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Identification of Chloramphenicol-Binding Protein in *Escherichia coli* Ribosomes by Affinity Labeling*

(antibiotics/ribosomal proteins/two-dimensional gel electrophoresis/equilibrium dialysis)

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ABSTRACT **Monoiodoamphenicol, a synthetic analogue of chloramphenicol, has been shown by competition experiments with chloramphenicol and lincomycin to bind at the same site of 70S ribosomes as chloramphenicol. At −2° it forms a 1:1 complex with 70S ribosomes having a value of K (7.5×10^4 M^{-1}) that is one order of magnitude lower than that of chloramphenicol. At 37°, monoiodoamphenicol irreversibly inhibits the protein-synthesizing activity of *E. coli* ribosomes. It is shown that the analogue reacted preferentially with protein L16 of *E. coli* 70S ribosomes, and we therefore conclude that protein L16 belongs to the chloramphenicol-binding site of *E. coli* ribosomes.**

Since the chemically reactive group of monoiodoamphenicol resembles iodoacetamide, the reaction of *E. coli* 70S ribosomes with monoiodoamphenicol was compared to that with iodoacetamide. Iodoacetamide did not react with protein L16, but it predominantly reacted with proteins S18 and S21 of the 30S subunit. Furthermore, monoiodoamphenicol was reacted with *E. coli* ribosomal subunits. Isolated 50S subunits bound monoiodoamphenicol by about one order of magnitude less than 70S ribosomes. Again, protein L16 reacted with the affinity label. Monoiodoamphenicol reacted with protein S18 in isolated 30S subunits; it also bound to 70S ribosomes of *Bacillus stearothermophilus*, however, it did not bind irreversibly to these 70S ribosomes.

A detailed understanding of structure–function relationship of the ribosome is not available (for review see ref. 1). The many elegant approaches that have been tried towards this goal have shown how difficult it is to assign unanimously specific functions to individual ribosomal proteins. Studies of mutants of ribosomal proteins (2), reconstitution of ribosomal particles missing one protein (3), and stimulation of ribosomal functions by addition of ribosomal proteins (4) have shed some light on the great complexity of the ribosomal particle. A promising approach for testing the function of individual ribosomal proteins is the technique of affinity labeling. This technique allows the direct identification of the specific function of an individual ribosomal protein.

Recently, the covalent attachment of peptidyl-tRNA analogues to *Escherichia coli* ribosomes was reported (5, 6). These experiments open the possibility to identify some of the ribosomal proteins involved in binding of peptidyl-tRNA. This report presents for the first time results of studies that probe chemically the chloramphenicol-binding region of *E. coli* ribosomes by reacting ribosomes with chloramphenicol analogues†. Chloramphenicol has been known for many years as a strong but reversible inhibitor of protein biosynthesis (7), which probably acts at or near the peptidyl-transferase center of the ribosome (8). Chloramphenicol was modified such that it did not lose its antibiotic specificity. The chemically reactive group introduced into the antibiotic was expected to react preferentially and irreversibly with a properly oriented amino-acid functional group in the binding region of the ribosome. The specificity of the labeling reaction by the chloramphenicol analogues monobromamphenicol and monoiodoamphenicol was tested (*a*) by comparison of the antibiotic activities of these analogues with chloramphenicol in a poly(U)-directed polyphenylalanine-synthesizing system, (*b*) by determination of the stoichiometry of the binding of the analogues to 70S ribosomes, (*c*) by competition experiments between monoidoamphenicol and chloramphenicol or lincomycin, (d) by purification of the affinity-labeled ribosomal subunit, followed by identification of the uniquely labeled ribosomal protein, and (*e*) by comparison of the affinity labeling reaction with other labeling reactions.

Part of this paper has already been communicated (9).

MATERIALS AND METHODS

Monoiodoamphenicol and monobromamphenicol were synthesized as described by Rebstock (10). Moniodo[^{14}C]amphenicol was synthesized as described (9). The affinity label had a specific radioactivity of 4.3 Ci/mol. Ribosomes of *E. coli* A19 were isolated and purified as described (11*a*). *Bacillus stearothermophilus* ribosomes were prepared according to Erdmann *et al.* (11*b*). The poly(U) assay was done according to the method of Nirenberg and Matthaei (12). Equilibrium dialysis experiments were done at −2° 50-μl chambers, as described (13).

