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The Attachment Site of Streptomycin to the 30S Ribosomal Subunit

(streptomycin–ribosomal binding/antibiotic/protein synthesis/16S RNA/protein P10)

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ABSTRACT **The 16S RNA dissociated from 30S ribosomal subunits of *Escherichia coli* strains either sensitive or resistant to streptomycin contains the attachment sites for two streptomycin molecules, as does the undissociated particle from a streptomycin-sensitive strain. Since no streptomycin binds to undissociated 30S subunits from a streptomycin-resistant strain, it is suggested that protein P10, specified by the *strA* locus—known to be responsible for drug sensitivity—controls the availability to streptomycin of the attachment sites. These sites remain exposed in the *strA*$^+$ wild-type, and become masked in *strA* streptomycin-resistant mutants.**

The 16S RNA molecule binds streptomycin specifically; it binds two drug molecules in its native state, binds many more after its secondary structure is unfolded by melting out, and again binds two molecules after reannealing. The binding is stable to exhaustive dialysis, but it is reversed by exposure of the streptomycin–RNA complex to high-salt concentration. The complex can not be used to reconstitute functional ribosomes, but the 16S RNA reacquires this property after streptomycin elimination.

The biological significance of this stable streptomycin binding is questioned, since in *strA* mutants exhibiting phenotypic masking, exposure to streptomycin induces a modified 30S behavior that persists even after streptomycin has been dialyzed away, and is reversed only by exposure of the modified RNA to high-salt concentration.

It is known from equilibrium dialysis studies that 30S ribosomal subunits extracted from wild-type, streptomycin-sensitive (Sm^S), *Escherichia coli* bind two molecules of streptomycin (Sm). In contrast, 30S ribosomal subunits extracted from a *strA* mutant, streptomycin-resistant (Sm^R), fail to bind the drug (1, 2). It is also known that P10, the 30S protein specified by the *strA* gene, is involved in this binding, since a mutation in *strA* confers resistance (2). From the inability of either P10, or of the artificially reconstituted P10-less subunit (2), to bind Sm, it has been surmised that the Sm attachment site is generated during the association process of the entire particle. In an attempt to determine the primary Sm-attachment site, we have dissociated the 30S subunit into proteins and 16S RNA and, upon exposure to [^{14}C]streptomycin, studied the ability of each component (*a*) to bind Sm, and (*b*) to reconstitute a functional ribosome. Binding of Sm to either 30S component was determined by measurement of the amount of radioactivity remaining after exhaustive dialysis. Under these conditions, two molecules of Sm bind to undissociated 30S subunits derived from a Sm^S strain, the same number found to be bound by equilibrium dialysis. In this paper, we report that the mixture of 30S proteins derived from either Sm^S or Sm^R strains does not bind Sm, as one might have expected. Instead, we found that naked 16S RNA binds the same number of Sm molecules as does the complete Sm^S 30S subunit. This binding is independent of the *strA* allele of the ribosomes from which the 16S RNA was derived.

We have also found that the two Sm molecules bound to 16S RNA prevent reconstitution of a functional ribosome, as long as they are not removed by a treatment more drastic than dialysis. This finding, together with the fact that Sm sensitivity is always associated with the firm binding of two Sm molecules to the 30S subunit, led to the assumption that this type of binding is responsible for the Sm action. We were able to challenge this conjecture by extending our study to a mutant of the drug-dependent class (Drug^D). A Drug^D strain—so-called (3) because it is dependent on either Sm, paromomycin (Pm), or ethanol—carries a mutation in the same *strA* gene as a Sm^R mutant (4). It is peculiar, however, because it is dependent on (and therefore resistant to) either Sm or Pm separately, but sensitive to these two drugs used in combination. Furthermore, cells grown in ethanol are resistant to Sm or Pm, but those grown in either Sm or Pm are killed by Pm or Sm, respectively, in spite of extensive intermediate washing. This phenomenon has been called "phenotypic masking" (3), and is paralleled (5) by a similar behavior of the ribosomes. In other words, ribosomes extracted from cells grown in ethanol do not misread artificial messengers in an *in vitro* incorporation system in the presence of either Sm or Pm, but those extracted from cells grown in Sm or Pm do misread when tested against Pm or Sm, respectively. The phenomenon of phenotypic masking displayed by the Drug^D strains suggests that Sm may modify the behavior of the 30S subunit under conditions in which the firm binding of two Sm molecules does not occur. The results presented in this paper confirm this suggestion.

