Volume 4 Number 10 October 1977 **Nucleic Acids Research**

Ribosomal protein - nucleic acid interactions. I. Isolation of a polypeptide fragment from 30S protein S8 which binds to 16S rRNA

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Received 19 July 1977

ABSTRACT

Within the bacterial ribosome a large number of specific protein and rRNA interactions appear to be required for assembly of the particle and its subsequent function in protein synthesis. In this communication it is shown that it is possible to isolate cyanogen bromide digestion products from ribosomal 30S protein S8 which will interact stoichiometrically with 16S rRNA. In addition to this a small binding polypeptide was generated from S8-16S rRNA complexes which were treated with proteinase K. The digestion of the complex yields a "protected" fragment of protein S8 which binds to 16S-rRNA. The isolated fragment will reassociate with 16S rRNA. It is not displaced by other 30S ribosomal proteins and blocks the binding of intact S8 to 16S rRNA. The size and possible structure of the S8 protein binding site are discussed and compared with the binding of cyanogen bromide digestion products which bind to 16S rRNA.

INTRODUCTION

The 30S subunit of the bacterial ribosome from E. coli is composed of 21 unique proteins and a single ribosomal RNA (16S rRNA) (Hardy, et al. 1969). It is possible to separate these proteins from the rRNA and effect an in vitro reassembly of a functional particle (Traub and Nomura, 1968). The initial stages of the assembly process appear to involve extensive protein-nucleic acid interactions followed by or in coordination with protein-protein interactions (Mizushima and Nomura 1970; Schaup, et al. 1970). The specificity of the protein-nucleic acid interactions has been extensively documented and the portions of the 16S rRNA to which four ribosomal proteins bind have been characterized at the primary structural level and located within the 16S rRNA near the 5' terminus of the molecule (Schaup, et al. 1971; Schaup, et al. 1973; Ungewickell, et al. 1975). Examination of the rRNA binding sites for these proteins

has not revealed common features which can be used to formulate general principles for understanding these interactions or the molecular mechanism which generates the high level of binding specificity observed.

Reconstitution experiments in which proteins from one organism and the rRNA from another have been successful (Nomura, et al. 1968). However a comparison of rRNAs from the different organisms reveals that there can be significant variation in the primary structure of the rRNA binding regions for specific proteins (Woese, et al. 1975). This implies that the nucleic acid recognition point for the protein must be determined at the secondary and/or tertiary structural level. One approach which would provide information useful in understanding the elements inherent in the specificity of such interactions would be to identify the region within a given ribosomal protein which interacts with 16S rRNA. The isolation and characterization at the primary and secondary structural level would permit the development of models for protein-nucleic acid complexes within the ribosome.

In this communication we wish to report the isolation of a polypeptide fragment derived from 30S protein S8 which will reassociate with and bind specifically to 16S rRNA. The isolated binding polypeptide is approximately 40% as large as the intact protein. The size of the fragment has been estimated to be of the same order as the previously identified rRNA binding site for protein S8 (Schaup, et al. 1973).

MATERIALS AND METHODS

a) Proteins and rRNA

Ribosomal proteins and rRNAs were prepared from exponentially growing E. coli B236 cell cultures as described by Hardy, et al. (1969). Isotopically labeled proteins were obtained using [^{3}H]lysine and phosphorus-32 as orthophosphoric acid was used to prepare [^{32}P] labeled-RNA as described by Voynow and Kurland (1971), Schaup, et al. (1971) and Pace, et al. (1970). The nomenclature for ribosomal proteins is that of Wittmann, et al. (1971). Specific activities of proteins and rRNA were determined as previously described (Schaup, et al. 1970).

b) Digestion conditions and complex isolation

The generation of cyanogen bromide fragments from protein S8 was performed by the method of Gross and Witkop (1961). The sample was treated for 20 hr in 70% formic acid with 2 µgm of CNBr/µgm of protein. Analysis of the S8 digestion products on polyacrylamide gels, as described by Hardy, et al. (1969), indicated that the digestions were approximately 80 to 90% complete on the basis of the relative staining intensity of the digestion products and tritium recovery when labeled proteins were utilized. In the case of labeled digestion products unlabeled S8 was added as marker and its position was determined by staining with amido schwartz as described by Hardy et al. (1969). Cyanogen Bromide digestion products were taken up after lyophilization in 1 M Urea (Mann Ultrapure), 0.01 M Tris HCl, pH 7. (UT buffer). The final concentration of protein was 0.1 mg/ml. Aliquots of this mixture of fragments were incubated with 16S rRNA under conditions for specific reassociation of intact protein (Schaup, et al., 1970).

