AFFINITY BINDING OF *ESCHERICHIA COLI* RIBOSOMAL PROTEINS TO IMMOBILIZED RNA*

Harry R. BURRELL and Jack HOROWITZ
Department of Biochemistry and Biophysics, Iowa State University, Ames, Iowa 50010 USA

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1. Introduction

The ribosome consists of a complex array of proteins and RNA arranged in a specific three-dimensional structure. Interactions between ribosomal proteins and ribosomal RNA are essential for the maintenance of this structure in an active conformation [1]. As part of a study of the recognition process between ribosomal proteins and RNA molecules, we have explored the use of affinity chromatography as a means of isolating and identifying the ribosomal proteins that form specific complexes with RNA or polynucleotide fragments. In this preliminary communication, we report an improved method, based on the nucleotide coupling procedure of Lamed et al. [2], for covalently linking RNA molecules, via their 3′-terminus, to an agarose gel and, as an example of the utility of this material, describe its use to probe the interactions between matrix-bound 5S RNA and *E. coli* ribosomal proteins. In addition, we have made a simple and inexpensive modification of the basic Kaltschmidt and Wittmann two-dimensional gel electrophoresis apparatus [3], which permits the use of reduced-size gel slabs.

2. Experimental

2.1. Preparation of Ribosomes, Ribosomal Proteins and RNA.

Ribosomes, prepared from *E. coli* B [4], were washed once in standard buffer (0.01 M Tris–HCl, pH 7.4; 0.01 M $MgCl_2$; 0.06 M NH_2Cl; 0.006 M 2-mercaptoethanol), once in the same buffer containing 0.5 M NH_4 Cl, and then dialyzed against standard buffer. Purified 50S and 30S subunits were separated by zonal centrifugation in the B IV rotor of the Beckman Model L-4 ultracentrifuge and their proteins extracted with 4 M urea –2 M LiCl [5]. Endogenous 5S RNA was removed from 50S ribosomal proteins with DEAE-cellulose [6], and the recovered proteins were dialyzed against 1% acetic acid, lyophylized, and taken up in binding buffer (0.005 M potassium phosphate, pH 7.4; 0.3 M KCl; 0.02 M $MgCl_2$; 0.006 M 2-mercaptoethanol). RNA was prepared by a phenol-sodium dodecyl sulfate method [4] and individual RNA species separated on Sephadex G-100 [7].

2.2 Preparation of Agarose - RNA Gel

RNA was linked to CNBr-activated Sepharose 4B (Pharmacia) through an adipic acid dihydrazide spacer. Adipic acid dihydrazide was prepared and attached to CNBr-Sepharose according to the procedure of Lamed et al. [2]. RNA preparations were oxidized with sodium periodate [8] and the oxidized RNA, dissolved in 0.1 M sodium acetate, pH 5.0 (1–2 mg/ml), was added to dihydrazide–Sepharose. The coupling reaction was allowed to proceed for 24 hr at 4°C, and the resulting RNA-agarose washed repeatedly with 2 M KCl and then equilibrated with binding buffer.

To examine the binding of ribosomal proteins to RNA-agarose, the two preparations, both in binding buffer, were mixed and permitted to interact for 24 hr at 4°C. Unbound proteins were removed by exhaustive washing with binding buffer. Bound proteins were then eluted with 0.005 M potassium phosphate buffer, pH 7.4, containing 2 M KCl, 0.005 M EDTA, and 0.006 M 2-mercaptoethanol.

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2.3 Gel Electrophoresis of Ribosomal Proteins

Disc gel electrophoresis at pH 4.5 was carried out according to the procedure of Leboy et al. [9] by using 10% acrylamide gels. Samples were treated with 0.06 M 2-mercaptoethanol at pH 8.1 to avoid artifacts arising from sulfhydryl oxidation products [10]. Two-dimensional gel electrophoresis, for the identification of individual ribosomal proteins, was performed by use of the gel and buffer systems of Howard and Traut [11] in the apparatus described by Kaltschmidt and Wittmann [3] modified by the insertion of plexiglass adapters to reduce gel thickness. The adapters consist of a plexiglass sheet 8 1/4″ X 7 7/8″ X 1/8″ with a 45° bevel, 3/16″ wide, along one 8 1/4″ edge. A plexiglass strip 3/32″ X 7 9/16″ X 1/16″ is glued flush with each 7 7/8″edge, leaving a 1/8″ gap at the bottem of the sheet. The spacers allow the preparation of gel slabs 1/16″ thick. With this simple, inexpensive modification, two different samples, electrophoresed on 0.5 cm X 10.0 cm 1-D gels, can be run in parallel on a single gel slab without loss of resolution. This facilitates comparison between different protein samples while doubling the sample capacity of the original apparatus. The thinner gel sheets allow a shorter electrophoresis time in both the first dimension (5–6 hr compared with 20–30 hr) and the second dimension (12 hr compared with 26 hr), and permit the use of microgram amounts of sample.

