AFFINITY LABELING OF *E. COLI* RIBOSOMES WITH A STREPTOMYCIN-ANALOGUE†

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1. Introduction

Streptomycin inhibits protein synthesis in sensitive bacteria [1]. It has been shown that the inhibitory action of this antibiotic is due to a distortion of the ribosomal-tRNA binding site [2]. Binding of aminoacyl-tRNA and of polypeptidyl-tRNA is impaired, which is followed by a subsequent polysome breakdown [2–4]. Genetic data [5] as well as partial reconstitution experiments [6, 7] have shown in one way or the other that several ribosomal proteins are involved in streptomycin binding.

Protein S 12 is the ribosomal component which confers resistance against or dependence on streptomycin. Phenotypic reversion from streptomycin dependence to independence can be caused by an altered S 4 or S 5 protein [8]. As suggested by reconstitution experiments, proteins S 3 and S 5 are directly involved in streptomycin binding. Furthermore, the S 5 dependent binding is stimulated by protein S 9 and S 14 [7].

This report presents the first results of studies which probe chemically the streptomycin binding site of *E. coli* ribosomes by reacting ribosomes with a streptomycin-affinity label. This label was constructed in such a way, that it did not lose its antibiotic specificity, but contained a chemically reactive group, which was expected to react preferentially with a properly oriented amino acid side chain at or near the streptomycin binding region of the ribosome.

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2. Experimental

2.1. *Synthesis of the 4-aminobenzhydrazone of streptomycin*

A solution of 100 μmoles of streptomycin sulfate (Boehringer, Mannheim, Germany) was added to a solution of 120 μmoles of 4-aminobenzhydrazine resulting in a final reaction volume of 1 ml in 0.01 M phosphate buffer, pH 7.0. After 1 hr at room temp., the total reaction mixture was applied to PLC aluminium plates (silica gel F254, neutral, Merck) with chloroform–methanol (7:3) as solvent system. Unreacted 4-aminobenzhydrazine migrated with the solvent front. The 4-aminobenzhydrazone of streptomycin had an R_f-value of 0.05–0.08. The band corresponding to it was cut out of the PLC plate and extracted with water. The solid material was removed by centrifugation and the supernatant lyophilized.

2.2. *Synthesis of 4-iodoacetamino-benzhydrazone of streptomycin*

A solution of 58.15 μmoles of *N,N'*-dicyclohexylcarbodiimide in 100 μl of dioxane was added to a solution of 58.12 μmoles iodoacetic acid and 65.16 μmoles *N*-hydroxysucciminide in 200 μl of anhydrous dioxane. The *N,N'*-dicyclohexylurea precipitated immediately; after 1 hr at room temp., the urea was removed by centrifugation. The dioxane solution was evaporated to dryness at 30°C *in vacuo*. The residue was dissolved in 100 μl of dioxane and under rapid mixing added to a solution of 25 μmoles of streptomycin-benzhydrazone in 1 ml 0.5 M $NaHCO_3$ buffer, pH 7.5. After 1 hr at room temp., the total reaction mixture was applied to high voltage paper electrophoresis (60–70 V/cm) on Whatman 3 MM paper at pH 7.8 in 0.05 M phosphate buffer. Under these con-

ditions, only the reaction product migrated towards the cathode, having a relative R_e value of −0.20 as compared to picric acid (taken as 1.0).

The 4-iodo-[^{14}C]acetaminobenzhydrazone of streptomycin was synthesized along similar lines. Monoiodo-[^{14}C]acetic acid (6 Ci/moles) was used instead of iodoacetic acid.

2.3. *Assays of affinity-label–ribosome interaction*

Ribosomes of *E. coli* A19 were isolated and purified as previously described [9]. Equilibrium dialysis experiments were carried out in essentially the same manner as described [10]. (*yeast*)tRNA$^{Phe}_{-y}$ was prepared according to Thiebe and Zachau [11]. The modified poly(U)-assay was carried out as described elsewhere [12].

2.4. *Purification of the affinity-labeled ribosomal subunits and proteins*

70 S ribosomes were incubated with a 10-fold excess of affintiy label for 2 hr at 37°C in TMA I buffer (10^{-2} M Tris–HCl (pH 7.8)–10^{-2} M $MgCl_2$–3×10^{-2} M NH_4Cl) and then centrifuged for 5 hr at 40 000 rpm in a Spinco Ti 50 rotor. The pellet was dissolved in TMA II buffer (same as TMA I, except 3×10^{-4} M $MgCl_2$). Ribosomal subunits were separated on a linear sucrose gradient as described [13]. Proteins were then extracted according to Kaltschmidt and Wittmann [14].

