IRREVERSIBLE BINDING OF CHLORAMPHENICOL ANALOGUES TO *E. COLI* RIBOSOMES

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1. Introduction

One of the most obvious difficulties in assigning specific functions to individual ribosomal proteins is the great complexity of the ribosomal particle. Consequently, many different approaches have been tried towards a detailed understanding of structure–function relationship of the ribosome (for review see [1]). A promising approach for testing the function of individual ribosomal proteins is the technique of affinity labeling as has been very recently demonstrated by the covalent attachment of peptidyl-tRNA analogues to *E. coli* ribosomes [2, 3].

This report presents the first results of studies which 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 inhibitor of protein biosynthesis [4], which probably acts at or near the peptidyl-transferase center of the ribosome [5]. Cloramphenicol was modified such that it did not lose its antibiotic specificity. The chemically reactive group introduced into the antibiotic was expected to react preferentially with a properly oriented amino acid functional group in the binding region of the ribosome. The specificity of the labelling reaction by the chloramphenicol analogues monobromamphenicol and monoiodoamphenicol was tested i) by comparing the antibiotic activities of these analogues with chloramphenicol in a polyU directed polyphenylalanine synthesizing system and ii) by purification of the affinity-labeled ribosomal subunits, followed by identification of their uniquely labeled ribosomal proteins.

† For the chloramphenicol analogues the following abbrevations 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.

2. Experimental

2.1. *Synthesis of monoiodo-[^{14}C]amphenicol and monobrom-[^{14}C]amphenicol*

A solution of 58.15 μmoles *N,N'*-dicyclohexylcarbodiimide in 100 μl of dioxane was added to a solutior of 58.12 μmoles iodo-[$^{14}C_2$]acetic acid (4.3 mCi/mmole) and 65.16 μmoles *N*-hydroxysuccinimide 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° *in vacuo*. The residue was dissolved in 250 μl of anhydrous *N,N'*-dimethylformamide and 57.06 μmoles of D-(−)-*threo*-1-*p*-nitrophenyl-2-amino-1,3-propanediol (Sigma, St. Louis, USA) were added. After 12 hr at room temp., th reaction mixture was applied to a 2 × 10 cm column of neutral alumina (Woelm A.G., Germany; Brockmanr Schodder activity III). Elution was carried out with chloroform/methanol (9:1). 5 ml fractions were collected and checked on TLC aluminium sheets (aluminium oxide F_{254}, neutral, Merck) with chloroform/methanol (9:1) as solvent system (R_f: 0.50). Unlabeled monoiodoamphenicol synthesized as described by Rebstock [6] was used as standard. Fractions 3–10, which contained the product, were combined, evapo-

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porated to dryness, and coevaporated twice with 1,2-dichloroethane. Recovery of the radioactivity was about 50%.

Monobrom-[^{14}C]amphenicol was synthesized along similar lines. Bromo-[$^{14}C_2$]acetic acid (1.5 mCi/mmole) was used instead of iodoacetic acid.

2.2. *PolyU-assay*

Ribosomes of *E. coli* A19 were isolated and purified as previously described [7]. The polyU-assay was carried out as described by Nirenberg and Matthaei [8].

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

70 S ribosomes were incubated with a 1000-fold excess of affinity label for 2 hr at 40° 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 overnight at 40,000 rpm in a Spinco Ti 60 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 [9].

3. Results

Chloramphenicol inhibits *in vivo* protein biosynthesis [4]. However, the effect of chloramphenicol is not as strong in the polyU directed *in vitro* protein synthesizing system [10]. We have found that chloramphenicol inhibits *in vitro* polyphenylalanine synthesis by about 50% (table 1) which is in agreement with previously published results [10, 11]. A similar degree of inhibition was observed when ribosomes were incubated with monobromamphenicol or monoiodoamphenicol instead of chloramphenicol. But, in the latter cases, the inhibition could be shown to be irreversible as summarized by the data in table 1. Incubation of 70 S ribosomes with chloramphenicol for 30 min and subsequent extensive dialysis of the ribosomes against TMA I buffer

Table 1
Inhibition of polyU directed polyphenylalanine synthesis of *E. coli* ribosomes by chloramphenicol and its analogues.

Antibiotic †	PolyU activity of *E. coli* 70 S ribosomes in % ††	
	Not dialysed	Dialysed
Chloramphenicol	48	90
Monobromamphenicol	65	70
Monoiodoamphenicol	40	50
None	100	100

† 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 2 mg antibiotic for 30 min at 37°. After incubation the samples were either placed on ice or dialysed agains TMA I (0°) for 12 hr.

†† PolyU activity of 1.5 A_{260} units 70 S ribosomes was determined as previously described [8]. The control samples gave 41,000 cpm corresponding to 60 moles of phenylalanir polymerized per mole of 70 S ribosome.