MATERIALS AND METHODS

Bacterial Strains. Three derivatives of *E. coli* B were used: (*a*) the Sm^S (Pm^S) *strA*$^+$ L1 (*argF40 argR15*) strain (6); (*b*) the Sm^R (Pm^S) *strA1* L1-401 strain, a spontaneous mutant of L1 that survives Sm exposure (7, 8); and (*c*) the Drug^D *strAd1* P48S23 strain (5), a spontaneous mutant of wild-type B that survives Pm exposure, isolated first as a dependent strain and then as an independent in the course of its purification. It is the result of two independent mutations, and carries the *strAd* allele of the Drug^D class. However, through a second mutation external to *strA*, the strain became drug independent; it retains the property of Sm^R and Pm^R when

Abbreviations: Sm, streptomycin; Pm, paromomycin.

TABLE 1. *Sm binding to Sm^S 30S subunits and to their dissociated components*

Sm* present during binding incubation	Sm* bound to		
	Undissociated 30S subunit	Dissociated 30S components: 16S RNA	Dissociated 30S components: Total proteins
1	0.34	—	—
10	1.70	1.10	0
100	2.20	2.00	0
300	—	2.20	—

* Molecules of Sm per 30S subunit or component thereof. Ribosomes extracted from Sm^S (L1) strain.

the two drugs are present separately, but is Sm^S Pm^S when the two drugs are in combination. Genetic study of the second mutation was not pursued further.

Growth Conditions. Cultures were grown in medium L (9) (Bacto-tryptone 1%, yeast extract 0.5%, sodium chloride 0.5%, glucose 0.2%) at 37° with vigorous aeration. Strain P48S23 was also grown in medium L containing 200 μg of Pm per ml. Cells were harvested at the end of the log phase (10^9 cells/ml), washed, and frozen (see ref. 5).

Preparation of Ribosomal Components. 70S Ribosomes were prepared from frozen cells as described (10); high-salt R1 buffer was used throughout the whole extraction procedure (1 M NH_4Cl–10 mM Tris·HCl, pH 7.6–10 mM $MgCl_2$–6 mM 2-mercaptoethanol). Subunit dissociation was performed by dialysis against low-Mg buffer II (10). Large-scale separation was done in a 7.5–35% sucrose gradient (11) by zonal centrifugation in a Ti15 rotor at 24,000 rpm for 15 hr. RNase activity was eliminated from the 30S subunits adsorbed on DEAE-cellulose by successive elution of the column with 0.25M and 0.5M NH_4Cl. 30S subunits (RNase-free by the test of [^{14}C]poly(U) hydrolysis) were finally eluted with 1M NH_4Cl. The total 30S protein fraction was prepared by 4 M LiCl–8 M urea treatment; 16S RNA was isolated either by the same method or by phenol extraction (for details of these manipulations and of the 30S reconstitution procedure, see ref. 10). Biological activity of reconstituted ribosomes was tested by measurement of poly(U)-directed phenylalanine incorporation and, when necessary, by determination of misreading (5).

