Isolation of ribosomal protein-RNA complexes by nitrocellulose membrane filtration: equilibrium binding studies

Eleanor Spicer⁺, Jean Schwarzbauer and Gary R. Craven

Laboratory of Molecular Biology and Department of Genetics, University of Wisconsin, Madison, WI 53706, USA

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ABSTRACT

E. coli ribosomal proteins are retained by nitrocellulose filters. In contrast, 16S RNA passes through nitrocellulose filters. We have found that specific protein-RNA complexes involving single proteins also pass through nitrocellulose filters. Thus, by utilizing radioactively labeled r-proteins, nitrocellulose filtration can be used to study directly and sensitively the stoichiometry of r-protein-RNA association. The filtration process maintains near equilibrium conditions¹, making it applicable to weak as well as strong protein-RNA associations.

We have used nitrocellulose filtration to obtain saturation binding curves for the association of proteins S4, S7, S8 and S20 with 16S RNA. In each case, the stoichiometry of binding was one mole of protein or less per mole of RNA. The stoichiometry of protein S8 binding to 16S RNA measured by filtration is comparable to that observed by sucrose gradient centrifugation. Association constants for the binding of proteins S4, S8 and S20 to 16S RNA have been determined by analysis of the saturation binding curves and were found to range from $.3-6 \times 10^7 \text{ M}^{-1}$.

INTRODUCTION

Studies on the interactions between protein and RNA in the 30S ribosome have been facilitated by the development of an in vitro reconstitution system for E. coli 30S ribosomes^{2,3}. A number of laboratories have utilized this in vitro system to examine the ability of the 21 30S ribosomal proteins to bind individually to 16S RNA and have found that up to 12 proteins possess this ability⁴⁻¹⁰. In these investigations, complexes between single proteins and 16S RNA were isolated by velocity sedimentation, sucrose gradient centrifugation or agarose gel filtration. Each of these techniques measures the stoichiometry of protein binding to RNA in the absence of unbound protein, i.e. under non-equilibrium conditions.

We have observed that there are variations in the qualitative strengths of the individual r-protein-RNA interactions in vitro¹¹. The use of non-equilibrium isolation techniques limits one to a study of the strong protein-RNA association. To facilitate examination of weak associations we have

investigated techniques which enable isolation of substrate-ligand complexes under equilibrium conditions. Filtration through nitrocellulose membranes was found to be a rapid, sensitive method for measuring r-protein binding to RNA. We described here the properties of r-protein and rRNA filtration and report binding constants determined by filtration for three r-protein-RNA association.

MATERIALS AND METHODS

Ribosomes were prepared from *E. coli* strain MRE600 (RNase deficient) in the manner described by Kurland¹². Ribosomes were collected from cell extracts by a series of ammonium sulfate precipitations. Purified 30S and 50S particles were isolated by zonal centrifugation (Beckman B1V rotor) of dissociated subunits through a 5-30% sucrose gradient containing 0.01 M Tris-HCl (pH 8.0) - 0.05 M KCl - 0.0005 M Mg acetate.

Proteins were extracted from ribosomes with 67% glacial acetic acid-0.2 M Mg acetate¹³. Individual 30S proteins were separated by phosphocellulose chromatography in 6 M urea-0.05 M Na phosphate (pH 5.8)-1 mM dithiothreitol¹³. Purified proteins were stored in the urea-phosphate buffer at -20°C. Protein identifications were determined by polyacrylamide gel electrophoresis, using the standard gel system described by Leboy *et al.*¹⁴, and as modified by Voynow and Kurland¹⁵. Proteins were radioactively labeled *in vitro* by reductive methylation^{16,17,9}, utilizing (³H-) NaBH₄. The specific activity of labeled proteins ranged from 0.2 to 4.0×10^8 cpm/umole. Specific activities were calculated by ninhydrin analysis, employing the molecular weights for 30S proteins determined by Craven *et al.*¹⁸.

E. coli 16S RNA was purified from ribosomal subunits by the phenol extraction procedure described by Traub *et al.*¹⁹. Following three exposures to sodium citrate buffer (SSC)-saturated phenol (pH 7.0), the RNA was dialyzed against 0.03 M Tricine (pH 7.4) and stored at -70°C. Yeast RNA (Calbiochem) was a mixture of 18S and 28S RNA and was highly dialyzed before use.

Protein-RNA complexes were prepared using the reconstitution system developed by Traub and Nomura³. Reactions were generally performed using 1 to 2 A₂₆₀ units of RNA in 0.2 to 0.5 ml reconstitution buffer (0.03 M Tricine (pH 7.4) -0.40 M KCl -0.02 M Mg acetate- 1 mM DTT). One A₂₆₀ unit of 16S RNA contains 75 pmoles of RNA. Protein-RNA complexes were isolated by centrifugation or filtration as described in the text.

