7.4) and 0.006 M β-mercaptoethanol. The suspension was mixed with 1.2 ml of CsCl (specific gravity 1.4) solution in

the above buffers and was called mixture 1. Another CsCl solution (specific gravity 1.8) in the same buffer was prepared and this was called mixture 2. A linear gradient (4.6 ml) was prepared using mixture 1 and mixture 2. The tube was centrifuged for 10 hrs at 35,000 rpm in Beckman Spinco SW-39 rotor. After the centrifugation, the top 0.5 ml solution containing split protein was carefully removed. The 3 drop fractions were then collected by puncturing the bottom of the tube. The core particle fraction appeared in the middle of the tube. The split protein and the 23S core particles thus prepared were stored in liquid nitrogen.

For the reconstitution of 30S ribosomal subunits from split protein and the 23S core particle, a modified method of Hosokawa et al., (1966) was used. Core particles (9.6 OD^{260} units in 2 ml) obtained from the CsCl centrifugation were mixed with the split protein 1.65 OD^{260} in 1.5 ml of the CsCl solution. The mixture was dialyzed against a buffer containing 0.2 M LiCl, 0.01 M magnesium acetate, 0.01 M Tris-HCl (pH 7.8), 0.03 M NH_4Cl and 6 mM β-mercaptoethanol for 5 hrs at 4°C. The mixture was then further dialyzed overnight at 4°C against the same buffer as above except for the omission of LiCl (this buffer was called buffer 1). To make the conditions comparable the core particles alone were subjected to the same treatment in the absence of split protein and the split protein alone was also subjected to the identical treatment except for the omission of the second dialysis step.

For the reconstitution of 23S core particle from 16S RNA and core protein, a modified method of Traub and Nomura (1968a) was used. For preparation of protein from 23S core particles, the core particles (212 OD^{260} units in 5.8 ml) obtained by the density gradient centrifugation were suspended in 5.8 ml of a solution containing 8 M urea and 4 M LiCl. The mixture was kept standing at 0°C for 48 hrs and the precipitated RNA was removed by centrifugation. The supernatant fluid was dialyzed overnight at 4°C against a buffer (pH 7.4) containing 5 mM K-phosphate, 17 mM $MgCl_2$, 3 mM $CaCl_2$, 0.5 M KCl and 6 mM β-mercaptoethanol (buffer 2).

For preparation of 16S RNA from 23S core particles, the core particles

(473 OD^{260} units in 14.6 ml) obtained by the density gradient centrifugation were dialyzed against the buffer 1 containing 0.01 M $MgCl_2$, instead of magnesium acetate for 2 hrs at 4°C. The suspension was further dialyzed against buffer 2 for 2 hrs. To this mixture equal volume of phenol saturated with buffer 2 was added, and shaken at 4°C for 10 minutes. The aqueous phase was mixed with 2 volumes of alcohol and the precipitated RNA was collected by centrifugation. The RNA was then dissolved in distilled water and dialyzed overnight against buffer 2 containing 0.25 M KCl, at 4°C. The split protein obtained from the CsCl gradient centrifugation was dialyzed against 6 M urea, 2 M LiCl, 0.01 M Tris-HCl (pH 7.5), 6 mM β-mercaptoethanol for 8 hrs at 4°C. The mixture was then dialyzed against buffer 2 for 9 hrs.

The reconstitution of 30S subunits from 16S RNA, 23S core protein, and split protein was performed as follows: 16S RNA (83 OD^{260} units in 0.6 ml) were mixed with 5.6 ml of buffer 2 except for the omission of KCl. The temperature of the mixture was raised to 37°C and the core protein solution (27 OD^{260} units in 1 ml) was added dropwise for 10 minutes. The mixture was incubated for 20 minutes at 37°C. The split protein solution (10 OD^{260} units in 3.5 ml) was then added dropwise to this mixture for five minutes and the total mixture was then incubated for 15 minutes at 37°C followed by incubation at 4°C for 20 minutes. The mixture was dialyzed for 9 hrs at 4°C against buffer 2 containing 1 M KCl. The mixture was dialyzed against buffer 1 for 8 hrs at 4°C. The reconstituted 30S subunits were centrifuged at 150,000 g for 8 hrs and the pellet was suspended in buffer 1. Specific activity of phenylaline was 300 μc/μmole. The labeled DHSM had a specific activity of 3.77×10^7 cts/min/μmole of material having the molecular weight which corresponds to $C_{21}H_{41}N_7O_{12}$.