Drug Binding Conditions. Sm sulfate or [^{14}C]Sm chloride (0.052 μCi/mg; a gift of Merck & Co., Rahway, N.J.) or Pm sulfate (a gift of Parke-Davis & Co., Detroit, Mich.) were added at the desired concentration to 1 ml of a solution containing RNA (about 1 mg/ml), or total proteins, or 30S subunits (about 3 mg/ml) in the following buffer: 30 mM Tris·HCl, pH 7–20 mM $MgCl_2$–0.3 mM KCl–6 mM 2-mercaptoethanol–0.1 mM EDTA. The mixture was incubated for 15 min at 2°. Some experiments were repeated at 37° with identical results. After incubation, the mixture was dialyzed at 2° for 16 hr against 20 volumes of the following buffer (three changes): 30 mM Tris·HCl, pH 7.4–20 mM $MgCl_2$–0.3 M KCl–6 mM 2-mercaptoethanol. When [^{14}C]Sm was used, no more detectable counts are released. A 0.2-ml sample of the dialyzed mixture was dissolved in Aquasol (New England Nuclear) and counted in a Beckman LS-230 liquid scintillation counter.

RNA Denaturation Conditions. RNA solution in Tris–$MgCl_2$–KCl–EDTA–mercaptoethanol buffer (1 mg/ml) was heated at 65 or 70° for 4 min (12), [^{14}C]Sm was added, and the sample was cooled immediately at 0°; sometimes the drug was added after cooling and storage at 0° for different times, as described in the experiments.

Calculations. A 1:1 molar ratio of Sm/16S RNA or Sm/30S subunit is equivalent to 1.17 μg of Sm/mg of 16S RNA (5.5 $\times$ 10^5 daltons) or 0.705 μg of Sm/mg of 30S (8.5 $\times$ 10^5 daltons). In our experiments, 1 μg of [^{14}C]Sm is equivalent to 120 cpm above the background of 30–40 cpm.

RESULTS AND DISCUSSION

The 30S ribosomal subunits and the 16S RNA extracted from L1, a Sm^S strain, bind Sm, while the total 30S proteins do not (Table 1). Dependence of Sm–30S subunit binding on Sm concentration is the same as that of Sm–16S RNA binding, and a maximum of two Sm molecules bound per subunit or per RNA molecule is reached. Results presented in Table 2 demonstrate that 30S subunits extracted from L1-401, a Sm^R strain, or from P48S23, a $Drug^D$ strain, do not bind any Sm, while 16S RNA isolated from these subunits behaves identically to Sm^S 16S RNA. Binding of [^{14}C]Sm to RNA is not a function of whether ribosomes were extracted with LiCl–urea or phenol. Moreover, the presence of Pm does not interfere with Sm binding. This result could indicate attachment site(s) for Pm different from those for Sm. Given ribosomal complexity, however, other possibilities

TABLE 2. *Binding of Sm to 16S RNA from different strains*

Extracted from strain	Extracted with	Prescence of Pm	(Sm)/(RNA)
L1(Sm^S)	Urea–LiCl	0	1.90
L1-401(Sm^R)	Urea–LiCl	0	2.70
P48S23($Drug^D$)	Urea–LiCl	0	2.04
L1(Sm^S)	Phenol	0	2.00
L1-401(Sm^R)	Phenol	0	2.10
P48S23($Drug^D$)	Phenol	0	2.14
L1-401(Sm^R)	Phenol	+	2.30
P48S23($Drug^D$)	Phenol	+	2.20

(Sm)/(RNA) = molar ratio of Sm to 16S RNA. Input molar ratio (for Sm and Pm when present) = 100.

TABLE 3. *Effect of denaturation on Sm binding to 16S RNA*

Denaturation temperature, °C	Renaturation time	(Sm)/(RNA)
70	0	10.40
65	0	6.80
65	5 min	6.37
65	15 hr	2.88
2	0	1.93

(Sm)/(RNA) = molar ratio of Sm to 16S RNA. Input molar ratio = 100. The RNA was extracted with phenol. After denaturation, Sm binding, and dialysis, the 16S RNA–Sm complex sediments as a single peak in the region of 16S RNA in a 5–20% sucrose gradient.

based on cooperative effects between 30S components cannot be excluded.

