Volume 9 Number 9 1981 Nucleic Acids Research

Apparent association constants for E. coli ribosomal proteins S4, S7, S8, S15, S17 and S20 binding to 16S RNA

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Received 20 January 1981

ABSTRACT

We have previously reported the development of a technique utilizing nitrocellulose filters, which rapidly separates ribosomal protein-ribosomal RNA complexes from unbound protein[1,2]. We have used this technique to obtain binding data for the association of proteins S4, S7, S8, S15, S17 and S20 with 16S RNA. With the exception of protein S17, the association behavior for each of these proteins exhibits a single binding site with a unique binding constant. The apparent association constants have been calculated and have been found to have a range from 1.6×10^6 M^{-1} for protein S7 to 7.1×10^7 M^{-1} for protein S17. The Scatchard plot for the protein S17 binding data is biphasic, suggesting that within the RNA population two different binding sites exist, each with a different apparent association constant.

INTRODUCTION

Many different proteins have been found capable of specific recognition of nucleic acid structure, yet in most cases the basic mechanism of this recognition process remains a complete mystery. Ribosomal components offer a unique system for the possible dissection of the underlying principles of specific protein-nucleic acid interactions. The ribosome is composed of over 50 proteins and many of these interact specifically with selected regions of ribosomal RNA. These proteins are very similar in size and general chemical structure, yet each one is unique in its ability to recognize and form a firm non-covalent bond with a precise region of the ribosomal RNA.

Several different approaches have been taken to elucidate some of the features of the protein-RNA interactions responsible for ribosome integrity. Ribonuclease digestion of protein-RNA complexes has led to a general knowledge of the specific regions

of RNA bound by individual proteins[3]. Chemical protection experiments have also proven to be very informative, especially in the utilization of the reagent kethoxl to identify specific guanine residues of the RNA that become unreactive due to the binding of ribosomal protein[4]. Similarly, the regions of the protein responsible for RNA recognition have been delimited by the use of enzymes and chemical cleavage reactions to yield smaller polypeptides which maintain the capacity to interact specifically with RNA[5,6]. A more precise knowledge of actual contact points between protein and RNA has been developed through the use of chemical crosslinkage[8]. Despite the rather detailed knowledge of the recognition domains for both protein and RNA, we still understand little about the underlying rules controlling this interaction.

The approaches outlined above are basically structural in nature and they unfortunately give us few clues about the chemical forces involved in protein-RNA complex formation. Information about these forces conceivably could be obtained from studies of the thermodynamics of these interactions. Before this can be done, however, it is essential to have a rapid and convenient method for measuring the binding reaction. In dealing with two interacting macromolecules this is not always readily accomplished. To obtain such measurements it is necessary to be able to separate the protein-RNA complex from the unbound protein[2]. To this end, protein-RNA complexes have been fractionated from free protein by sucrose gradient centrifugation, gel filtration, and gel electrophoresis[9,10,11]. Recently, we have developed a technique to achieve this separation under near-equilibrium conditions by nitrocellulose membrane filtration[1,2]. We report here the use of this technique to measure binding properties of proteins S4, S7, S8, S15, S17, and S20 with 16S RNA.

MATERIALS AND METHODS

Buffers

TKM: 0.01 M Tris-HCl, 0.05 M KCl, 0.52 mM Mg acetate, pH 8.0

RB: 0.01 M Tris-HCl, 0.33 M KCl, 0.02 M Mg acetate, 0.001 M dithiothreitol (DTT), pH 7.6

Preparation of Ribosomes

70S ribosomes were prepared from E. coli strain MRE 600 using the ammonium sulfate precipitation and washing procedure devised by Kurland[12] and modified as previously described[13]. 30S and 50S subunits were dissociated in low Mg buffer (TKM) and separated by zonal centrifugation with a Beckman T:15 rotor as described by Eikenberry et al.[14].

Preparation of Ribosomal Proteins and RNA

Ribosomal proteins were extracted from 30S subunits and purified by phosphocellulose column chromatography as described by Hardy et al.[15]. In certain cases proteins were further purified by carboxymethylcellulose chromatography or by Sephacryl S-200 gel filtration in 6 M urea and 0.05 M phosphate at pH 6.5. Proteins were identified by one dimensional and two dimensional polyacrylamide gel electrophoresis[16,17,18] as well as by double immunodiffusion[19]. The protein S17 preparation used showed no detectable contamination by protein S16.

