Evidence that *E. coli* ribosomal protein S13 has two separable functional domains involved in 16S RNA recognition and protein S19 binding

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ABSTRACT

We have found that *E. coli* ribosomal protein S13 recognizes multiple sites on 16S RNA. However, when protein S19 is included with a mixture of proteins S4, S7, S8, S16/S17 and S20, the S13 binds to the complex with measurably greater strength and with a stoichiometry of 1.5 copies per particle. This suggests that the protein may have two functional domains. We have tested this idea by cleaving the protein into two polypeptides. It was found that one of the fragments, composed of amino acid residues 84-117, retained the capacity to bind 16S RNA at multiple sites. Protein S19 had no effect on the strength or stoichiometry of the binding of this fragment. These data suggest that S13 has a C-terminal domain primarily responsible for RNA recognition and possibly that the N-terminal region is important for association with protein S19.

INTRODUCTION

It is becoming generally accepted that the ribosome owes its complex architecture to the multifunctional nature of its constituents. The RNA molecules appear to be constructed such that they offer specific interactions with themselves, with other ribosomal RNAs, with the ribosomal proteins and with the various factors and components of the protein synthetic machinery. Similarly, the ribosomal proteins in general have been shown to maintain specific sites for the recognition of ribosomal RNA, other ribosomal proteins, and the many macromolecules involved in the translation process. Proteins which have multifunctional properties can often be fragmented into structural domains which maintain the specific functions that reside within those individual domains. In our early studies of ribosomal protein S4, we found that this protein could be subdivided by a variety of enzymatic and chemical cleavages to yield a set of fragments which either maintained or

lost one or more of the functions attributable to the intact protein (1, 2, and 3). Other workers have used a similar approach to identify functional domains in proteins S8 (4) protein L18 (5) and protein S1 (6). Encouraged by our results as well as those by other investigators, we have extended this approach to other ribosomal proteins. Our results with protein S13 are presented in this report.

We chose to search for functional domains in protein S13 for a variety of reasons. First, S13 has been shown to weakly bind 16S RNA and to be dependent on proteins S4, S8 and S20 for assembly (7). Second, using a variety of bifunctional reagents on the 30S particle, it has been shown to form a relatively high yield crosslink with protein S19 as well as low yield crosslinks with 8 other 30S ribosomal proteins (8). Evidence has also been presented that a dimer can be formed with S19 that binds 16S RNA at a specific site (9). Third, omission of protein S13 from a reconstituted 30S particle results in about a 25% loss of ribosome activity in a variety of assay systems (10). Protein S13 also has been implicated in the binding of messenger RNA by both affinity label investigations (11) and chemical inactivation studies (12, 13). Furthermore, protein S13 has been identified as one of a group of proteins that crosslink to all three initiation factors (14). Finally, protein S13 has recently been found to crosslink 4 proteins in the 50S ribosome when the 70S complex is treated with 2-iminothiolane (15), suggesting that it may be involved in 30S-50S subunit interaction. Thus, protein S13 would seem to be a good candidate as a multifunctional protein. It most likely has a specific 16S RNA binding site as well as a recognition site for protein S19 and perhaps other proteins.

Protein S13 is composed of 117 amino acid residues with a single cysteine at position 84 (16). Cysteine residues can be readily cleaved using the reagent 2-nitro-5-thiocyanobenzoate (NTCB) which cyanylates cysteine sulfhydryl groups selectively and subsequently cleaves the peptide bond under relatively mild conditions (17). We have used NTCB to cleave protein S13 and have purified the resultant fragments comprising residues 1-83 and residues 84-117. Of the two fragments, we find that only

protein S13-fragment (84-117) retains 16S RNA binding activity. Additionally, this fragment can be incorporated into 30S subunits in place of the intact protein S13. However, the 30S particles containing the fragment are as defective in poly U dependent polyphenylalanine synthesis as 30S particles completely lacking protein S13. We also have found some indirect evidence that the N-terminal region of protein S13 is important for the interaction with S19. On the basis of these results we suggest that protein S13 is a multifunctional protein.

MATERIALS AND METHODS

Ribosomes and their subunits were isolated from *E. coli* MRE600 as previously described (18). Ribosomal proteins were extracted from the purified 30S subunits both by LiCl-urea (19) and by acetic acid (20), fractionated by phosphocellulose (20) or by carboxymethylcellulose column chromatography (2).

