IRREVERSIBLE BINDING OF *N*-IODOACETYLPUROMYCIN TO *E. COLI* RIBOSOMES*

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1. Introduction

The technique of affinity labeling has recently been demonstrated as being a promising tool for correlating specific functions to individual ribosomal proteins. Two laboratories have reported the covalent attachment of peptidyl-tRNA analogues to *E. coli* ribosomes [1, 2]. In our laboratory, we have previously succeeded to probe chemically the chloramphenicol binding region of *E. coli* ribosomes and identified protein L16 as the chloramphenicol binding protein by the affinity labeling technique [3, 4]. This finding was also verified by partial reconstitution experiments [5].

This report presents our first results of studies, which probe the puromycin binding site of *E. coli* ribosomes with a chemically reactive puromycin analogue. Puromycin is known to act in the peptidyltransferase center of the ribosome as an acceptor for the peptidyl moiety [6]. In the "fragment" reaction a peptide bond between peptidyl-tRNA and puromycin is formed to yield *N*-peptidylpuromycin [7, 8]. In analogy to this product, we synthesized *N*-iodoacetylpuromycin † and reacted it with ribosomes. The poly(U) dependent polyphenylalanine synthesizing activity and the peptidyltransferase activity of *E. coli* ribosomes was irreversibly inhibited by this reaction.

* Paper no. 3 on "Affinity Labeling of Ribosomes", preceding paper: Pongs, O., Bald, R. and Erdman, V.A. (1973) Proc. Natl. Acad. Sci. U.S., in press.

† *N*-iodoacetylpuromycin refers to 6-dimethylamino-9-[3-(*p*-methoxy-*N*-iodoacetyl-L-β-phenylalanylamino)-3-deoxy-β-D-ribofuranosyl] purine.

2. Experimental

2.1. *Synthesis of N-iodoacetylpuromycin*

A solution of 210 μmoles *N,N'*-dicyclohexylcarbodiimide in 1 ml of anhydrous dioxane was added to a solution of 200 μmoles iodoacetic acid and 220 μmoles *N*-hydroxysuccinimide in 1 ml of anhydrous dioxane. The *N,N'*-dicyclohexylurea precipitated immediately; after 1 hr at room temperature, the urea was removed by centrifugation. The dioxane solution was then evaporated to dryness at 25°C *in vacuo*. The residue was dissolved in 300 μl of anhydrous dioxane. It was added to a solution of 100 μmole puromycin (Serva, Heidelberg, Germany) in 2 ml 0.4 M $NaHCO_3$-buffer (pH 8.0), which contained 20% v/v methanol. The reaction mixture was shaken for 1 hr at room temp. The product was isolated by chromatography on PLC aluminium plates (aluminium oxide F_{254}, Type T, Merck, Germany) in chloroform/methanol (9:1) as solvent system (A). R_f of *N*-iodoacetylpuromycin on TLC aluminium sheets (aluminium oxide F_{254}, neutral, Merck, Germany) in solvent system A: 0.84 – relative mobility to puromycin: 1.50 –; R_f on TLC aluminium sheets (silica gel F_{254}, Merck, Germany) in solvent system A: 0.34. UV: λ_{max} 267.5 (in methanol). IR: ν_{sy} O=C–N of sec. amide: 1580 cm^{-1}; ν C=O : 1650 cm^{-1}.

N-iodo-[^{14}C]acetylpuromycin (28 mCi/mmole) was synthesized along similar lines. Iodo-[$^{14}C_2$]acetic acid (Amersham, England) was used instead of iodoacetic acid.

2.2. *Poly(U)-assay*

Ribosomes of *E. coli* A19 were isolated and purified

as previously described [9]. The poly(U)-assay was carried out as described by Nirenberg and Matthaei [10].

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

70 S ribosomes were incubated with a 30-fold excess of *N*-iodoacetylpuromycin overnight at 0°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 45 000 rpm in a Spinco Ti 50 rotor. The pellet was dissolved in TMA II buffer (same as TMA I, except 3×10^{-4} M $MgCl_2$). The ribosomal subunits were separated on a linear 10–30% sucrose gradient in TMA II buffer. Centrifugation was carried out in a Spinco SW27 rotor at 24 000 rpm for 14 hr. The fractions containing the 50 S and 30 S subunits, respectively, were collected and centrifuged overnight at 40 000 rpm in a Ti 60 rotor. The pellets were dissolved in TMA II buffer. Proteins were then extracted according to Kaltschmidt and Wittmann [11].