Proteins were made radioactive by in vitro reductive methylation of lysine residues using ^{3}H-$NaBH_4$ (Amersham) and formaldehyde[20]. In certain cases the more recent method of Jentoft and Dearborn[21] was employed. Protein concentrations were determined by the Lowry method[22] using lysozyme as the standard. Specific activities of the radioactive proteins ranged from 1.0 to 5.0 x 10^8 cpm/μmole.

Ribosomal RNA was prepared by phenol extraction of purified 30S or 50S subunits[23]. Alternatively, RNA was obtained by phenol extraction of intact 70S particles followed by zonal centrifugation. RNA was stored in 0.01 Tris, pH 7.0 at -70°C.

Formation and Isolation of Protein-RNA Complexes

Protein-RNA complexes were prepared using the reconstitution system of Traub et al.[23]. The reaction mixture containing protein and 0.5-1.0 A_{260} units of RNA in a maximum of 400 μl was incubated for 1 hr at 40°C and was chilled to 4°C before isolation of the complex. Protein to RNA molar ratios were varied assuming that 75 pmoles per ml of 16S RNA has an absorption at 260 nm of 1.0 using a 1 cm cell.

Protein-RNA complexes were purified from unbound protein by nitrocellulose membrane filtration[1,2]. Filtration was

carried out using an ultrafiltration cell[24]. Before filtration, an aliquot of the reaction mixture was removed, diluted and the absorbance at 260 nm determined. Another aliquot was taken and assayed for radioactivity to determine the total protein concentration. Up to 12 samples were filtered through pre-wetted nitrocellulose filters (Millipore, type HA, 0.45 μm pore size) with the aid of low nitrogen pressure (approximately 2 psi). The apparatus was inverted while pressure was maintained. The filtrate was assayed for RNA recovery and the radioactive protein. In all experiments the final concentration of bound protein was corrected for the approximate 5% of unbound protein which leaks through the filter. Control experiments were also done using non-cognate ribosomal RNA.

RESULTS

In earlier work we demonstrated that nitrocellulose filters can retain ribosomal proteins while allowing ribosomal RNA and protein-RNA complexes to pass through[1,2]. This technique allows the rapid and convenient measurement of the amount of protein bound to ribosomal RNA. We have used this method to study the 16S RNA binding characteristics of six 30S ribosomal proteins. Binding curves obtained by this method are shown in Figures 1 and 2.

The binding characteristics of proteins S4, S8, S20 and S15 (data not shown) are all very similar. Saturation of the RNA for these four proteins is reached at between 0.8 and 1.1 copies of protein bound per RNA molecule. The binding curves for proteins S7 and S17 are somewhat different from the other proteins we have studied. The initial slope of the binding curve for protein S7 is significantly more shallow with a less dramatic plateau, indicating a relatively weaker interaction with the RNA. On the other hand, the binding curve of protein S17 shows a steep initial slope and the binding begins to plateau at levels approaching 1.3 copies per RNA molecule. This suggests that protein S17 has more than one binding site on 16S RNA under these conditions and that the binding sites are relatively strong.

Although relative binding strengths of these proteins to RNA can be qualitively deduced from the binding curves, it is

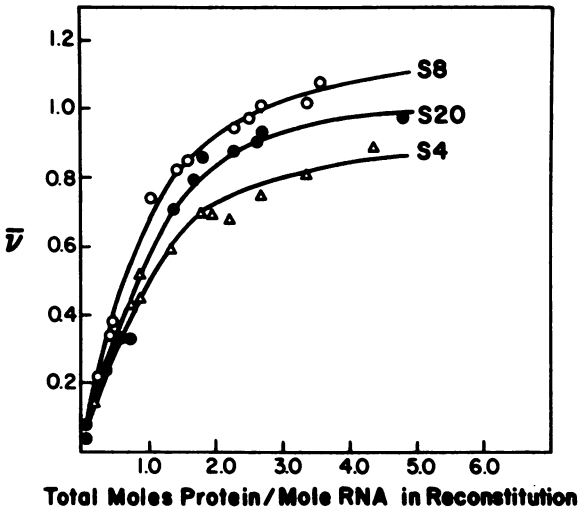

Figure 1. Saturation binding curves for ribosomal protein-16S RNA interactions. Increasing concentrations of ^{3}H-labelled protein were reconstituted with 16S RNA and the complexes were isolated by filtration. $\bar{\nu}$ = moles protein bound/mole RNA.