Purified protein S13 was tritium labeled by the *in vitro* reductive methylation of lysine residues using sodium [³H] borohydride (21). For the purposes of determining protein specific activities, protein concentrations were determined by the Lowry method (22) using hen lysozyme as the standard. Phenol extraction of 70S ribosomes, followed by separation of 16S RNA and 23S RNA by preparative sucrose gradient centrifugation, was used to prepare 16S RNA (19).

Treatment of protein S13 with 2-nitro-5-thiocyanobenzoic acid (NTCB) (Eastman) was carried out according to the procedure of Jacobson *et al.* (23). A 5-fold excess of NTCB over total thiol groups was added from a 0.1 M stock solution containing 0.2 M Tris acetate, pH 8.0. The cyanylation reaction was allowed to proceed for 15 min at 37 °C and then terminated by acidification to pH 4.0 with glacial acetic acid. The mixture was cooled to 4 °C, dialyzed into 10% acetic acid and lyophilized.

Cleavage of the cyanylated protein S13 was carried out by redissolving the lyophilized protein in 6M guanidine-HCl, 0.1 M sodium borate (pH 9.0) and incubating the solution at 37 °C for 16 hr. Extent of cleavage, as judged by relative staining of the fragments observed on one dimensional polyacrylamide gel

electrophoresis, was approximately 50%. The reaction products were dialyzed into 10% acetic acid and lyophilized.

Protein S13 and the two fragments produced by NTCB cleavage were separated by gel filtration on a Sephadex G-50 superfine column (1.5 cm X 120 cm) equilibrated with 0.25 M ammonium formate, pH 2.5. The column was run at room temperature with a flow rate of 12 ml/hr. Purity of the fractions was determined by gel electrophoresis. Specific activities were calculated from the known specific activity of the S13 and the known number of lysine residues in each fragment. Final specific activities ranged from 0.5 to 7.3×10^8 cpm/ μ mole.

Protein-RNA complexes, as well as reconstituted 30S subunits, were prepared using the reconstitution system of Traub et al. (19). A solution of RNA was preincubated at 40° for 10 min in a buffer containing 0.01 M Tris-HCl (pH 7.6) 0.02 M MgCl₂ and 1 mM DTT (buffer RB-A). Protein preparations were dissolved in a buffer consisting of the same constituents as RB-A with the addition of 1.0 M KCl (buffer RB-B), and aliquots were added to the RNA solution along with a sufficient volume of RB-A to give a final KCl concentration of 0.33 M. The reaction mixtures were incubated for 1 hr at 40° C and then chilled on ice prior to isolation. Concentrations of labeled protein were varied while RNA and unlabeled protein concentrations were held constant.

Binding of the radioactive proteins to protein-RNA complexes was analyzed by the centrifugation of the particles through a sucrose gradient using either a Beckman SW 56 rotor for 2.5hr at 54,000 rpm or a Beckman VTi 65 vertical rotor for 45 min at 50,000 rpm. The sucrose gradients used were either a linear 5-17.5% gradient in the SW 56 rotor or a 10-25% linear gradient in the VTi 65 rotor. The sucrose solutions were prepared with 0.01 M Tris-HCl (pH 7.6), 0.01 M MgCl₂, 0.03 M NH₄Cl and 1 mM DTT (buffer TMA). This lower ionic strength buffer was used to help stabilize weak protein-RNA interactions. It was found that if RB-A buffer was used in the sucrose gradient, considerable "stripping" of protein S13 and its fragments took place. In control experiments using 23S RNA we found no evidence that the use of TMA promotes non-specific protein-RNA interactions. Gradients were fractionated from the

bottom of the tube into equal fractions of approximately 200 μ l each. The fractions were measured for absorbance at 260nm and analyzed for radioactivity by scintillation counting.

For the analysis of protein composition by gel electrophoresis, larger quantities of the protein-RNA complexes were prepared. For this purpose usually 7.0 A₂₆₀ units of RNA were reconstituted with a 1.2 fold molar excess of protein.