Gel slabs were stained for 3 hr in 0.1% Coomassie Bleu R250 in methanol–acetic acid [11] and destained by dialysis.

3. Results and discussion

The procedure described for the coupling of RNA to agarose is simple, and rapid and permits the immobilization of rather large amounts of RNA. Use of a one step method for preparing agarose–hydrazide [2] has the advantage over the three-step procedure described by Robberson and Davidson [12], of avoiding the introduction of carboxyl groups which interfere with RNA binding. As much as 3.0×10^{-7} mol (12 mg) of periodate oxidized 5S RNA can be bound to 1 gram (3.5 ml settled vol) of the modified agarose gel (table 1); unoxidized 5S RNA does not bind (table 1).

Steric factors seem to play a role in the coupling of oxidized RNA molecules to dihydrazide–agarose; the gel is capable of binding approximately 10 times more benzaldehyde than oxidized 5S RNA on a molar basis (table 1). Oxidized tRNA, as well as 16S and 23S ribosomal RNA, can be linked to agarose by the procedure described, and here too the molar amount coupled decreases with molecular size (results not shown). The coupling reaction is relatively rapid. Although in the experiments described here, coupling is allowed to

Table 1
Coupling of RNA to agarose–hydrazide

	Mg added per g gel	Mg bound per g gel[a]	Moles bound per g gel
Benzaldehyde	2.6	0.5	4.7×10^{-6}
Oxidized 5S RNA	7.2	7.2	1.8×10^{-7}
	14.4	6.9	1.7×10^{-7}
	43.3	10.0	2.5×10^{-7}
	108.2	12.0	3.0×10^{-7}
Unoxidized 5S RNA	8.5	Not detectable	Not detectable

[a] The uptake of benzaldehyde and RNA was determined indirectly be measuring the absorbance of the supernatent and 2 M KCl washes (see Experimental) and subtracting the amount recovered from the amount originally added. Benzaldehyde concentration was calculated by using a molar extinction coefficient of 12×10^3 at 248 nm (12); an $E_{260}^{0.1\%} = 24$ was used to calculate RNA concentration. Values determined by this procedure agreed well with those obtained by measuring the absorbance released after hydrolyzing a sample of 5S RNA–agarose with 1.0 M KOH at 25°C for 12 hr. Data from alkaline digestion were corrected for hyperchromicity due to hydrolysis of approx. 40%.

proceed for 24 hr, kinetic studies have shown that the reaction is 90% complete in 4 hr. The linkage of RNA to the agarose gel is quite stable in binding buffer at 4°C; less than 1% of the A_{260} units bound are released after storage for several months. In addition, we have found that 5S RNA–agarose can be reutilized for the binding of 50S ribosomal proteins at least twice with no loss of specificity.

When 50S ribosomal proteins interact with 5S RNA linked to agarose, a small fraction of the protein is retained on the gel (table 2). These proteins bind strongly to the 5S RNA matrix; they are not removed by exhaustive washing with binding buffer, but require both EDTA and high salt concentrations for complete elution. Specific binding requires the presence of RNA; little or no protein is retained on unsubstituted agarose–hydrazide gels (table 2).

Polyacrylamide disc-gel electrophoresis of the 50S ribosomal proteins that bind to 5S RNA–agarose shows two heavily stained protein bands and one lightly stained band (fig.1). These were identified as L18 and L25 (heavily stained) and L5 (light stain) by two-dimensional gel electrophoresis (fig.2, left). Positive

Table 2
Binding of ribosomal proteins to 5S RNA–agarose[a]

	Mg added per g gel[b]	Mg bound per g gel
50S ribosomal proteins	17.0	0.37
50S ribosomal proteins	87.5	1.7
50S ribosomal proteins	12.4[c]	Not detectable
30S ribosomal proteins	40.5	Not detectable
30S ribosomal proteins	14.7[c]	Not detectable

[a] 6.8 mg and 10.4 mg of 5S RNA were bound per g gel, in the experiments with 50S and 30S ribosomal subunit proteins respectively.
[b] Protein concentration was determined by either the procedure of Lowry et al. [16] or that of Geiger and Bessman [17].
[c] An agarose–hydrazide gel containing no coupled 5S RNA was used.