RESULTS AND DISCUSSION

Binding of ^{3}H-DHSM and ^{3}H-phenylalanyl tRNA to the 30S subunit which has been reconstituted from CsCl core and split protein. Table 1 shows the DHSM binding capacity of 30S ribosomal subunits which have been reconstituted from

TABLE I

Binding of ^{3}H-DHSM and ^{3}H-phe-tRNA to the 30S subunit which has been reconstituted from CsCl core and split protein

Ribosome components	Bound to subparticles	
	^{3}H-DHSM	^{3}H-phe-tRNA
	(cpm)	
SM-sensitive core alone	32	33
SM-sensitive core and SM-sensitive split protein	135	730
SM-resistant core alone	13	33
SM-resistant core and SM-resistant split protein	40	420
SM-sensitive core and SM-resistant split protein	165	805
SM-sensitive split protein and SM-resistant core	41	733

The reaction mixture (0.4 ml) for the binding of ^{3}H-DHSM or ^{3}H-phenylalanyl tRNA contained 0.24 OD_{260} unit of ribosomes, or their components, 80 µg of poly U 23,000 cpm of ^{3}H-DHSM or 24,000 cpm of ^{3}H-phenylalanyl tRNA, 0.02M magnesium acetate, 0.01 M Tris-HCl buffer (pH 7.4), 0.03 M NH_4Cl, and 6 mM β-mercaptoethanol. The mixture was incubated at 23°C for 20 minutes and the bound radioactivity was measured by the Millipore Filter paper technique.

core particles and split proteins. It is noted the SM-sensitive core alone did not bind DHSM, but when combined with split protein it gained the binding activity. On the other hand, when SM-resistant core was used, even in the presence of split protein, the binding capacity of the reconstituted 30S ribosomal subunits was very low. The split protein from the SM-sensitive 30S ribosomal subunits gave much less DHSM binding when combined with core particles obtained from SM-resistant 30S subunits. These results indicate that the SM-binding activity is determined by the core particle despite the fact that both core particle and split protein were necessary for the binding of DHSM. The

data in this table are consistent with the previous conclusion that the SM-sensitivity resides in the 23S core particle (Staehelin and Meselson, 1966; Traub et al., 1966).

It is noted in this table that the binding capacity of the reconstituted 30S subunits for phenylalanyl tRNA was about the same with various preparations regardless of whether 23S core particles were derived from streptomycin-resistant ribosomes or not. One exception to this was the case where 30S ribosomal subunits were reconstituted from core and split protein, which were both derived from SM-resistant ribosomes. It should be pointed out that the 30S subunits from the SM-resistant strain are occasionally lower in their binding capacity for phenylalanyl tRNA.

Binding of ^{3}H-DHSM and ^{14}C-phenylalanyl tRNA to the 30S ribosomal subunits which were reconstituted from RNA and protein. The data presented in Table II show the binding of DHSM to the 30S ribosomal subunits, which were reconstituted from RNA and protein. It is clear from this table that maximum binding of the ^{3}H-DHSM was observed when core protein was derived from the SM-sensitive strain. It should be noted that even when 16S RNA derived from the SM-sensitive strain was used, the reconstituted particles were not able to bind the labeled DHSM if the protein from the resistant 23S particle was used. This indicates that the capacity to bind labeled DHSM resides in the protein, and not RNA, of 30S ribosomal subunits. On the other hand for the binding to take place, total 30S particle is necessary because the SM-sensitive 23S core particle alone, in the absence of split protein, bound no appreciable amount of DHSM. Recently, Traub and Nomura (1968b) reported that polyphenylalanine formation by reconstituted 30S ribosomal subunits and 50S ribosomal subunits was sensitive to streptomycin only when protein of the sensitive core particle was used. This observation, together with the observation reported in this paper, clearly establishes that the sensitivity of the ribosomal function to streptomycin resides in the protein derived from 23S core particles. In the experiment shown in Table II, the origin of split protein did not make any difference as to the

TABLE II

Binding of ^{3}H-DHSM and ^{14}C-phe-tRNA to reconstituted 30S subunits

SM-sensitive strain		SM-resistant strain			Bound	
Core protein	16S RNA	Core protein	16S RNA	Split protein	^{3}H-DHSM	^{14}C-phe-tRNA
					(cpm)	
+	+	-	-	+	400	289
-	+	+	-	+	124	351
+	-	-	+	+	367	345
-	-	+	+	+	82	346

The reaction mixture for the binding of ^{3}H-DHSM or ^{3}H-phenylalanyl tRNA contained 1.66 OD_{260} units of reconstituted 30S ribosomal subunits, 8 μg of poly U, 15,000 cpm of ^{3}H-phenylalanyl tRNA or 10,300 cpm of ^{3}H-DHSM, 0.02 M magnesium acetate, 0.01 M Tris-HCl buffer (pH 7.4), 0.03 M NH_4Cl and 6 mM β-mercaptoethanol. The reaction mixture was incubated at 23°C for 20 minutes and the bound radioactivity was measured by the Millipore Filter paper technique.

capacity of the reconstituted 30S subunits to bind DHSM.

As can be seen from the last column of this table, the amount of bound ^{14}C-phenylalanyl tRNA was similar with all these reconstituted 30S ribosomal subunits. The somewhat low value with the 30S subunits reconstituted from sensitive RNA and protein remains to be explained.

SUMMARY

Binding of ^{3}H-dihydrostreptomycin to reconstituted 30S subunits was studied. It was found that protein from a 23S core particle is responsible for the sensitivity of ribosomes to streptomycin.

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