RESULTS

The Binding of Protein S13 to 16S RNA is Multisited

Mizushima and Nomura (7) were the first to observe independent binding of protein S13 to 16S RNA. The specificity of this binding was later disputed by other workers on the basis of a binding isotherm which failed to reach a discernible plateau (24). Subsequently, the specificity of protein S13 binding was demonstrated by showing that it binds selectively to the 5' two thirds of the 16S RNA molecule (25). In view of these conflicting data, we felt it to be necessary to re-investigate the characteristics of the S13-16S RNA interaction. Figure 1A shows the typical binding isotherm we obtained for the reaction of protein S13 with 16S RNA. This binding curve is similar to that determined by Muto *et al* (25). No binding plateau is reached even at high molar ratios of protein to RNA, suggesting binding to more than one site. Analysis of these data by a double-reciprocal plot substantiates this conclusion (figure 1B). Unfortunately the data are not sufficiently precise to permit a meaningful estimation of the number of binding sites. We can only say that there must be more than 5. These puzzling data cannot be explained by a concentration dependent aggregation of the protein. If protein S13 tended to self-associate as well as weakly bind 16S RNA, the binding isotherm would have a pronounced sigmodal character reflecting the cooperativity of the system. Since this is not the case, we are forced to conclude that the 16S RNA molecule contains multiple sites for binding S13.

Also included in figure 1A are the data obtained from similar binding experiments using 23S RNA. It is clear that under the same conditions as those used for the 16S RNA binding

Fig. 1A. Binding isotherms for the interaction of protein S13 with 16S RNA (o) or with 23S RNA (●). Increasing amounts of tritium labeled protein were incubated with a constant concentration of RNA. The protein-RNA complexes were separated from unbound protein by sucrose gradient centrifugation. Figure 1B. is a double reciprocal plot of the data in figure 1A. $\bar{v}$ = moles protein S13 bound per mole RNA; $[A]$ = molar concentration of unbound protein.

experiments, no significant binding of protein S13 takes place with 23S RNA. Thus the multisite binding we observe to 16S RNA is unique for that RNA and must in some sense be specific.

The Stoichiometry of Protein S13 Binding to 16S RNA-protein Complexes is Altered by Protein S19

The amount of protein S13 incorporated into the 30S subunit has been shown to be enhanced by proteins S4, S7, S8, and S20 (7, 24). We investigated the binding of S13 to a 16S RNA-protein complex containing these proteins as well as the additional proteins S15, S16 and S17 (hereafter referred to as the S4-20 mix). Reconstitution of increasing amounts of radioactive S13 with a constant concentration of non-radioactive S4-20 mix and 16S RNA gave a binding isotherm (see figure 2A) nearly coincident with the S13-16S RNA binding experiments (figure 1A). The points generally lie on a straight line with no plateau and with more than two copies of S13 incorporated per RNA molecule at the highest molar input ratio analyzed. The effect of ribosomal protein S19 was also tested. With the addition of protein S19 to this reaction mixture, a very different binding curve is observed. The data obtained from this series of experiments are also plotted in figure 2A. As

Fig. 2A The effect of protein S19 on the binding of protein S13 to a 16S RNA-protein complex. The binding of tritium labeled protein S13 to a complex of 16S RNA and proteins S4, S7, S8, S15, S16, S17 and S20 was done as described in figure 1A in the presence (●) and absence (○) of protein S19.

Figure 2B is a double reciprocal plot of the data in figure 2A. (●) are data obtained in the absence of protein S19 and (○) represent data obtained in the presence of protein S19.

can be seen the slope of the isotherm is initially considerably steeper, suggesting that the interaction of S13 is much stronger in the presence of S19. Furthermore, the binding apparently begins to reach saturation in the presence of a 4-fold excess of protein S13 over the 16S RNA-protein complex. These data were subjected to a double reciprocal plot analysis as seen in figure 2B. Multisite binding of protein S13 to the 16S RNA-(S4-20 mix) particle is suggested by the intercept approaching zero on the ordinate. With the inclusion of S19 in the reaction mixture, the binding stoichiometry of S13 is dramatically reduced to approximately 1.5 sites per RNA-protein sub-particle. The association constant for the S13 binding in the presence of S19 estimated from these data is $3.0 \times 10^6 \text{ M}^{-1}$. Association constants for S13 binding either to 16S RNA alone or in complex with the S4-20 mix can not be estimated due to the uncertainty in the number of binding sites. However, if one assumes an arbitrary 10 binding sites per RNA molecule, the estimated association constant would be approximately 100-fold lower in the presence of protein S19 than in its absence.