Affinity-Labeling of Ribosomes. 70S ribosomes were incubated with a 10-fold excess of affinity label for 2 hr at 37° in TMA I buffer [10 mM Tris·HCl (pH 7.8)–10 mM $MgCl_2$–30 mM NH_4Cl] and then centrifuged overnight at 40,000 rpm in a Spinco Ti 60 rotor. The pellet was dissolved in TMA II buffer (same as TMA I, except 0.3 mM $MgCl_2$). The ribo-

* Paper no. 51 on *Ribosomal Proteins*. Preceding paper is by D. Nierhaus and K. Nierhaus (1973) *Proc. Nat. Acad. Sci. USA* 70, 2224–2228.

† For the chloramphenicol analogues the following abbreviations are used: monobrom-[^{14}C]amphenicol, D-(−)-*threo*-1-*p*-nitrophenyl-2-(bromo-[$^{14}C_2$']acetamido)-1,3-propanediol; monoiodo-[^{14}C]amphenicol, D-(−)-*threo*-1-*p*-nitrophenyl-2-(iodo-[$^{14}C_2$']-acetamido)-1,3-propanediol. Monobromamphenicol and monoiodoamphenicol correspond to the unlabeled compounds.

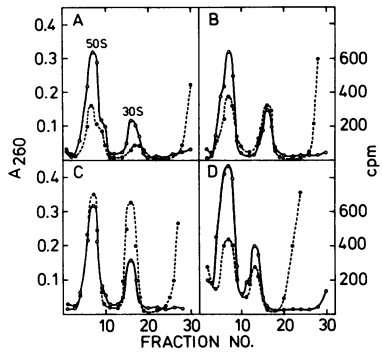

FIG. 1. Sucrose gradient of 70S ribosomes labeled with monoiodo[^{14}C]amphenicol as described in *Methods*. On top of the gradient 60 A_{260} units of 70S *E. coli* ribosomes were layered and centrifuged at 24,000 rpm for 14 hr at 4° in a Spinco SW27 rotor. 1.3-ml Fractions were collected. 40-μl aliquots of each fraction were diluted with 1 ml of TMA II buffer and absorbance was read at 260 nm. Radioactivity was monitored by mixing 1 ml of each fraction with 12 ml of Bray's counting solution. (O——O) A_{260}; (O- - -O) cpm. Incubation of (*A*) *E. coli* ribosomes at 4°; (*B*) *E. coli* ribosomes at 22°; (*C*) *E. coli* ribosomes at 37°; (*D*) *B. stearothermophilus* ribosomes (90 A_{260} units) at 37°.

somal subunits were separated on a linear 10–30% sucrose gradient in TMA II buffer. Centrifugation was done in a Spinco SW27 rotor at 24,000 rpm for 14 hr. Fractions containing the 50S and 30S subunits were collected. Proteins were then extracted and separated by two-dimensional gel electrophoresis according to Kaltschmidt and Wittmann (14).

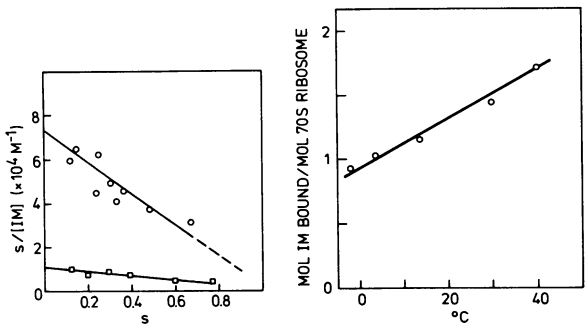

FIG. 2 (*left*). Scatchard plot of data for monoiodo[^{14}C]amphenicol binding to *E. coli* 70S and 50S ribosomes at −2°. Equilibrium dialysis experiments were done as described (13) in TMA I buffer. The concentration of 70S ribosomes was 4.4 μM and that of 50S subunits was 16.4 μM. Concentrations of added monoiodo[^{14}C]amphenicol were 2–15 μM. Incubation times were 24 hr at −2°. *s* corresponds to the moles of monoiodo[^{14}C]amphenicol bound per mole of ribosome; [*IM*] to the concentration of unbound monoiodo[^{14}C]amphenicol. (O—— O) data of binding IM to 70S ribosomes; (□— —□) data of binding IM to 50S subunits.