Table 3 shows that the amount of Sm bound is limited by the three-dimensional structure of the RNA, since 16S RNA acquires a reversible ability to bind more than two Sm molecules after heat denaturation. Under identical experimental conditions, we have found that 50S ribosomal subunits, and the 23S RNA thereof, bind 0.7 and 1.3 molecules of Sm per subunit or per RNA molecule, respectively (average of four determinations). In contrast to the 30S subunit and 16S RNA, however, Sm binding by the 50S subunit is independent of the strain, whether Sm^S, Sm^R, or $Drug^D$, and the binding of Sm by 23S RNA is independent of heat denaturation. This finding supports the conclusion that Sm binds to specific sites of 16S RNA. These sites are easily amenable to structural changes, and become masked or exposed in the 30S architecture depending on whether protein P10 is mutant or wild-type.

Functional 30S ribosomes were reconstituted from 16S RNA and 30S proteins extracted from Sm^S and Sm^R strains. Table 4 shows the effect on reconstitutions of exposure to Sm of either 16S RNA or 30S proteins. Treatment of 16S RNA with Sm, regardless of its origin, prevents reconstitution, while complete reconstitution was obtained with Sm-treated proteins, regardless of their origin. Furthermore, the binding, stable to dialysis, of Sm to complete Sm^S 30S subunit is reversed after LiCl–urea treatment, dialysis, and reconstitution. The results reported in Table 5 show that Sm may also be removed from its complex with isolated 16S RNA if the complex is exposed to LiCl–urea at the same concentrations and under the same conditions; in this way, the ability of 16S RNA to reconstitute functional 30S subunits is largely restored. The 16S RNA–Sm complex obtained after reversible denaturation at 65° also regains, by the LiCl–urea treatment, some ability to reconstitute functional 30S subunits. This result indicates that the presence of Sm is needed to produce and maintain in the 16S RNA the structural alteration that prevents reconstitution of active ribosomes, but which is reversed in the absence of Sm by high-salt concentration.

TABLE 4. *Reconstitution of functional 30S subunits from either RNA or proteins exposed to Sm*

Sm phenotype of the strain yielding		30S component exposed to Sm		
RNA	Proteins	RNA	Proteins	Reconstitution
S	S	✓		0.09
R	S	✓		0.12
S	R	✓		0.53
R	R	✓		0.67
S	S		✓	104.0
R	S		✓	93.0
S	R		✓	122.0
R	R		✓	93.0

The Sm^S and Sm^R strains were L1 and L1-401, respectively. Separation of 16S RNA from total proteins by the LiCl–urea procedure. Exposure to Sm and subsequent dialysis by the *Drug binding procedure* (*Methods*). 50S subunits are from the respective strains. Reconstitution is measured by the amount of poly(U)-directed phenylalanine incorporation, and is expressed as % of that obtained with RNA and proteins not exposed to Sm. Activity of 30S subunits reconstituted from untreated components was equal to that of the original 30S subunit whether the cell was Sm^S or Sm^R.

TABLE 5. *Reconstitution of 30S subunits after removal of bound Sm*

Denaturation temperature of 16S RNA, °C	Sm* initially bound	Subsequent urea–LiCl treatment	Sm* remaining	Reconstitution
0	2.1		2.0	0.2
0	1.9	✓	0	85.0
65	6.7		6.7	0.1
65	6.8	✓	0	21.0
65 + renaturation	2.7	✓	0	57.0
0 (not exposed to Sm)		✓	—	100.0
65 (not exposed to Sm)		✓	—	80.0

16S RNA is from strain L1(Sm^S). Denaturation of 16S RNA, Sm exposure, and dialysis are as in Table 4. Renaturation is by quick cooling and storage at 0° overnight before exposure to Sm, subsequent urea–LiCl treatment, and dialysis. Reconstitution is expressed as % of that obtained with undenatured 16S RNA not exposed to Sm. Reconstituted 30S subunits from untreated components possessed 50% of the activity of the original undissociated 30S subunits.

* Molecules of Sm per molecule of 16S RNA.