We did attempt to test the effect of protein S19 directly on S13 binding. Unfortunately, with our protein preparations,

Fig. 3 Separation of protein S13 cleavage products produced by the reaction with NTCE. Separation was achieved by gel filtration using Sephadex G-50 superfine as described in the Materials and Methods. The insert shows gel electrophoreograms of pooled fractions. Slot 0 is the NTCE reaction mixture applied to the column. Slots 1, 2 and 3 are column fractions 1, 2, and 3 respectively. Fraction 1 was identified as uncleaved S13, fraction 2 as S13 fragment (1-83) and fraction 3 as S13 fragment (84-117) (see text). Note that fragment (84-117) migrates with the electrophoretic front using our gel system.

we failed to observe any reproducible effect in the absence of the S4-20 protein mix. We conclude from these experiments that protein S13 is probably a multifunctional protein, having a site which recognizes 16S RNA and perhaps a separate site which responds to protein S19.

Cleavage, Purification and Identification of S13 Fragments

Having found evidence that protein S13 is composed of several functional domains, we attempted to add weight to this conclusion by the isolation of fragments of the protein comprising only one of the measurable activities. The approach which has yielded the most information was to cleave the protein at the position 84 cysteine residue using the reagent NTCE (17). Tritium labeled S13 of known specific activity was cleaved at

Table 1. Amino Acid Compositions of Protein S13 Fragments

Amino Acid	Protein S13		Fraction A		Fraction B	
	Expt.	Seq.	Expt.	Seq.	Expt.	Seq.
Aspartic	8.0	8	6.7	7	1.0	1
Threonine	6.8	6	4.1	3	3.0	3
Serine	5.7	6	5.4	6	0.4	0
Glutamic	8.8	8	7.1	7	1.4	1
Proline	4.6	4	1.6	1	2.7	3
Glycine	11.8	11	7.7	7	4.0	4
Alanine	10.7	11	9.2	10	1.4	1
Valine	7.8	7	5.7	6	1.3	1
Cysteine	0.1	1	0.0	0	0.0	0
Methionine	1.4	2	1.0	2	0.1	0
Isoleucine	8.8	12	9.0	11	1.2	1
Leucine	8.8	9	7.1	7	2.2	2
Tyrosine	2.3	2	1.3	1	1.0	1
Phenylalanine	1.6	1	1.3	1	0.1	0
Lysine	10.5	11	5.9	6	4.2	5
Histidine	3.9	3	2.6	2	1.4	1
Arginine	15.4	15	7.3	6	8.4	9
Total	117.0	117	83.0	83	33.8	33

Analyses were performed on pooled fractions from the gel filtration separation illustrated in figure 3. All determinations were done in duplicate and the data presented here represents the average values. Residue compositions were obtained from the sequence (16).

this cysteine and the products were purified by gel filtration (figure 3). Fractions were analyzed for purity by one dimensional polyacrylamide gel electrophoresis, pooled and concentrated. Identification of the fractions could be deduced from the relative molecular weights estimated from the gel filtration column profile. The molecular weight of fraction 2 was estimated to be 9300 and fraction 3 approximately 3500 daltons. This compares with the molecular weights calculated from the known sequence of 8930 and 4038 for the fragment (1-83) and the fragment (84-117) respectively. The chemical identity of the fractions was also determined by amino acid analysis as summarized in Table 1. The analysis of fraction 2 agrees extremely well with that predicted from the sequence for the N-terminal section of residues 1-83. Similarly the analysis of fraction 3 identifies it as the C-terminal fragment (84-117).

It should be noted that the fragment (1-83) consistently

Fig. 4 Binding isotherms for the interaction of protein S13 fragment (84-117) with 16S RNA (○) and with 23S RNA (●). The experiments were carried out as outlined in figure 1A.

ran as one major band and one relatively minor band (figure 3). We are confident, however, that both bands represent different forms of fragment 1-83, as this fraction runs as a single species in SDS gel electrophoresis (data not shown) and the amino acid composition agrees well with the predicted analysis.

Protein S13 Fragment (84-117) Retains 16S RNA Binding Function Whereas Fragment (1-83) Does Not

The purified tritium labeled fragments were tested for their capacity to bind 16S RNA and 23S RNA. The S13 fragment (1-83) was found to have absolutely no residual RNA binding activity under our conditions. The S13 fragment (84-117), on the other hand, did form a complex with 16S RNA, but not with 23S RNA. When a complete series of binding experiments was performed, it was found that the 16S RNA binding isotherm was nearly identical to that determined for the intact protein S13 (see figure 4). In addition the S13 fragment (84-117) failed to associate with 23S RNA even up to a ratio of 5 moles of polypeptide per mole of RNA. Thus the C-terminal domain of protein S13 retains the multisite 16S RNA binding capacity of the parent protein.