FIG. 3 (*right*). Temperature dependence of monoiodoamphenicol binding to *E. coli* 70S ribosomes. *E. coli* 70S ribosomes (5 μM) were incubated with monoiodo[^{14}C]amphenicol (20 μM) in TMA I buffer. After 2 hr the incubation mixtures were transferred to 50-μl dialysis chambers for equilibrium dialysis at −2°. After 24–48 hr equilibration time, samples were withdrawn in triplicate and the radioactivity was measured. Specific radioactivity of monoiodo[^{14}C]amphenicol was 4.3 Ci/mol.

TABLE 1. *Inhibition of poly(U)-directed polyphenylalanine synthesis of E. coli and B. stearothermophilus ribosomes by chloramphenicol and its analogues*

	% activity of 70S ribosomes† of			
	E. coli		*B. stearothermophilus*	
Antibiotic*	Not dialyzed	Dialyzed	Not dialyzed	Dialyzed
Chloramphenicol	48	90	70	98
Monobromamphenicol	65	70	72	94
Monoiodoamphenicol	40	50	69	86
None	100	100	100	100

* Incubation of 70S ribosomes with antibiotic: In 500 μl of TMA I, 15 A_{260} units of 70S ribosomes were incubated with 0.2 mg of antibiotic for 30 min at 37°. After incubation the samples were either placed on ice or dialyzed against TMA I (0°) for 12 hr.

† Poly(U) activity of 1.5 A_{260} units of 70S ribosomes was determined as described (12). The control samples of *E. coli* ribosomes gave 41,000 cpm and those of *B. stearothermophilus* 33,000 cpm. This corresponds to 64 and 50 mol, respectively, of phenylalanine polymerized per mol of 70S ribosome.

Iodoacetamide Treatment of Ribosomes. 70S ribosomes were incubated with a 10-fold excess of iodo[^{14}C]acetamide (50 Ci/mol) for 2 hr at 37° in TMA I buffer. Isolation of ribosomal subunits and purification of ribosomal proteins were done as described above.

RESULTS

Characterization of the labeling reaction

Chloramphenicol inhibits *in vivo* protein biosynthesis completely (7). However, the inhibitory effect of chloramphenicol is not so strong in the poly(U)-directed *in vitro* protein-synthesizing system (15). We have found that chloramphenicol inhibits polyphenylalanine synthesis *in vitro* by about 50% (Table 1), which is in agreement with published results (15, 16). A similar degree of inhibition was observed when ribosomes of *E. coli* and of *B. stearothermophilus* were incubated at 37° with monobromamphenicol or monoiodoamphenicol instead of chloramphenicol. But, for *E. coli* ribosomes, the inhibition was irreversible (Table 1). Incubation of 70S ribosomes

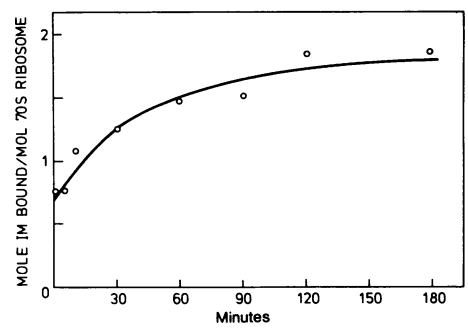

FIG. 4. Time dependence of monoiodoamphenicol binding to *E. coli* 70S ribosomes at 37°. *E. coli* 70S ribosomes (5 μM) were incubated with monoiodo[^{14}C]amphenicol (20 μM) in TMA I buffer. After the times indicated, the incubation mixtures were transferred to 50-μl dialysis chambers for equilibrium dialysis at −2°. After 24–48 hr equilibrium time, samples were withdrawn in triplicate and their radioactivity was measured. Specific radioactivity of monoiodo[^{14}C]amphenicol was 4.3 Ci/mol.