3. Results

The formyl-residue in position 3 of the streptose moiety of streptomycin is known to be unimportant for the action of this antibiotic on ribosomal activity [15]. Therefore, it seemed to be feasible to derivatize streptomycin in this position to an affinity label. First, the aldehyde was reacted with 4-aminobenzhydrazine to yield the hydrazone of streptomycin as described under Experimental. Then this compound was checked if it had retained the antibiotic activity of streptomycin, i.e. distortion of the ribosomal-tRNA binding site [2–4]. As can be seen from table 1, this was indeed the case. Transfer-activity of (*yeast*)tRNA^{Phe} in poly(U) directed polyphenylalanine synthesis was slightly depressed, whereas that of (*yeast*)tRNA$^{Phe}_{-y}$ was greatly stimulated. As observed with streptomycin, the

Table 1

Influence of streptomycin and its 4-aminobenzhydrazone on poly(U) directed polyphenylalanine synthesis of *E. coli* ribosomes.

Antibiotic†	Source of tRNA	Poly(U) activity of *E. coli* 70 S ribosomes in %††
None	(*yeast*)tRNA^{Phe}	100
	(*yeast*)tRNA$^{Phe}_{-y}$	8
Streptomycin	(*yeast*)tRNA^{Phe}	80
	(*yeast*)tRNA$^{Phe}_{-y}$	30
4-Aminobenzhydrazone of streptomycin	(*yeast*)tRNA^{Phe}	75
	(*yeast*)tRNA$^{Phe}_{-y}$	32

† Incubation of 70 S *E. coli* ribosomes with antibiotic and (*yeast*)tRNA: In 500 μl TMA I, 1.5 A_{260} units of 70 S ribosomes were incubated with 10 μg antibiotic and 0.75 A_{260} unit (*yeast*)tRNA^{Phe} or (*yeast*)tRNA$^{Phe}_{-y}$. S-100 enzymes of *E. coli* and crude synthetase of *yeast* were added as described [9, 12].

†† Poly(U) activity of 1.5 A_{260} units 70 S ribosomes was determined as previously described [9]. The control samples gave 30 000 cpm corresponding to 45 moles of phenylalanine polymerized per mole of 70 S ribosome.

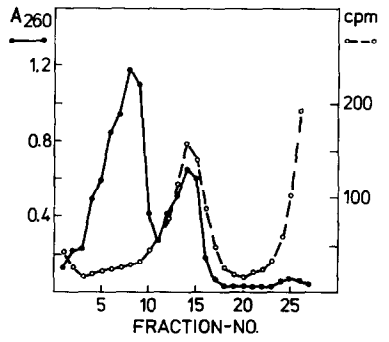

Fig. 1. Sucrose gradient of 70 S ribosomes labeled with the 4-iodo[^{14}C]acetaminobenzhydrazone of streptomycin as described under Experimental. On top of the gradient 200 A_{260} units 70 S ribosomes were layered and centrifuged at 24 000 rpm for 14 hr at 4°C in a Spinco SW27 rotor. 1.3 ml fractions were collected. 50 μl fractions of each fraction were diluted with 0.8 ml TMA II buffer and absorbance was read at 260 nm. Radioactivity was monitored by mixing 50 μl of each fraction with 10 ml of Bray's counting solution: (●——●——●) A_{260}; (○– – –○– – –○) cpm.

Table 2
Comparison of the reaction of 4-iodoacetaminobenzhydrazino-streptomycin with *E. coli* 70 S ribosomes to that of other antibiotic affinity labels.

Antibiotic	Affinity label	Poly(U) activity of *E. coli* 70 S ribosomes in %†
Chloramphenicol		48
	Monoiodoamphenicol	50
Puromycin		18
	N-iodoacetylpuromycin	27
Streptomycin		80* 30**
	4-Iodoacetamino-benzhydrazino-streptomycin	90* 25**

† Poly(U) activity of 1.5 A_{260} units 70 S ribosomes was determined as previously described [9]. The control samples gave 30 000 cpm corresponding to 45 moles of phenylalanine polymerized per mole of 70 S ribosome. Data for chloramphenicol and monoiodoamphenicol are taken from ref. [13], data for puromycin and *N*-iodoacetylpuromycin are taken from ref. [17]. 70 S ribosomes were incubated with a 10-fold molar excess of affinity label for 2 hr at 37°C. After incubation the samples were chilled and dialysed exhaustively against TMA I (0°C) for 12 hr. Then poly(U) activity was determined *after* the dialysis as described [9, 12].