Binding of Sm to 16S RNA, naked or embedded into a 30S subunit, might not be identical so that *in vitro* artifacts might be introduced in working with isolated 16S RNA. It is a commonly held assumption, however, that the stable binding of two Sm molecules to the 30S subunit is responsible for Sm action. This assumption is now questioned by the existence of the phenomenon of phenotypic masking. Strain P48S23 is resistant to Sm or Pm separately, but is sensitive to Sm and Pm in combination. It has been reported (5) that growth in either drug yields ribosomes altered (tagged) in such a way that they allow misreading when tested *in vitro* in the presence of the second drug. The tag could be either the drug itself firmly bound to the ribosome, some ribosomal modification induced by the drug-binding, or both. Our first approach was to grow the cells in the presence of either drug, split the ribosome, and determine with which component the tag is associated. In preliminary experiments in which 30S and 50S subunits from cells grown with and without Sm or Pm were interchanged, we found that the 30S subunit was tagged, as was expected, since the pertinent mutation carried by P48S23 is in the *strA* gene. We had hoped to continue this analysis by splitting 16S RNA from the protein. This procedure was prevented, however, because during the splitting procedure the tag was lost, i.e., the reconstituted ribosome behaved normally, without apparent memory of the previous growth. A completely *in vitro* approach was attempted next. We tagged the 30S subunit by exposing it first to one drug and then, after exhaustive dialysis, to the second. Results presented in Table 6 show that with cells grown without drugs (*a*) the 30S subunits bind [^{14}C]Sm (and

TABLE 6. *Influence of Pm on Sm binding to 30S subunits from a phenotypic masked strain*

Strain	First incubation and dialysis	Second incubation and dialysis	Sm* bound	Index of misreading
P48S23	None	[^{14}C]Sm	0	104
P48S23	Pm	[^{14}C]Sm	2.2	340
P48S23	[^{14}C]Sm	Pm	0	384
P48S23	None	[^{14}C]Sm + Pm	2.0	360
P48S23(Pm grown)	Any above condition		2.3 ± 0.3	300
L1(Sm^S)	Any above condition		2.2 ± 0.4	—
L1401(Sm^R)	Any above condition		0	—

Standard exhaustive dialysis after each incubation. "*None*" means incubation without drug. When Sm was present in the first incubation, its binding was determined after the first dialysis. The P48S23 strain was grown in the absence (L medium) or in the presence of Pm. Index of misreading is the ratio of misreading (isoleucine) per normal reading (phenylalanine). With 30S subunits from P48S23 grown on L medium, the index of misreading, when the drugs are added during the amino-acid incorporation test only, are: no addition = 15; in the presence of either Sm or Pm = 105; in the presence of both = 356 (for more details, see ref. 5).

* Molecules of Sm per 30S subunit.

misread) only when this drug is used in combination with Pm or after an exposure to Pm; (*b*) although exposure to Sm alone does not result in detectable binding, the 30S subunit is modified, because it misreads when later exposed to Pm; (*c*) since labeled Pm was not available, direct measurement of Pm binding was not possible, but results *in vivo* (3, 5) indicate that the behavior with Pm is exactly analogous to that found with Sm. All controls were consistent: (*a*) 30S subunits from a Sm^S strain (L1) tested under any of the four conditions of Table 6, bind Sm (2.2 ± 0.4 molecules); (*b*) 30S subunits from a Sm^R strain (L1-401) do not bind Sm under any of the four conditions; and (*c*) 30S subunits from P48S23 grown with Pm bind Sm (2.3 ± 0.3 molecules) and misread under any of the four conditions, because the 30S subunits were tagged with Pm during growth. It is concluded that in a $Drug^D$ strain, Sm (or Pm) do not need to be stably bound to the 30S subunit to change its behavior toward Pm (or Sm). Thus, the tag attached to the 30S subunit by growth of the cells in the presence of the drug is not the drug itself (5), but is a structural modification that is not readily reversible. We have been able to reverse this modification and thus erase the ribosomal memory for drug exposure only by dissolving the ribosome in buffers of high-salt concentration.

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