Proteinase K (EM Biochemicals, Darmstatt, Germany) digestion was performed on protein S8 after incubation of the protein with 16S rRNA under conditions which yield a specific and stable complex with 16S rRNA (Schaup, et al., 1970). After complex formation the sample was divided into aliquots and treated with the desired amount of proteinase K. The polypeptide fragment reported in this work was generated by a 5 µgm Proteinase K/µgm S8 treatment. Following treatment with proteinase K the reaction was stopped by the addition of 1 λ of 0.3 mM phenylmethylsulfonylfluoride/10 µgm of enzyme (PMSF).

Analysis of either CNBr fragment binding or intact complexes derived from proteinase K digestion was performed by zone ultracentrifugation or gel filtration on BioGel P-150 columns (65 cm x 1 cm) followed by scintillation counting in a Packard 3300 scintillation spectrophotometer using a TX-100 counting cocktail as previously described (Schaup, et al., 1970). Ribosome reconstitution buffer (0.005M Na_2HPO_4, 0.02m $MgCl_2$, 0.36M KCl, pH 7.6) was the running buffer in the columns and was the buffer used in sucrose gradients.

c) Extraction of the S8 Protease fragment

Complexes of 16S rRNA with the proteinase K generated and attached fragment (K_{S8}) were precipitated by the addition of 2 volumes of absolute ethanol at 4°C for 8 hr and then taken up in a buffer (TSM) containing 0.01 M Tris HCl, 0.005M Succinc acid, 0.01M $MgCl_2$, pH 8. The proteinase K fragment, K_{S8}, was then removed from the complex by the addition of 2 volumes of glacial acetic acid as described by Hardy, et al. (1969). The K_{S8} was then dialyzed into UT buffer to be stored and used in subsequent reassociation experiments.

When direct analysis on acrylamide gels was to be performed on tritium labeled protein, the sample of 16S rRNA-protein complexes was made to 9 mg/l Cleland's reagent, 1% SDS (British Drug Houses) and immersed in a boiling water bath for 1 min. The sample was then applied to 15% polyacrylamide gels (.5mm x 80mm) and run at 6 mA/gel for 6 hours as described by Laemmli (1970). Unlabeled samples were analyzed on polyacrylamide gels using the fluorescent method of Talbot and Yphantis, (1971). The samples were treated with a 10% solution of dansylchloride in acetone in the amount of 20 λ/ml of protein solution. Unreacted dansylchloride was removed by passage of the sample through a Sephadex G-25 column (0.7cm x 8cm) and the fractions containing the protein were layered onto polyacrylamide gels for analysis.

d) Amino acid composition

Protein-nucleic acid complexes were lypholysed in ampules after which 0.05 ml of constant boiling HCl (~5.7 N) was added to the sample. The evacuated sealed ampule was placed at 110°C for 18 hr. The hydrolysate was taken to dryness under vacum and amino acid analyses were performed utilizing a 0.3 x 30 cm column (Durrum DC-4A resin), an o-phthalaldehyde fluorometric reagent, as described by Roth et al., 1973, and a detector system described by Ayres et al. (1974).