TABLE 2. *Competition between binding of monoiodoamphenicol and chloramphenicol or lincomycin to E. coli ribosomes*

Ribosome	Antibiotic	Competitor	K_o (M^{-1})	K_{app} (M^{-1})	K_c (M^{-1})
70S	Monoiodoamphenicol	Chloramphenicol	7.5×10^4	4.5×10^3	1.6×10^6
	Monoiodoamphenicol	Lincomycin	7.5×10^4	1.2×10^4	4.5×10^4
	Chloramphenicol	Monoiodoamphenicol	2.0×10^6	4.1×10^5	6.5×10^4
50S	Chloramphenicol	Monoiodoamphenicol	2.0×10^5	1.2×10^4	1.9×10^4

Competition experiments at $-2°$ between an antibiotic, which binds to the ribosome with K_o, and a competitor. A reduction of the measured K_{app} indicates competition, and the K_c of the competitor is calculated by assuming equimolar competition for the same site. Concentrations were: 4.4 μM 70S ribosomes; 10 μM 50S ribosomes; 20 μM monoiodoamphenicol; 8 μM chloramphenicol; 60 μM monoiodoamphenicol as competitor; 10 μM chloramphenicol as competitor; 120 μM lincomycin.

with chloramphenicol for 30 min and subsequent extensive dialysis of the ribosomes against TMA I buffer restored ribosomal activity. However, the original ribosomal activity could not be restored after incubation of 70S ribosomes of *E. coli* with monobromamphenicol or monoiodoamphenicol followed by extensive dialysis against TMA I buffer. This indicated that the chloramphenicol analogues had irreversibly reacted with *E. coli* ribosomes, but not with *B. stearothermophilus* ribosomes.

The specificity and stoichiometry of the affinity labeling reaction of monoiodo[^{14}C]amphenicol with *E. coli* 70S ribosomes was determined by sucrose-gradient analysis and equilibrium dialysis experiments. After *E. coli* 70S ribosomes had been incubated for 2 hr at 4° with monoiodo[^{14}C]amphenicol, most of the radioactivity migrated with the 50S peak in linear sucrose gradients (Fig. 1). This finding indicated that the affinity label bound to its specific target, the 50S subunit. However, after incubation at higher temperatures an increasing amount of radioactivity migrated with the 30S part of the ribosome (Fig. 1*A–C*). Though the activity of *B. stearothermophilus* ribosomes was not irreversibly inhibited by monoiodoamphenicol, sucrose-gradient analysis (Fig. 1*D*) showed that monoiodoamphenicol also migrated with the 50S and 30S subunits of *B. stearothermophilus* ribosomes. The radioactivity per A_{260} unit was similar to that per A_{260} unit of ribosomal subunits of *E. coli*, which had been incubated with label at 4°.

Equilibrium dialysis experiments showed that binding of monoiodoamphenicol to *E. coli* ribosomes was still reversible at $-2°$. Under these experimental conditions the binding was further characterized. The Scatchard plot of the monoiodoamphenicol-binding data (Fig. 2) shows that monoiodoamphenicol forms a 1:1 complex with *E. coli* 70S ribosomes at low saturation levels with a binding constant of 7.5×10^4 M^{-1}. This value of K is one order of magnitude lower than that reported for chloramphenicol binding (8). The value of K of binding of monoiodoamphenicol to 50S subunits was 1.1×10^4 M^{-1}. This value indicates that the affinity of monoiodoamphenicol to 50S subunits is considerably lower than to 70S ribosomes.