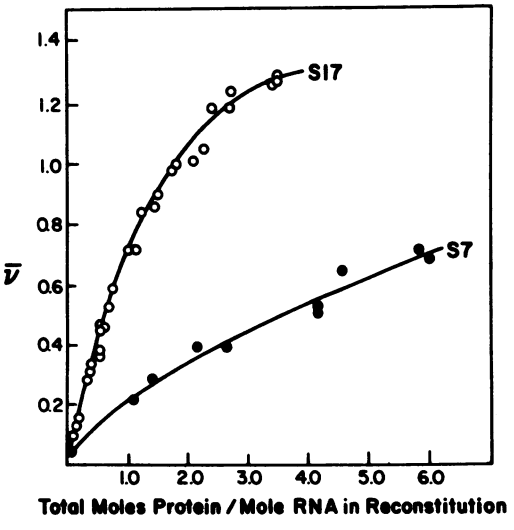

Figure 2. Saturation binding curves for protein S17 and S7.

more meaningful to derive actual apparent binding constants for these interactions. To this end we have analyzed the binding data by applying the double reciprocal and the Scatchard equations[25,26]. Double reciprocal plots for proteins S4, S7, S8 and S20 are presented in Figure 3. Scatchard plots for these four proteins appear in Figure 4. All lines were determined by linear regression analysis of the binding data. The data presented in Figures 3 and 4 reveal the relative binding strengths of the proteins in a more readily recognizable way than simple saturation binding curves. In fact, these analyses permit the calculation of apparent binding constants along with the actual number of binding sites per RNA molecule. These data are summarized in Table 1. They demonstrate that proteins S4, S8, S15 and S20 have very similar, strong binding characteristics. Protein S7, on the other hand, is considerably weaker in its association with 16S RNA.

In addition to proteins S4, S7, S8, S15 and S20, we have also studied the independent binding characteristics of protein S17. Referring back to Figure 2 it can be seen that as protein S17 approaches saturation of its 16S RNA binding site $\bar{\nu}$ exceeds

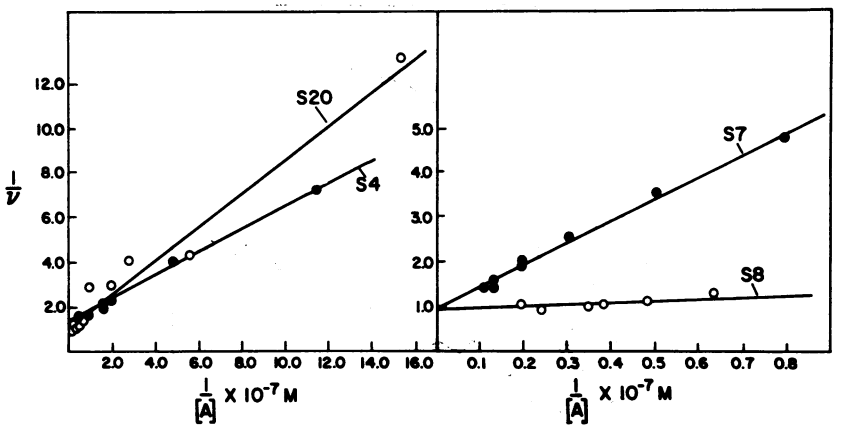

Figure 3. Double reciprocal plots of binding data for proteins S4, S7, S8 and S20. $\bar{\nu}$ = moles protein bound/mole RNA; [A] = molar concentration of unbound protein. $1/\bar{\nu}$ intercept = $1/n$; slope = $1/nK$ where n is number of binding sites and K the apparent association constant.