Membrane filtrations were carried out in a Paulus ultra-filtration

cell¹, enabling eight samples to be filtered simultaneously. Small triangles of nitrocellulose membranes were cut from pre-wetted millipore filters (Millipore Corp., type HA) and positioned over 5 mm diameter sample channels in the lower block. Samples in 200 to 400 μ l volumes were pushed through the filters by low N₂ pressure (approximately 2 psi) in 10 to 30 seconds. The filtrate was collected by inverting the apparatus (while N₂ pressure was maintained) and removing the sample from the filtrate channels with a pasteur pipette.

RESULTS AND DISCUSSION

Filtration Properties

Nitrocellulose filters were tested for their ability to retain ribosomal proteins by filtering solutions of (³H-) S7 in reconstitution buffer. Figure 1 shows that 96-98% of the protein was retained by the filters when up to 400 pmoles of protein were applied. Similar results were obtained with protein S4. Subsequent experiments employed less than 400 pmoles of protein per sample. When 16S RNA was filtered, in the presence or absence of ribosomal proteins, 90-95% of the RNA A₂₆₀ units were recovered in the filtrate. Additionally, when an individual protein was pre-incubated with a two-fold excess of 16S RNA, 80% of the protein was recovered from the filtrate. Thus, single-protein-RNA complexes and 16S RNA pass through nitrocellulose filters while unbound protein is selectively retained by the filter. Through the use of radioactively labeled proteins the stoichiometry of protein binding to RNA can be measured directly by assaying the filtrate for radioactivity and A₂₆₀ units.

We have utilized these properties of nitrocellulose filters to obtain saturation binding curves for the interactions of individual 30S proteins with 16S RNA. Figure 2 shows the results of reconstituting increasing amounts of (³H-) S20 with 16S RNA, followed by filtration through millipore filters. A plateau in binding was observed when a three molar excess of S20 was reconstituted with 16S RNA. In this case, the stoichiometry of binding was 0.93 moles S20 bound per mole of 16S RNA. Similar binding curves were obtained with proteins S4 and S8 (data not shown).

To test the specificity of the filtration method, yeast ribosomal RNA was reconstituted with (³H-) S20 and the results are shown in Figure 2. Only a slight amount of protein was pulled through the filter by yeast RNA. The filtration process was also judged to be specific by the fact that a plateau in binding was observed when one mole of protein or less was bound per mole RNA. Additionally, the filtration of (³H-) lysozyme in the

Figure 1. Filtration of (^3H -) S7 through nitrocellulose filters, in the absence of 16S RNA. Increasing amounts of S7 in reconstitution buffer (0.3 ml volume) were applied to the filter and the amount of protein in the filtrate (----) and on the filter (—) was determined. The protein specific activity was 1.7×10^8 CPM/ μmole .

presence of 16S RNA resulted in no enrichment of lysozyme in the filtrate.

The accuracy of the filtration technique was evaluated by dividing reconstituted samples of protein S8 and 16S RNA into two portions. Protein-RNA complexes were then isolated by sucrose gradient centrifugation and by filtration. Table 1 shows the stoichiometry of protein bound to 16S RNA as measured by the two techniques. There is good correspondence between the two sets of data, indicating the filtration technique is as accurate and reliable as the commonly used technique of gradient centrifugation.

The technique of nitrocellulose filtration has a number of advantages over centrifugation and gel filtration methods. It provides a very sensitive method for isolating protein-RNA complexes, enabling detection of approximately 3×10^{-11} moles of complex. More importantly, it makes quantitative examination of protein-RNA interactions possible, since one can calculate an (apparent) equilibrium association constant from the data contained in a saturation binding curve (see following section). Additional advantages are the requirement for small quantities of protein and RNA and the speed with which the complexes are isolated.

It should be noted that the filtration properties of r-protein-rRNA complexes are unusual in that, apparently, the RNA dominates the behavior of the complex, pulling bound protein into the filtrate.

Figure 2. Saturation binding curve for S20-16S RNA interaction. Increasing concentrations of (^3H -) S20 were reconstituted with 16S RNA (—) or yeast RNA (----). The solid curve is a composite of two experiments (indicated as O and ●), utilizing the same preparations of protein but different preparations of RNA. Protein-RNA complexes were isolated by nitrocellulose filtration. Stoichiometry of binding was determined by assaying the filtrate for A_{260} units (RNA and (^3H -) counts (protein)).

TABLE 1. Comparison of the Stoichiometry of S8 Binding to 16S RNA, using 2 Methods to Isolate Protein-RNA Complexes.