Competition experiments were done in order to determine

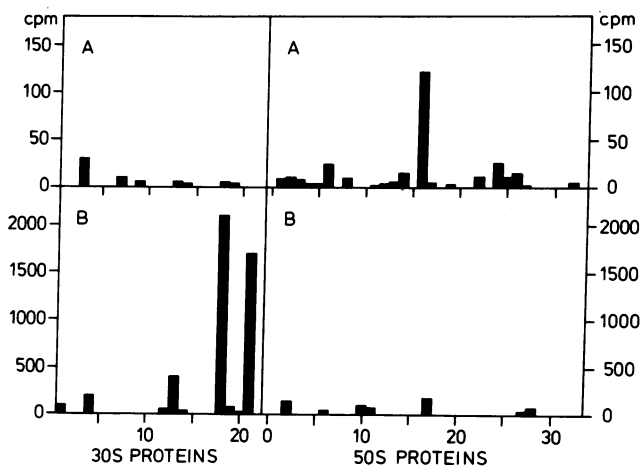

FIG. 5. Radioactivity detected in ribosomal proteins after incubation of 70S ribosomes with monoiodo[^{14}C]amphenicol or with iodo[^{14}C]acetamide. (*A*) After incubation of *E. coli* 70S ribosomes with a 10-fold molar excess of monoiodo[^{14}C]amphenicol (4.3 Ci/mol) for 2 hr at 37°, the ribosomal subunits were isolated as described in *Methods*. Proteins were extracted and separated by two-dimensional gel electrophoresis according to Kaltschmidt and Wittmann (15). Each spot, which contained ribosomal protein, was cut into 0.15-cm^3 aliquots and dissolved overnight in 1 ml of soluene TM 100 (Packard, Lot no. 34053). Radioactivity was counted after the addition of 10 ml of toluene-based scintillation fluid. (*B*) After incubation of *E. coli* 70S ribosomes with a 10-fold molar excess of iodo[^{14}C]acetamide (50 Ci/mol) for 2 hr at 37°, the ribosomal subunits were isolated. Extraction and separation of proteins and counting of radioactivity was done as described in (*A*).

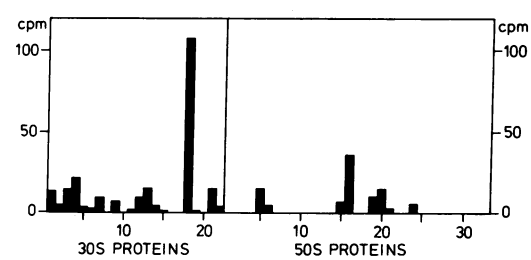

FIG. 6. Radioactivity detected in ribosomal proteins after incubation of 30S and 50S subunits with monoiodo[^{14}C]amphenicol. 30S and 50S subunits of *E. coli* ribosomes were incubated with a 10-fold molar excess of affinity label for 2 hr at 37° in TMA I buffer. Then the subunits were spun down by high-speed centrifugation (50 Ti; 40,000 rpm; 6 hr). Ribosomal proteins were extracted and separated as described (15). Each spot, which contained ribosomal protein, was cut into 0.15-cm^3 aliquots and dissolved overnight in 1 ml soluene TM (Packard, Lot No. 34053). Radioactivity was counted after the addition of 10 ml of toluene-based scintillation fluid.

whether monoiodoamphenicol bound to the same site on the ribosome as chloramphenicol. Binding data were evaluated according to the expression from Edsall and Wyman (17):

$$K_{app} = \frac{K_o}{1 + K_c [c]}$$

K_{app} is the measured association constant, which is related to the association constant of the competitor K_c, the free competitor concentration [c], and the actual association constant K_o. Data in Table 2 show that monoiodoamphenicol and chloramphenicol or lincomycin compete with one another. These data suggest that monoiodoamphenicol binds to the chloramphenicol-binding site on the 70S ribosome as well as on the 50S subunits.

When *E. coli* 70S ribosomes were incubated with monoiodoamphenicol for 2 hr in the temperature range of −2°–40°, it was found that the amount of monoiodoamphenicol bound to 70S ribosomes increased 2.5-fold (Fig. 3). The higher molar ratios, found at higher temperatures, are probably due to an increasing incorporation of label into the 30S subunit, as demonstrated by the sucrose-gradient analysis of the ribosomes reported in Fig. 1. After a period of 2 hr a plateau of monoiodoamphenicol binding to *E. coli* 70S ribosomes was reached, which yielded a ratio of about 2 mol of label bound per mol of 70S ribosomes (Fig. 4). These conditions were chosen for further analysis in order to determine which of the ribosomal proteins had reacted with monoiodoamphenicol. After incubation of 70S ribosomes for 2 hr at 37° with monoiodoamphenicol, binding of chloramphenicol to 70S ribosomes could not be detected anymore by equilibrium dialysis experiments. In summary, all these data (Figs. 1–4; Tables 1 and 2) showed that monoiodoamphenicol bound to the same site of 70S ribosomes as chloramphenicol and that it reacted irreversibly with this site.