3. Results

As previously reported, chloramphenicol inhibits *in vitro* polyphenylalanine synthesis by about 50% (table 1) [3]. A similar degree of inhibition was observed when ribosomes were incubated with *N*-iodoacetylpuromycin, whereas puromycin inhibited the ribosomal activity more strongly (about 80%). The inhibition by *N*-iodoacetylpuromycin could be shown to be irreversible as summarized by the data in table 1. Incubation of 70 S ribosomes with chloramphenicol or puromycin for 2 hr 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 70 S ribosomes with *N*-iodoacetylpuromycin followed by extensive dialysis against TMA I buffer. Preincubation of 70 S ribosomes with *N*-iodoacetylpuromycin at 37°C yielded a more extensive irreversible inhibition (∿70%) than preincubation at 0°C (∿55%).

Incubation of 70 S ribosomes (8 μM) with a 30-fold molar excess of *N*-iodo-[^{14}C]acetylpuromycin in the presence of 10^{-2} M puromycin for 2 hr and subsequent equilibrium dialysis at –2°C revealed that puromycin inhibited the recation of the affinity label with the ribosome by ∿40% under these experimental conditions. Then 70 S ribosomes were incubated with *N*-iodo-[^{14}C]acetylpuromycin on a preparative scale. 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 with the 50 S and 30 S peak indicating that both subunits had reacted with the affinity label. The fractions containing the 50 S and 30 S subunits, respectively, were collected. 50 S and 30 S ribosomal proteins were extracted as described in Experimental, and run in the first dimension of the 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 50 S and 30 S ribosomal proteins is shown at the bottom of fig. 2 and fig. 3, respectively. The gels were cut into 4 mm slices, dissolved in 30% hydrogen peroxide (60°C overnight) and counted. As seen in figs. 2 and 3, radioactivity was found in several peaks. Since *N*-iodoacetylpuromycin was expected to react specifically in the peptidyltransferase center of the 50 S part of the ribosome, it is suggested that a protein contained in one of the

Table 1
Inhibition of polyU directed polyphenylalanine synthesis of *E. coli* ribosomes by puromycin and *N*-iodoacetylpuromycin.

Antibiotic*	Poly U activity of *E. coli* 70 S ribosomes in %†		Incubation temperature
	Not dialysed	Dialysed	(°C)
Chloramphenicol	48	90	37
Puromycin	23	72	0
	18	90	37
N-iodoacetyl-	53	44	0
puromycin	36	27	37
None	100	100	0
	73	100	37

* Incubation of 70 S *E. coli* ribosomes with antibiotic: In 500 μl TMA I, 15 A_{260} units of 70 S ribosomes were incubated with 50 nmoles antibiotic for 2 hr. After incubation the samples were either placed on ice or dialysed against TMA I (0°C) for 12 hr.

† Poly U activity of 1.5 A_{260} units of 70 S ribosomes was determined as previously described [9]. The control samples gave 41 000 cpm corresponding to 60 moles of phenylalanine polymerized per mole of 70 S ribosome.