Moles S8 per mole RNA in recon- stitution	^3H -CPM bound/ A_{260} unit		Moles S8 bound/mole RNA	
	filtra- tion assay	sucrose gradient analysis	filtra- tion assay	sucrose gradient analysis
1.0	2040	2210	0.17	0.18
2.0	5500	4970	0.46	0.40
2.7	6270	6210	0.52	0.52

The data from sucrose gradient analysis reflect an average of 5 points on a gradient curve. Filtration data are the average of two points for each.

Most other filtration studies report the retention of nucleic acid-protein complexes on membranes (e.g. lac repressor-operator complexes²⁰, RNA polymerase-promoter complexes²¹, and t-RNA synthetase-t-RNA complexes²²). The fact that the complex is recovered in the filtrate eliminates the need for washing the filter (thus reducing error and

decreasing the disturbance of the equilibrium) and increases the amount of complex which is recovered for use in further experiments.

Equilibrium Binding Constants

In order to calculate association constants from the data obtained by filtration, the process should not disturb the equilibrium between free and bound protein. Paulus¹ has studied the binding of radioactive ligands to proteins by filtration through Diaflo (Amicon) membranes and has found that the concentration of free ligand above the filter does not change during the filtration. Similarly, Riggs *et al.*²⁰ have found that during nitrocellulose filtration of *lac* repressor-operator complexes the filter does not perturb the equilibrium. We have not directly examined this question for the filtration of r-protein-rRNA complexes. However, the speed of the filtration process (completed within 10 to 30 seconds) suggests that the membrane would have little effect on the equilibrium.

We have determined apparent association constants ($K_{\text{assoc.}}$) by applying double reciprocal plot analysis to the data contained in the saturation binding curves. Figure 3 presents such an analysis of the binding of S20 to 16S RNA, where the reciprocal of the number of moles of protein bound per mole of RNA ($1/v$) is plotted versus the reciprocal of the concentration of free protein. The $1/v$ intercept indicates that n , the number of protein binding sites of RNA, is 0.93 for S20. Determination of the slope of this plot shows that the $K_{\text{assoc.}}$ is $1.4 \times 10^7 \text{ M}^{-1}$ for this reaction.

Table 2 presents a summary of the association constants derived for proteins S4, S8 and S20. These values are surprising in two respects. First, we anticipated the binding strength of S4 to be greater than that of S8 in view of the (proposed) substantial difference in their manner of interaction with 16S RNA (e.g. S4 protects from RNase digestion approximately 500 bases, while S8 protects only 36 bases^{23,24}). However, the association constant for S8-16S RNA interaction is greater than $K_{\text{assoc.}}$ for S4. Secondly, the association constants are significantly lower than those reported for other protein-nucleic acid association of similar specificity (e.g. $K_{\text{assoc.}}$ for *lac* repressor-operator interaction is 10^{11} to 10^{13} M^{-1}). One explanation for the lower values is that the $K_{\text{obs.}}$ for the binding of a ligand to a polyelectrolyte is strongly dependent on the ionic strength of the environment²⁵ and the concentration of counterions in our reconstitution buffer ($\bar{\mu} = 0.39$) is higher than that of the

Figure 3. Double reciprocal plot analysis of the binding of S20 to 16S RNA (from Figure 2). V = moles S20 bound per mole 16S RNA; A = concentration of unbound protein. The $1/v$ intercept indicates $n = 0.93$. The slope of the plot indicates $K = 1.4 \times 10^7 \text{ M}^{-1}$.

Table 2. Binding constants for 30S Protein-16S RNA Interactions in Reconstitution Buffer.

PROTEIN	Expt. no.	$K_{\text{assoc.}} (\text{M}^{-1})$	n
S4	1	3.5×10^6	1.10*
S8	1	2.6×10^7	1.16
	2	5.3×10^7	1.09
S20	1	1.7×10^7	1.00
	2	1.6×10^7	0.80

Binding parameters were calculated by linear regression analysis of double reciprocal plots.

*S4 data was corrected for a systematic loss of protein and RNA.

binding buffer employed for the lac repressor-operator studies ($\bar{u} = 0.04$ to 0.2). In addition, the strength of the protein-RNA interaction may be lower when a single protein binds to RNA than when the same protein interacts with RNA in the intact ribosome.

In summary we have developed a rapid and convenient technique which permits the accurate determination of association constants between ribosomal proteins and rRNA. This approach has numerous potential applications. For example, many important parameters such as temperature, ionic conditions, and solvent conditions can be quantitatively analyzed for their effect on protein-RNA recognition. In addition, ribosomal proteins which have not previously been shown to interact with rRNA can be surveyed for possible specific associations too weak to measure by the traditional techniques.

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⁺Present Address: Department of Molecular Biophysics
and Biochemistry
Yale University, 333 Cedar Street
New Haven, Connecticut 06510

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