* The incubation mixture contained (*yeast*)tRNA^{Phe}.

** The incubation mixture contained (*yeast*)tRNA$^{Phe}_{-y}$, experimental conditions as described in table 1.

derivated streptomycin renders the ribosomal-tRNA recognition screen less sensitive towards altered anticodon-loop structure [12, 16]. Derivatization of streptomycin in the described manner apparently had no effect on its action.

Then, the 4-monoiodoacetylaminobenzhydrazone of streptomycin was synthesized (see Experimental). Its reaction with 70 S ribosomes was checked by equilibrium dialysis experiments, by assays of poly(U)-directed polyphenylalanine synthesis and by comparison with other antibiotic affinity labels. The results are reported in table 2. Binding of the streptomycin-label to 70 S ribosomes is inhibited by streptomycin to about 50% as revealed by equilibrium dialysis experiments. Whereas monoiodoamphenicol and *N*-iodoacetylpuromycin irreversibly inhibit poly(U)-dependent polyphenylalanine synthesis by *E. coli* ribosomes, this is not the case, when 70 S ribosomes are incubated with streptomycin-label. The ribosomal-tRNA binding site, however, is irreversibly distorted as shown by the increased levels of polyphenylalanine synthesis with tRNA$^{Phe}_{-y}$. These data suggest that the streptomycin-label indeed competed with streptomycin for the same binding site and reacted irreversibly and specifically with this site on 70 S ribosomes.

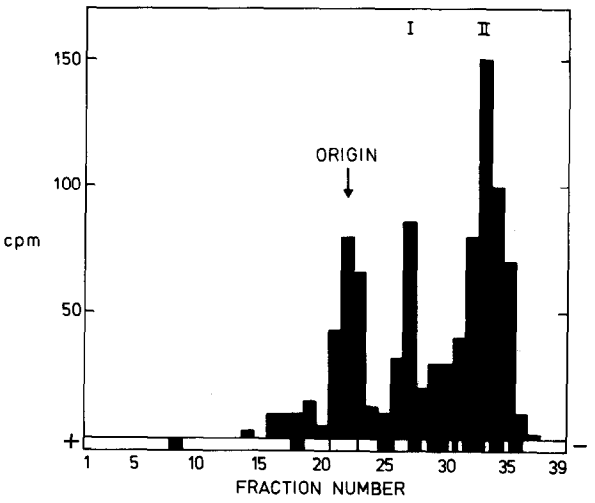

Fig. 2. Polyacrylamide gel electrophoresis of 30 S proteins (9 A_{260} units) was carried out as described by Kaltschmidt and Wittmann [14] for the first dimension. The gel was sliced into 4 mm sections. Each section was hydrolyzed by 1 ml of 30% hydrogen peroxide overnight at 60°C. Radioactivity was measured in Bray's solution.

E. coli 70 S ribosomes were incubated with a 10-fold molar excess of 4-monoiodo[^{14}C]acetylbenzaminohydrazone of streptomycin. After high speed centrifugation the ribosomes were dissolved in TMA II buffer and ribosomal subunits were separated by sucrose gradient centrifugation. As can be seen from fig. 1, radioactivity migrated almost exclusively with the 30 S peak indicating that 30 S protein(s) had reacted with the affinity-label. The 30 S and 50 S ribosomal proteins were extracted as described, and applied to the first dimension of the two-dimensional gel electrophoresis according to Kaltschmidt and Wittmann [14]. 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 30 S ribosomal proteins is shown in

fig. 2. The gels were cut into 5 mm slices, dissolved in 30% hydrogen peroxide and counted. As seen in fig. 2, radioactivity was found in two major peaks. Peak I tentatively contains ribosomal proteins S 3, S 4, and Peak II S 14, S 18, S 19. 50 S ribosomal proteins were analyzed similarly, but did not exhibit any significant radioactivity.

4. Concluding remarks

Since it is possible to label irreversibly *E. coli* ribosomes with a streptomycin-label in the 30 S subunit, further analysis of the labeling reaction should identify a ribosomal protein(s) located at or near the streptomycin-binding region. Experiments with streptomycin-resistant ribosomal mutants as well as two-dimensional gel electrophoresis should answer these questions.

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