Identification of ribosomal proteins labeled with monoiodoamphenicol

1000 A_{260} units of *E. coli* 70S ribosomes were incubated with a 10-fold molar excess of monoiodo[^{14}C]amphenicol for 2 hr at 37°. After high-speed centrifugation (100,000 × g), the ribosomes were dissolved in TMA II buffer, and ribosomal subunits were separated by sucrose-gradient centrifugation. The 30S and 50S ribosomal proteins were extracted and then separated by two-dimensional polyacrylamide–gel electrophoresis. It is important to note that no radioactivity could be detected in the RNA material isolated from the labeled subunits. The staining pattern of the gels obtained from the 30S and 50S ribosomal proteins was identical to that of ribosomal proteins of untreated ribosomes, which has been published by Kaltschmidt and Wittmann (14), except for that of protein L16. As a result of the labeling reaction protein L16 was found in a new position, which was between that of unmodified L16 and S9. The radioactivity contained in each ribosomal protein was determined. The results are illustrated in Fig. 5*A*. As can be seen, almost all of the radioactivity was contained in protein L16 of the 50S subunit. In addition, some radioactivity (10% of that found in protein L16) was detected in proteins L6 and L24. The amount of radioactivity that could be detected in 30S proteins was low. Protein S3 contained 25% of the radioactivity found in protein L16. These data strongly indicate that monoiodoamphenicol specifically labeled protein L16. Hence, it is suggested that protein L16 belongs to the chloramphenicol-binding site of *E. coli* 70S ribosomes.

Labeling of ribosomes by iodoacetamide

Monoiodoamphenicol cannot only be regarded as an analogue of chloramphenicol, but also as an N-substituted iodoacetamide. Therefore, *E. coli* 70S ribosomes were incubated with a 10-fold excess of iodo[^{14}C]acetamide under conditions similar to the affinity-labeling reaction. After high-speed centrifugation (100,000 × g), the ribosomes were dissolved in TMA II buffer, and ribosomal subunits were separated by sucrose-gradient centrifugation. The 30S and 50S ribosomal proteins were extracted and separated as described above. The radioactivity contained in each ribosomal protein was determined (Fig. 5*B*). Clearly, iodoacetamide did not react with protein L16 and S3. Moreover, ten-times more radioactivity was incorporated into 30S proteins than into 50S proteins. S18 and S21 were the primary targets of the iodoacetamide reaction. This is again supporting evidence for the specificity of the labeling reaction used.

Labeling of ribosomal subunits with monoiodoamphenicol

Furthermore, the reaction of monoiodoamphenicol with isolated 30S and 50S subunits of *E. coli* ribosomes was investigated. The subunits were incubated with a 10-fold excess of label at 37° for 2 hr. Then they were isolated by high-speed centrifugation. Ribosomal proteins were extracted and separated as described above. In the 50S subunit, protein L16 was again the protein that had preferentially reacted with monoiodoamphenicol (Fig. 6). Protein S18 was the most reactive 30S ribosomal protein towards monoiodoamphenicol (Fig. 6). It is suggested that this represents an unspecific labeling of the 30S subunit. This is not entirely surprising, since S18 is also one of the primary targets for the reaction with iodoacetamide.

Incubation of *B. stearothermophilus* with monoiodoamphenicol

The data of Table 1 indicated that monoiodoamphenicol did not inhibit *B. stearothermophilus* 70S ribosomes irreversibly. These 70S ribosomes were incubated with a 100-fold excess of monoiodo[^{14}C]amphenicol for 2 hr at 40°. After high-speed centrifugation, the ribosomes were dissolved in TMA II buffer, and ribosomal subunits were separated by sucrose-gradient centrifugation. The 30S and 50S ribosomal proteins were extracted and separated as described above. Analysis of the proteins revealed that none contained any significant radioactivity. This would indicate that monoiodoamphenicol binds to the 50S subunit of *B. stearothermophilus* 70S ribosomes, but that the binding protein does not contain a properly oriented amino-acid side chain that could react with the affinity label.

DISCUSSION

Conclusive evidence has been presented that the chloramphenicol analogue, monoiodoamphenicol, reacts with an amino-acid functional group in the chloramphenicol-binding region of *E. coli* ribosomes. The protein, to which the affinity label was preferentially attached, was identified as protein L16. This observation finds strong support by reconstitution experiments, which are reported by D. Nierhaus and K. Nier-

haus in the preceding paper, pages 2224–2228. By these experiments it was demonstrated that *E. coli* 50S ribosomal subunits bind chloramphenicol, only if protein L16 is present in the subunit.

The data of Fig. 5 shows that some monoiodoamphenicol reacted with protein S3. This finding might indicate that protein S3 is very close to protein L16 in 70S ribosomes. Protein S3 stimulates the tRNA-binding capacity of *E. coli* ribosomes (4). Hence, it is suggested that protein S3 belongs to the 30S proteins, which are part of the ribosomal interface between the 30S and the 50S subunit, and that it is located in the near neighborhood of the peptidyltransferase center.

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