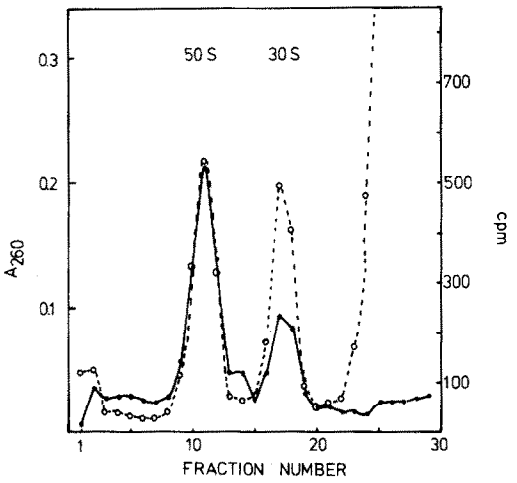

Fig. 1. Sucrose gradient of 70 S ribosomes labeled with monobromamphenicol as described in Experimental. On top of the gradient 40 A_{260} units 70 S ribosomes were layered and centrifuged at 24,000 rpm for 14 hr at 4° in a Spinco SW27 rotor. 1.3 ml fractions were collected. 40 µl aliquots of each fraction were diluted with 1 ml of TMA II buffer and adsorbance was read at 260 nm. Radioactivity was monitored by mixing 1 ml of each fraction with 12 ml of Bray's counting solution. (•—•—•) A_{260}; (o - - o - - o) cpm.

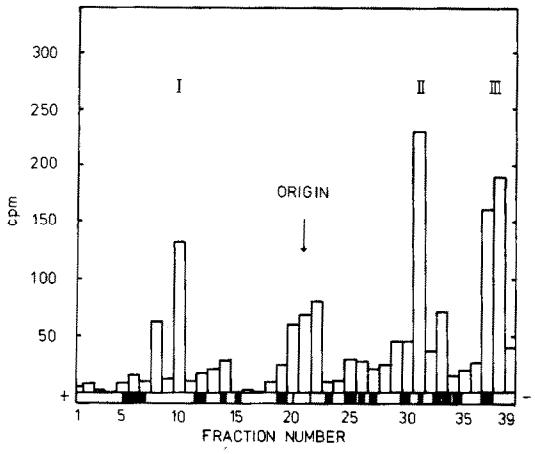

Fig. 2. Polyacrylamide gel electrophoresis of 30 S proteins (9 A_{260} units) was carried out as described by Kaltschmidt and Wittmann [12] for the first dimension. The gel was sliced into 5 mm sections. Each section was hydrolyzed by 1 ml of 30% hydrogen peroxide overnight at 60°. Radioactivity was measured in Bray's solution.

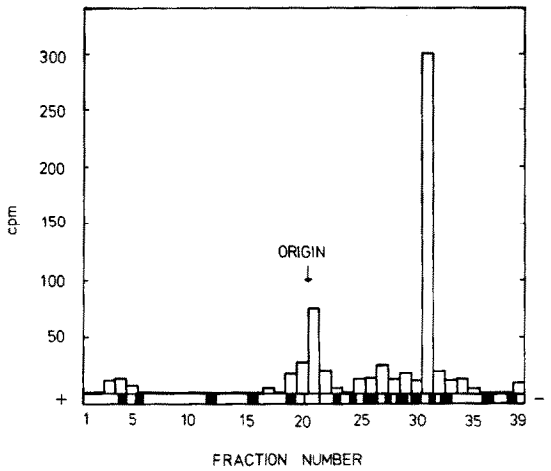

Fig. 3. Polyacrylamide gel electrophoresis of 50 S proteins (25 A_{260} units), carried out as described in fig. 2.

restored ribosomal activity. However, the original ribosomal activity could not be restored after incubation of 70 S ribosomes with monobromamphenicol or monoiodoamphenicol followed by extensive dialysis against TMA I buffer.

Next, we incubated *E. coli* 70 S ribosomes with monobrom-[^{14}C]amphenicol. 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 chloramphenicol analogue. The same experiment was repeated with monoiodo-[^{14}C]amphenicol on a large scale, giving a result similar to that reported in fig. 1. Since monoiodo-[^{14}C]amphenicol was prepared with a higher specific radioactivity (4.3 mCi/mmole), the specific radioactivity per A_{260} unit of labeled ribosomal subunit was three times higher than that reported in fig. 1. Thus, the iodoamphenicol labeled ribosomal subunits were more suitable to further analysis. The 30 S and 50 S ribosomal proteins were extracted as described in Experimental, and applied to one-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 30 S and 50 S ribosomal proteins is shown in fig. 2 and fig. 3, respectively. The gels were cut into 5 mm slices, dissolved in 30% hydrogen peroxide and counted. As seen in fig. 2, radioactivity was found in three major peaks. Peak II tentatively contains ribosomal protein S9 and peak III ribosomal proteins S 20 and S 21 [12]. Peak I has not been assigned yet. Much clearer is the pattern of the 50 S ribosomal proteins. All the radioactivity is contained in only one peak, which tentatively corresponds to proteins L16 and L24 [12]. Since the reaction of monoiodoamphenicol with the 50 S subunit is unique, it is suggested that one of the proteins contained in this peak corresponds to the chloramphenicol binding site on the 50 S subunit of *E. coli* ribosomes.

4. Concluding remarks

Since it is possible to label irreversibly *E. coli* ribosomes with chloramphenicol analogues, further analysis of the labeling reaction should exhibit the chloramphenicol binding region. Present investigations are carried out in order to test 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 presented in fig. 1–3. Competition of the labeling reaction

with chloramphenicol and other antibiotics as well as two-dimensional polyacrylamide gel electrophoresis should answer these questions.

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