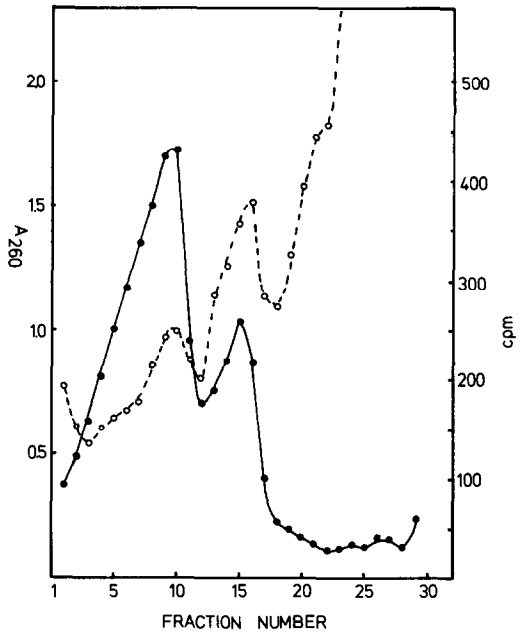

Fig. 1. Sucrose gradient of 70 S ribosomes labeled with *N*-iodo-[^{14}C]acetylpuromycin as described in Experimental. On top of the gradient 300 A_{260} units of 70 S ribosomes were layered and centrifuged at 24 000 rpm for 14 hr at 4°C in a Spinco SW27 rotor. 1.3 ml fractions were collected. 50 µl aliquots of each fraction were diluted with 0.8 ml of TMA II buffer and absorbance was read at 260 nm. Radioactivity was monitored by mixing 50 µl of each fraction with 12 ml of Bray's counting solution. (•—•—•) A_{260}; (○ - - ○ - - ○) cpm.

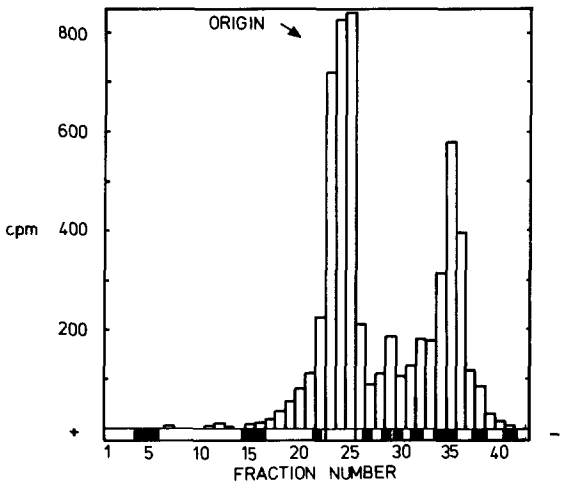

Fig. 2. Polyacrylamide gel electrophoresis of 30 S proteins (9 A_{260} units) was carried out as described by Kaltschmidt and Wittmann [11] for the first dimension. The gel was sliced into 4 mm sections. Each section was hydrolyzed by 1 ml of 30% hydrogen peroxide overnight at 60°C. Radioactivity was measured in Bray's solution.

two major radioactive peaks of fig. 3 belongs to the peptidyltransferase center of the ribosome.

4. Concluding remarks

Since it is possible to label irreversibly *E. coli* ribosomes with the puromycin analogue *N*-iodoacetylpuromycin, further analysis of the labeling reaction should exhibit one or several ribosomal proteins, which belong to the peptidyltransferase center and, thus, are directly involved in peptide bond formation. Present investigations are carried out in order to test the specificity of the affinity labeling reaction and whether this binding region is exclusively located on the 50 S subunit or also includes the 30 S subunit as might have been indicated by the data in figs. 1–3.

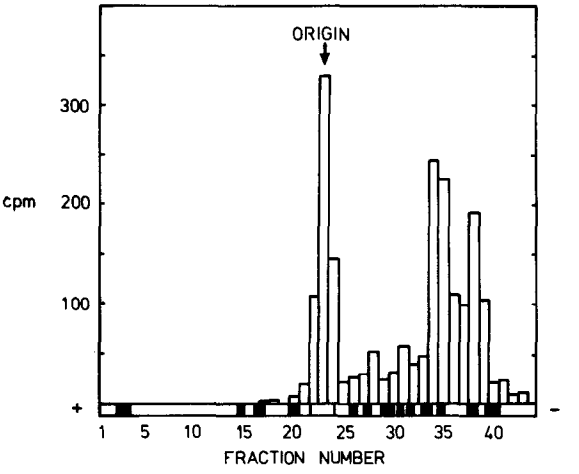

Fig. 3. Polyacrylamide gel electrophoresis of 50 S proteins (25 A_{260} units), carried out as described in fig. 2.

Competition of the labeling reaction with antibiotics, investigation of the "fragment" reaction as well as two-dimensional polyacrylamide gel electrophoresis should answer these questions.

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