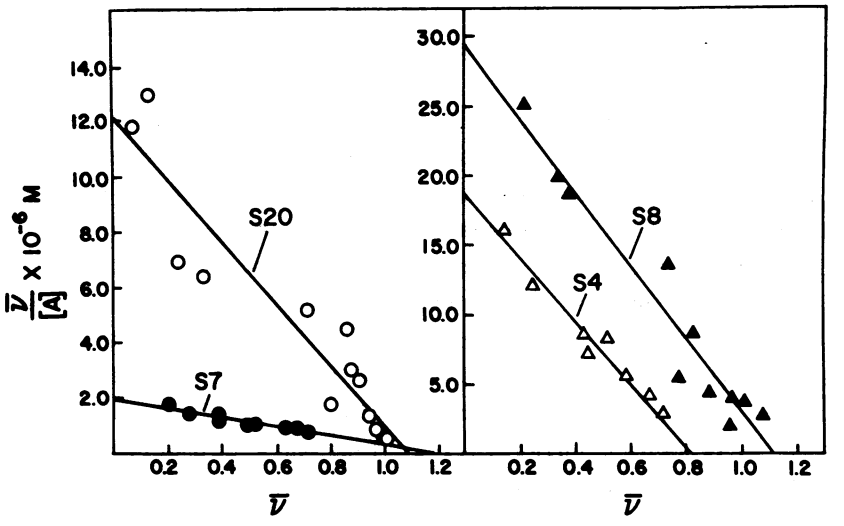

Figure 4. Scatchard plot for the association of proteins S4, S7, S8 and S20. $\bar{\nu}/[A]$ intercept = nK; $\bar{\nu}$ intercept = n; slope = -K.

the value of 1.0. Since protein S17 is present in the ribosome at levels of only one copy per particle[27], these results might imply that at least a component of the binding we observe involves a non-specific interaction. However, using the same conditions for binding, protein S17 fails to associate with 23S RNA even at input ratios of 3 moles of protein per mole RNA.

Table 1: Association Constants and Numbers of Binding Sites for Ribosomal Protein-16S RNA Interactions

Protein	Double Reciprocal		Scatchard	
	$K_{ave}(10^6 M^{-1})$	n_{ave}	$K_{ave}(10^6 M^{-1})$	n_{ave}
S4	18.0 ± 10.0	0.9 ± 0.2	17.0 ± 8.0	0.9 ± 0.1
S7	1.8 ± 0.2	1.1 ± 0.1	1.6 ± 0.1	1.2 ± 0.0
S8	26.8 ± 1.3	1.2 ± 0.1	28.5 ± 3.3	1.2 ± 0.1
S15	9.0 ± 0.7	0.8 ± 0.1	8.1 ± 1.4	0.8 ± 0.1
S20	15.1 ± 4.2	1.1 ± 0.2	13.4 ± 3.5	1.2 ± 0.2
$S17_1$			71	0.6
$S17_2$			4	1.2

Thus, the formation of this complex in the absence of other ribosomal proteins exhibits characteristics of both specific and non-specific binding.

Scatchard analysis of the S17 binding data clarifies the situation considerably. Figure 5A shows the plot of $\bar{\nu}/[A]$ vs. $\bar{\nu}$. The data form a smooth curve with a decreasing slope and binding affinity as $\bar{\nu}$ increases. This type of binding behavior is consistent with the presence of two independent sites on the RNA which are recognized by protein S17. Furthermore, these two sites would have to have substantially different binding affinities for the protein to produce the type of curve seen in Figure 5A. The association constants for both the strong (K_1) and the weak (K_2) binding interactions were extracted from the data in Figure 5A using a graphical method originally described by Scatchard et al.[26]. It was assumed that at low protein to RNA ratios, protein S17 primarily binds to the high affinity sites on 16S RNA. A straight line was determined for the points obtained at low $\bar{\nu}$ values. The binding constant (K_1) and number of binding sites (n_1) were calculated from the slope of this

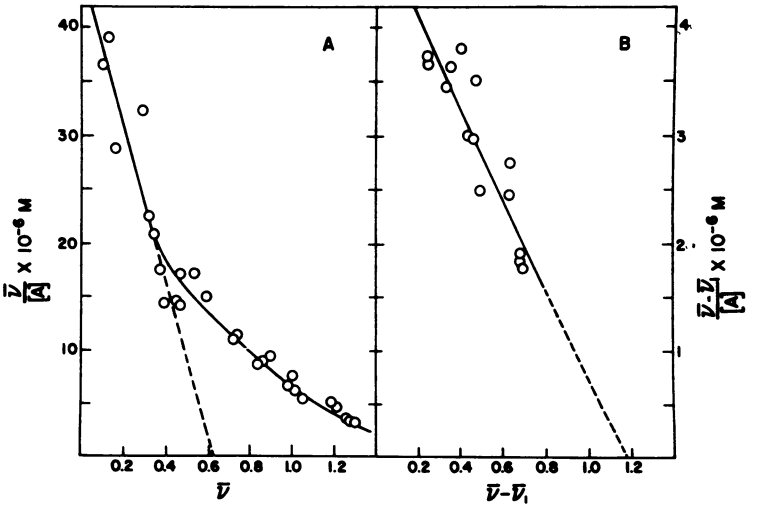

Figure 5A. Scatchard plot for the association of protein S17 with 16S RNA. The dashed line represents the strong binding and was drawn through points of $\bar{\nu}$ less than 0.4.
Figure 5B. Plot of the weak binding of S17 to 16S RNA (see text).