RESULTS

Cyanogen Bromide Binding Fragments

Cyanogen bromide fragments derived from tritium labeled protein S8 were incubated with 16S rRNA under conditions pre-

viously reported for specific binding of intact protein S8. Figure 1A shows the results of such a binding experiment on sucrose gradients. Figure 1B shows the results when increasing amounts of CNBr fragments are added to a fixed level of 16S rRNA. The first experiment was performed in the presence of a three fold molar excess of 16S rRNA and indicates that approximately 70% of the label in the protein fragments will form a complex with the rRNA. This level of binding is much higher than would be expected if only intact S8 which escapes digestion were reassociating with the RNA. The saturation experiment, Figure 1B, shows that the binding of protein reaches a plateau at a point which, in terms of the mass of protein present, does not exceed that which would be expected for 1:1 binding of intact protein to rRNA. The polypeptide extracted from the complex runs primarily as a single band on SDS polyacrylamide gels

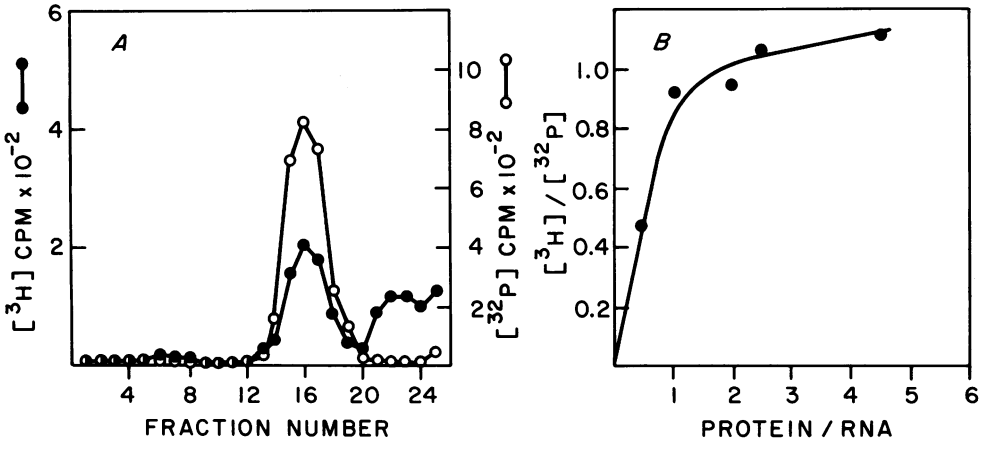

Figure 1. Binding of CNBr fragments of protein S8 to 16S rRNA. A. A three molar excess of [^{32}P]-16S (O O 34 cpm/µgm) incubated with fragmented [^{3}H]-S8 (• •, 1820 cpm/µgm) was separated by zone ultracentrifugation in a Beckman SW50.1 rotor at 50,000 rpm for 2 hr in a 15% to 30% sucrose gradient made up in ribosome reconstitution buffer. The tubes were fractionated through the bottom into 180 µl fractions and prepared for scintillation counting as described in Materials and Methods. B. Samples with a fixed amount of [^{32}P]-16S rRNA were incubated with increasing quantities of [^{3}H]-S8 fragments and analyzed as described above. The ratio of [^{3}H]/[^{32}P] in the zone containing 16S rRNA is plotted as a function of the number of moles of S8 to moles of 16S rRNA in the mixture before centrifugation.

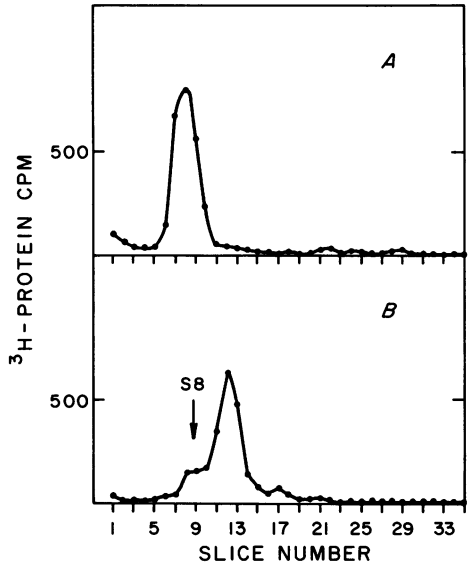

Figure 2. Polyacrylamide gel separation of A) protein S8 utilized in these experiments and B) CNBr digestion products which are found associated with 16S rRNA after zone centrifugation. Unlabeled S8 was added to both samples prior to electrophoresis. The samples were stained and destained prior to analysis for radioisotope. The arrows indicate the position of stained material.