Fig. 5 Sucrose gradient analytical ultracentrifugation profiles of protein-RNA complexes digested with T1 RNase. Reconstitution mixtures consisting of 16S RNA and either protein S13 or protein S13 fragment (84-117) were treated with T1 RNase as described in Materials and Methods. The digested mixtures were layered on 10-20% linear sucrose gradients containing TMA buffer and centrifuged in a Beckman VTi65 rotor for 85 min at 50,000 rpm. Gradients were fractionated and the fractions were assayed for radioactivity (●) and absorbance at 260 nm (○). Regions of the profile corresponding to 8S, 12S, and 16S sedimenting species are indicated. Profile A was obtained for the S13-RNA complex and profile B for the S13 fragment (84-117)-RNA complex.

Protein S13 and S13 Fragment (84-117) Bind the 5' Two-thirds of 16S RNA

Perhaps the best evidence that the binding of protein S13 to 16S RNA is specific comes from the demonstration by Muto *et al.* (25) that it associates selectively with a discrete fragment of the RNA. These workers found that careful digestion of 16S RNA with ribonuclease T1 yields two sub-fragments which sediment as a 12S species and an 8S species. The 12S fragment and the 8S fragment were shown to encompass the 5' section and the 3' section respectively of 16S RNA. Incubation of radioactive S13 with the RNase T1 digestion product, revealed that the protein bound only the 12S fragment. We attempted to repeat this work

Fig. 6 Binding isotherms for the interaction of protein S13 fragment (84-117) with protein-RNA complexes. Reconstitution systems containing 16S RNA and the S4-20 protein mixture (see legend to figure 2A), were analyzed for binding tritium labeled fragment in the presence (●) and absence (○) of protein S19.

and to determine whether S13 fragment (84-117) retains the capacity to bind the 12S fragment of 16S RNA. Figure 5 shows the results. Although our digestion conditions failed to produce a well defined 8S RNA peak, a readily discernible 12S RNA species was observed that bound both S13 and S13 fragment (84-117). We suggest that the S13 fragment (84-117) retains the capacity to associate with the same section of 16S RNA that the intact S13 molecule binds.

Protein S19 Has No Effect on the Binding of Protein S13 Fragment (84-117) to 16S RNA-protein Complexes

As shown above, the presence of proteins S4, S7, S8, S15, S16, S17 and S20 in the reconstitution mixture had no appreciable influence on the binding of protein S13. However, inclusion of protein S19 in the reaction system greatly increased the strength of S13 binding to the protein-RNA complex and changed the number of binding sites from many to something approaching stoichiometric. We tested the effect of S19 on the binding of S13 fragment (84-117) to a 16S RNA complex containing the S4-20 mix. The results are presented in figure 6. The data

Table 2. Incorporation of protein S13, protein S13 fragment (1-83) and protein S13 fragment (84-117) into 30S ribosomal subunits.

Protein added	Moles Protein Incorporated per Mole 16S RNA	
	TP ₃₀ minus S13	TP ₃₀
S13	0.75	0.48
S13 frag. (1-83)	0.09	n.d.
S13 frag. (84-117)	0.50	0.35
S13 frag. (1-83) + frag. (84-117)	0.20	n.d.

Tritium labeled protein was incubated with 16S RNA and either TP₃₀ or a protein mixture of TP₃₀ minus S13. Reconstituted particles were isolated by sucrose gradient centrifugation. Gradient fractions were assayed for absorbance at 260 nm and for radioactivity. The total moles of protein per mole of RNA in the reaction mixture was 1.5 for protein S13, 3.3 for S13 fragment (1-83), 3.5 for S13 fragment (84-117) and 2.8 for the mixture of the two fragments. n.d. = not determined

obtained for S13 binding to the RNA-protein complex with and without protein S19 are plotted together to demonstrate that there was no detectable change in the S13 fragment (84-117) binding characteristics due to the presence of S19.