line and the x-interecept, respectively. A plot of $(\bar{\nu}-\bar{\nu}_1)/[A]$ vs. $(\bar{\nu}-\bar{\nu}_1)$ was constructed (Figure 5B) and the slope and x-intercept of that curve were used to compute K_2 and n_2. The results of these calculations are presented in Table 1. One interpretation of these data is that about half of the 16S RNA population consists of molecules which possess a very strong recognition site for protein S17 while the other half of the molecules contain a relatively weak S17 binding site.

DISCUSSION

The nitrocellulose membrane filtration technique we have used to study ribosomal protein-RNA interactions has a number of important advantages over techniques employed by other workers. Our method provides a very sensitive procedure which requires small quantities of protein and RNA, enabling the detection of as little as 30 pmoles of complex. In addition to being very sensitive, the technique is extremely rapid, allowing isolation of the complexes without any demonstrable perturbation of equilibrium[41] and can be used to determine the percentage of protein that is competent for RNA binding[2]. These advantages make it possible to rapidly and conveniently obtain a large amount of quantitative data for a given protein-nucleic acid interaction.

The binding isotherms obtained from the nitrocellulose filtration assay compare favorably with those obtained by sucrose gradient centrifugation, and gel filtration[10,11,28,29]. In these studies strong binding of proteins S4, S8, S15 and S20 to 16S RNA was observed at low total protein to RNA ratios and much weaker binding by protein S7, which only reached a plateau above a molar input of 3 per 16S RNA. However, none of these investigations produced sufficient quantitative data to permit the calculation of association constants.

Our nitrocellulose filtration technique allows the direct determination of association constants. Precise knowledge of the concentration of RNA, the amount of bound protein, the amount of total protein in the system and, by deduction, the amount of unbound protein permits the calculation of an apparent association constant as well as the number of RNA binding sites

for the protein. The values of K we have determined range from a low of 1.6×10^6 M^{-1} for protein S7 up to 7.1×10^7 M^{-1} for the strong binding site of protein S17. This range of values for K compares well with those determined for the binding of individual 50S ribosomal proteins to 5S RNA[30]. In those experiments the amount of radiolabeled 5S RNA retained by nitrocellulose filters in the presence of ribosomal proteins was measured and binding constants of 2.3×10^6 M^{-1}, 1.5×10^7 M^{-1} and 2.3×10^8 M^{-1} for proteins L5, L25 and L18, respectively, were calculated.

Several of the RNA binding sites for the proteins we have studied have been identified. Protein S8, which we find has a relatively high affinity for 16S RNA, binds to two non-contiguous, complementary pieces of RNA totaling 34 nucleotides. This section of RNA is located in the central region of the molecule forming the stem of a hairpin loop[31,32]. This small combining site is extremely stable, remaining intact even after depleting the RNA of Mg^{2+} by dialysis against distilled water[33]. The binding site for protein S15 also is within this region of the 16S RNA, but covers a second hairpin as well to encompass a total of 150 nucleotides[34].

Proteins S4 and S20 show roughly comparable binding affinities for their respective combining sites. Both proteins have been shown to protect rather substantial regions of the 5' domain of 16S RNA from nuclease digestion[35]. The domain protected by protein S4 covers about 400 nucleotides from three non-continuous pieces of 16S RNA. This region has a rather complex secondary structure, probably involving several "long range" interactions[36]. Protein S20, on the other hand, protects a much smaller section of RNA, approximately 100 nucleotides, which lies within the same region of RNA protected by protein S4[31].

With all reconstitution conditions equal, the differences in the binding affinities of ribosomal proteins for RNA are probably a consequence of the size and complexity of the binding domain as well as the conformational integrity or stability of the interacting regions of both protein and RNA.