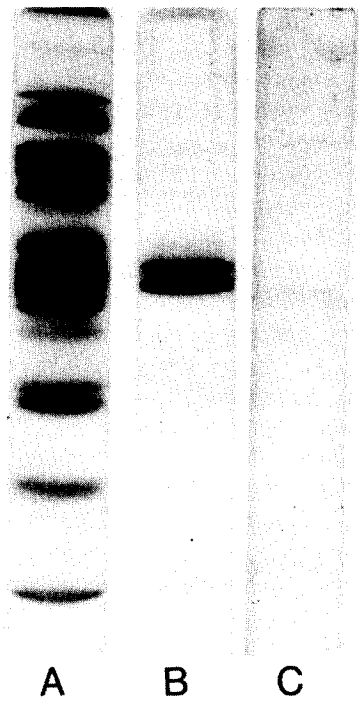

Fig. 1. Disc-gel electrophoresis of ribosomal proteins bound to 5S RNA–agarose. Conditions for electrophoresis are described in Experimental. (A) 50S ribosomal proteins not bound to 5S RNA–agarose; (B) 50S ribosomal proteins bound to 5S RNA–agarose; (C) 50S ribosomal proteins bound to tRNA–agarose.

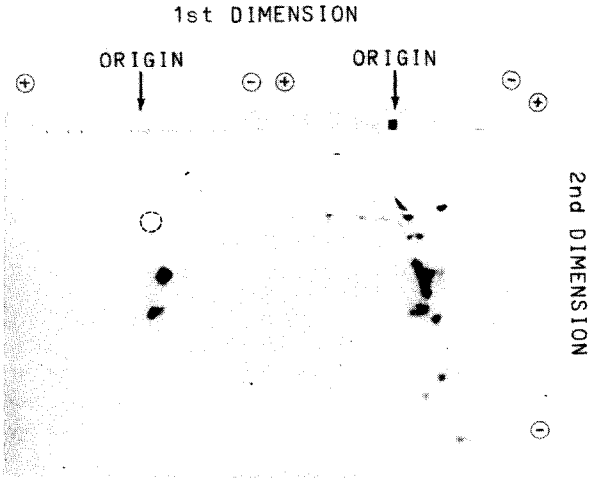

Fig. 2. Two-dimensional gel electrophoresis of 50S ribosomal protein fractions from affinity-binding to 5S RNA–agarose, Electrophoresis was carried out in the apparatus of Kaltschmidt and Wittmann modified with the plexiglass inserts described in the Experimental section. Left: 50S ribosomal proteins that bind to 5S RNA–agarose. The dotted circle indicates position of the small amount of L5 present. Right: 50S ribosomal proteins not bound to 5S RNA–agarose.

identification was made by coelectrophoresis of a mixture of 5S RNA-binding proteins together with a small amount of total 50S ribosomal proteins (results not shown). The three unknown proteins were then readily identifiable against the light background of total 50S ribosomal proteins. Essentially all L18 and L25 are retained on 5S RNA–agarose; these proteins cannot be detected among the proteins removed by washing tne gel with binding buffer (fig.2, right). L5 is less firmly bound to 5S RNA, and some is found in the protein fraction not bound to matrix-linked 5S RNA (fig.2). Binding of L18, L25, and L5 to the gel specifically requires 5S RNA. These proteins are not retained on unsubstituted agarose–hydrazide nor are they bound to agarose linked tRNA (fig.1C).

Our results are consistent with those of others who have detected specific complexes between 5S RNA and ribosomal proteins by nitrocellulose filtration [13], sucrose gradient centrifugation [13,14] and gel electrophoresis techniques [14,15]. The major ribosomal proteins found associated with 5S RNA in these complexes were L18 and L25 [13–15]; lesser amounts of L5 [13,14] and traces of L20 and L30 [14] also have been reported.

The data presented demonstrate the feasibility of using affinity binding techniques to probe the interactions between ribosomal proteins and RNA. We are exploring the application of the method to studies of other ribosomal protein-RNA interactions important for the structure and function of the ribosome using tRNA as well as 16S and 23S ribosomal RNA covalently coupled to agarose.

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