It could be argued that the loss of response of protein S13 fragment (84-117) to the presence of protein S19 or the loss of binding activity for S13 fragment (1-83) is due to inactivation by the NTCB treatment and cleavage conditions. We tested this possibility by examining the binding of the residual, uncleaved protein S13 recovered from the gel filtration purification as seen in figure 3. This preparation of protein S13 essentially received the same series of conditions experienced by the two fragment preparations. It was found that S13 treated in this way behaved in a nearly identical manner to untreated S13 in binding 16S RNA and in binding the 16S RNA-S4-20 complex, in the presence and absence of protein S19 (data not shown). We conclude that the loss of the N-terminal domain from the protein results in an concomitant loss of capacity to respond to the presence of protein S19, without affecting the inherent 16S RNA binding function of the protein.

Table 3. Polyuridylic acid dependent polyphenylalanine synthesis promoted by 30S ribosomes containing protein S13 fragments.

Particle Protein Composition	Percent Activity
(TP30 - S13) + S13	100
(TP30 - S13)	74
(TP30 - S13) + S13 Fragment (1-83)	70
(TP30 - S13) + S13 Fragment (84-117)	73
(TP30 - S13) + S13 Fragments (1-83) & (84-117)	76

Partiacles were prepared and isolated as described in Materials and Methods. After overnight centrifugation, pelleted subunits were resuspended in buffer TMA and assayed for activity in a poly U translation system. Particles were also analyzed by gel electrophoresis for protein composition.

Protein S13 and S13 Fragment (84-117) are Incorporated into 30S Subunits, but S13 Fragment (1-83) is Not

Although protein S13 fragment (1-83) failed to bind 16S RNA, it is conceivable that it could still become incorporated into a reconstituted 30S particle via interactions with constituent proteins. We examined this possibility by constructing a complete equimolar mixture of 30S proteins minus protein S13 (TP30-S13) and using this mixture, with the addition of radioactive S13 or its fragments, for re-assembly of the 30S ribosome. The results of these experiments are summarized in Table 2. Both protein S13 and S13 fragment (84-117) were individually incorporated into the reconstituted subunits, but S13 fragment (1-83) was not. To investigate the specificity of this incorporation, the experiments were also done with a complete mixture of 30S proteins (TP30) which contain non-radioactive S13. As seen in Table 2, the non-radioactive S13 effectively competed with both the radioactive S13 and S13 fragment (84-117). An equimolar mixture of the two fragments was also tested for incorporation in the presence of the TP30-S13 mix. It was found that the amount of radioactivity incorporated was less than one half that expected for the sum of the two fragments. This implies that the S13 fragment (1-83) and the S13

Fig. 7 One dimensional polyacrylamide gel electrophoresis analysis of reconstituted 30S ribosomes. Reconstitutions were performed with 16S RNA, a mixture of purified proteins consisting of TP30-S13, and either protein S13, (slot 1), protein S13 fragment (84-117) (slot 2) or no protein S13 or its fragment (slot 3). The levels of incorporation of protein S13 and the protein S13 fragment were estimated, from the amount of bound tritium label, to be 0.8 and 1.1 copies per RNA molecule respectively. Slot 4 contained RNase digested 30S ribosomes (0.5 A260 units) and slot 5 the NTCB cleavage products of protein S13 for reference.

fragment (84-117) do not interact in a manner that promotes incorporation of both polypeptides. We conclude that S13 fragment (1-83) has lost the capacity to form stable complexes with all components of the 30S ribosome.

Protein S13 Fragment (84-117) Cannot Replace Intact Protein S13 in 30S Ribosome Dependent Protein Synthesis

In 1969 Nomura *et al.* (10), studied the protein synthetic activities of ribosomes reconstituted in the absence of single

proteins. These workers found that omission of protein S13 yielded particles which displayed a sedimentation velocity of 30S. When these S13 deficient particles were assayed for polyuridylic acid directed polyphenylalanine synthesis, they were found to have an activity reduced by approximately 25%. We have repeated those experiments and find essentially the same results. In addition we have prepared particles containing the fragments of S13 to determine specifically whether the C-terminal domain (84-117) can restore the activity lost by the absence of the whole protein S13. Table 3 summarizes the data obtained from these experiments. Clearly, the addition of either fragment (1-83), fragment (84-117) or a mixture of the two to the reconstitution system failed to restore the protein synthesis activity. Thus, none of the fragments of S13 can substitute for the intact protein in whatever role it plays in ribosome function.