The weakest protein-RNA interaction we have found is that of protein S7. This confirms earlier work suggesting the in-

stability of this interaction[28,29]. In studies on interdependent assembly relationships, Mizushima and Nomura[9] found that maximum binding of protein S7 was only achieved in the presence of five other ribosomal proteins (S4, S8, S9, S19 and S20). This could be explained in one of two ways. Either the auxiliary proteins increase the number of RNA binding sites in the population or they directly or indirectly increase the stability of the S7-16S RNA complex. Since we find that the 16S RNA population has one binding site for protein S7, we must conclude that the other proteins exert their effect by somehow increasing the binding affinity of protein S7, either via direct protein-protein contacts or indirectly induced, new RNA-protein interactions.

The interaction of protein S17 with 16S RNA was originally thought to be non-specific[10,11], based on the observations that it apparently bound 23S RNA as well as 16S RNA and that the saturation binding curve failed to reach a plateau. Later work, however, contradicted these results showing that this protein binds only 16S RNA and that a plateau is actually achieved[29,37]. Our results corroborate the conclusion that S17 binds 16S RNA with specificity. However, we find that the binding is not a simple direct formation of a one to one complex. In our experiments, protein S17 exhibited two levels of interaction. At low molar input ratios of S17 to RNA, the binding is very strong as reflected by the steepness of the saturation curve. However, it appears that the strong binding site becomes fully occupied early, and that a weaker binding process becomes dominant. This binding behavior is most clearly seen in the Scatchard analysis of the data. From the Scatchard plot we extracted the weak and strong binding constants as well as the respective numbers of binding sites. By this analysis the total number of protein S17 binding sites per RNA molecule was determined to be 1.8. Extrapolation of the curve representing the strong binding to the abscissa yields a value of 0.6 binding sites. This suggests that roughly 60% of the RNA molecules prepared by our procedures contain a strong binding site and that the remaining RNA molecules are capable of only a weaker binding. Extracting the strong binding values produces a Scatchard plot for the

weak interaction, from which we calculate that approximately 1.2 weak S17 binding sites exist within the RNA population. There are several possible explanations of these observations. For example, one could imagine that the RNA population consists of molecules each of which contains a single independent weak binding site. With this model one must then postulate that roughly half of the RNA population contains a second, relatively strong S17 binding site.

An alternative model to explain the S17 binding behavior can be suggested. It is possible the S17 binding site of our 16S RNA preparation consists of two domains brought together via "long range" RNA-RNA interactions. Roughly half of the RNA population might contain the complete S17 binding site with an intact long range interaction, and the other half could have incompletely renatured to produce molecules containing two weak binding domains devoid of the long range interactions.

Although there are a number of other possible hypotheses which could explain our results with protein S17, several published investigations tend to support the second proposal involving long range RNA-RNA interactions. Protein S17 is thought to interact with 16S RNA within the S4 binding domain (i.e. the 5' region)[35]. This region is structurally complex and has been shown to contain domains of long range interaction[38]. Furthermore, Woese *et al.*[39] have recently proposed a secondary structure for 16S RNA, based on chemical and nuclease susceptibility studies as well as comparative sequence analysis. Their proposed structure contains several striking points of long range interaction. One of these involves the 5' end of the RNA and a region downstream around the first one-third section. It seems likely that in fact long-range interactions do exist within the S17 binding domain. In addition, it has been observed that the 5' region of 16S RNA has a definite tendency to incompletely renature[40]. Although these observations do not prove the hypothesis, they certainly are consistent. Clearly much more needs to be done to fully explain the binding characteristics of protein S17.

In summary, we have utilized a rapid, quantitative technique for the isolation of protein-RNA complexes and have used this technique for the analysis of the interactions of ribosomal proteins with 16S RNA. Using double reciprocal and Scatchard analyses, we found that proteins S4, S7, S8, S15, S17 and S20 bind specifically and independently to 16S RNA. Association constants for these protein-RNA interactions cluster around 10^7 M^{-1}, except for protein S7 which binds more weakly at about 10^6 M^{-1}. We anticipate that this precise knowledge of protein-RNA binding parameters will allow us to develop a better understanding of the thermodynamics and the chemistry of protein-RNA recognition in the ribosome.

ACKNOWLEDGEMENTS

We would like to acknowledge Donald Winkelmann for immunochemical identification of protein S17. This work was supported by the College of Agricultural and Life Sciences as well as the Graduate School, University of Wisconson, Madison. Primary support was derived from research grant GM-24019 from the National Institutes of Health.

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