It could be argued that the approximately 25% reduction of protein synthetic activity of ribosomes deficient in S13 or carrying the S13 fragment (84-117) is not due to a direct effect of S13 but rather to the loss of a second protein dependent on S13 for correct assembly. We have examined, therefore, the protein composition of particles reconstituted in the absence of protein S13 and in the presence of S13 fragment (84-117). Figure 7 is a photograph of a one-dimensional polyacrylamide gel electrophoresis analysis of the proteins extracted from such particles. By comparison to the protein composition of 30S ribosomes, appropriate amounts of all ribosomal proteins, except S1 and S2, were incorporated. Thus, the assembly of all other ribosomal proteins is not perturbed by the absence of protein S13 or by the presence of S13 fragment (84-117). This result agrees well with the work of Mizushima and Nomura (7).

DISCUSSION

It was our objective in this study to investigate the multifunctional nature of protein S13 and to search for stable structural domains which retain one or more of the functions of the protein. We decided to concentrate our efforts on two activities implicated for protein S13, namely binding to

16S RNA and interaction with protein S19. To this end we characterized the binding of the protein to ribosomal RNAs and to various protein-16S RNA complexes. The binding data we obtained indicate that the protein weakly interacts with a number of separate sites on the 16S RNA. Since this multi-site binding behavior was observed only in the case of 16S RNA and not 23S RNA, we are forced to conclude that these multi-recognition sites are specific. It is important to note that this type of binding is unprecedented in the studies of ribosomal protein-RNA interactions. The significance of these observations is not understood, but we believe it is unlikely that this weak, multisited binding operates during the actual process of 30S ribosome assembly. In any event we found it possible to use this 16S RNA binding activity as an assay for a functional domain.

The original experiments by Mizushima and Nomura (7) demonstrated that the binding of protein S13 to 16S RNA is augmented by the presence of proteins S4, S8 and S20. We therefore examined the binding characteristics of S13 to a 16S RNA complex containing these proteins as well as proteins S15, S16, S17 and S20. Under our conditions for measuring the degree of S13 binding, we found absolutely no detectable change in the binding isotherm when the set of 7 additional proteins was included in the reaction mixture. Thus, these proteins do not prevent binding of multiple copies of S13 nor do they strengthen the interaction of S13 with the complex. However, the inclusion of protein S19 into the reaction system greatly strengthened the binding of S13 and the number of copies bound per RNA molecule was dramatically reduced, approaching a 1:1 stoichiometry. It should be noted that this facilitating effect of S19 on the binding of S13 could only be demonstrated in the presence of the set of 7 proteins designated S4-20. Nonetheless, these results gave us an assay for a second S13 functional domain, namely a response to the presence of protein S19 in the incorporation into a protein-RNA complex.

In searching for structural domains which might retain one or both of protein S13's activities, we prepared two fragments of the protein encompassing residues 1-83 and 84-117. We found

that S13 fragment (1-83) was completely devoid of any residual capacity to form stable complexes with either 16S RNA, 16S RNA-protein complexes or a 30S ribosome reconstituted in the absence of intact S13. We conclude that if this section of the protein contains a functional domain, and we believe that it does, the polypeptide must have become unfolded sufficiently to prevent expression of its activity.

The protein S13 fragment (84-117), on the other hand, was found to have retained essentially all of the 16S RNA binding properties of the parent protein. It weakly bound multiple copies to the RNA as well as to a 16S RNA-protein complex containing proteins S4, S7, S8, S15, S16, S17 and S20. In contrast to the behavior of intact S13, the S13 fragment (84-117) failed to respond to the presence of protein S19 when tested for binding to the 16S RNA-protein S4-20 complex. This suggests that the N-terminal two-thirds of protein S13 is required for the cooperative association with S19, either by direct interaction with S19 or an S19 induced interaction with other components of the complex. In view of the observation that S19 and S13 can be isolated as a 1:1 dimer which is capable of binding 16S RNA (9), we prefer the hypothesis that the N-terminal domain of S13 is responsible for recognition and binding protein S19 whereas the C-terminal domain comprises the RNA binding function.

Not only is the N-terminal domain of protein S13 necessary for the cooperative assembly relationship with protein S19, it also is required for the full expression of 30S ribosomal activity. This is evidenced by our observation that S13 deficient ribosomes are reduced in poly U translation activity by approximately 25% and the S13 fragment (84-117) fails to restore the activity to that seen for ribosomes containing intact S13.

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