(Figure 2). However some lower and higher molecular weight material appear to be present. We believe the major component represents a large fragment typically generated when protein S8 is treated with CNBr as described in Materials and Methods. The primary structure of protein S8 has been reported (Stadler, 1974) and such a fragment representing amino acid residues 27-94 (approximately 63% of the intact protein) would be expected in our CNBr digest of S8. The other material in the gel may represent incomplete digestion products as well as other smaller CNBr fragments which may bind to the RNA fortuitously. This will be discussed later.

Proteinase K digestions

On the basis of the CNBr fragment binding data alone it is not possible to unambigously identify the polypeptide binding site of protein S8, although it does appear in part to be associated with residues 27 to 94. Therefore, complexes of 16S rRNA

and protein S8 were prepared and treated with increasing amounts of proteinase K which was free of RNase activity. Figure 3A shows the results of such a digestion when the 16S rRNAprotein complex is separated from S8 digestion products by ultracentrifugation. Figure 3B shows that the digest rapidly reaches a point where approximately 50% of the radioactivity in the S8-16S rRNA complex have been removed. This result implies that the portion of the protein associated with the rRNA is protected from further attack.

Samples of the 16S rRNA complexed with the protected fragment (called K_{S8}) were prepared in which approximately 50% digestion of the protein had occurred. Fractions containing the K_{S8}-16S rRNA zone in the sucrose gradients were pooled and

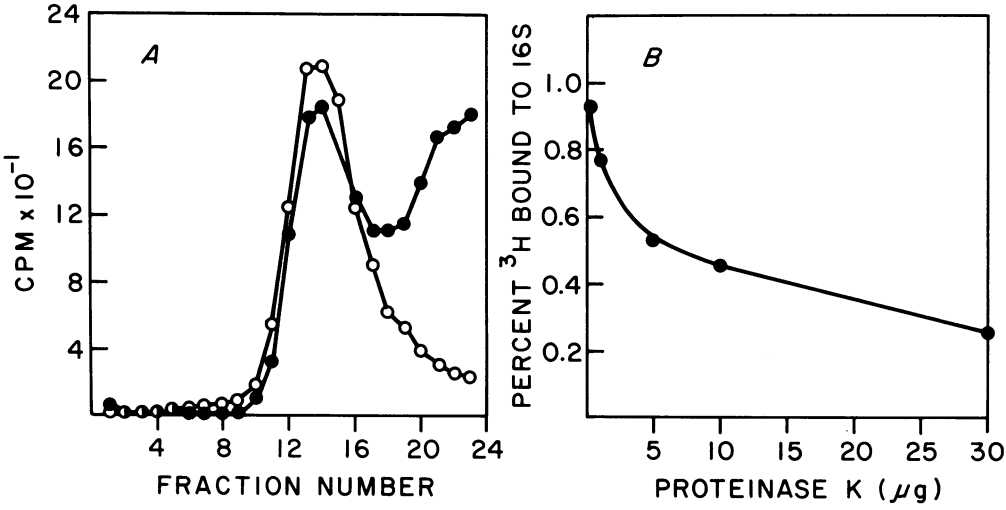

Figure 3. Digestion of S8-16S rRNA complexes with proteinase K. A. A sample of protein-rRNA complex ([^{3}H]-S8 • •, 1820 cpm/μgm; [^{32}P]-16S O O, 28 cpm/μgm) was digested by the addition of 10 μgm proteinase K, 22°C, 5 min. The digestion products were separated form the remaining rRNA complex by ultracentrifugation and analyzed as described in Figure 1. B. A S8-16S rRNA complex was prepared as described in Materials and Methods. The sample was divided into aliquots and each was treated with increasing amounts of proteinase K. The plot shows the amount of [^{3}H]-protein associated with 16S rRNA after ultracentrifugation relative to an untreated aliquot of the [^{3}H]-S8-16S rRNA complex.

the complex precipitated by the addition of 2 volumes of absolute ethanol at 4°C. The precipitate was resuspended in TSM and treated with two volumes of glacial acetic acid to extract

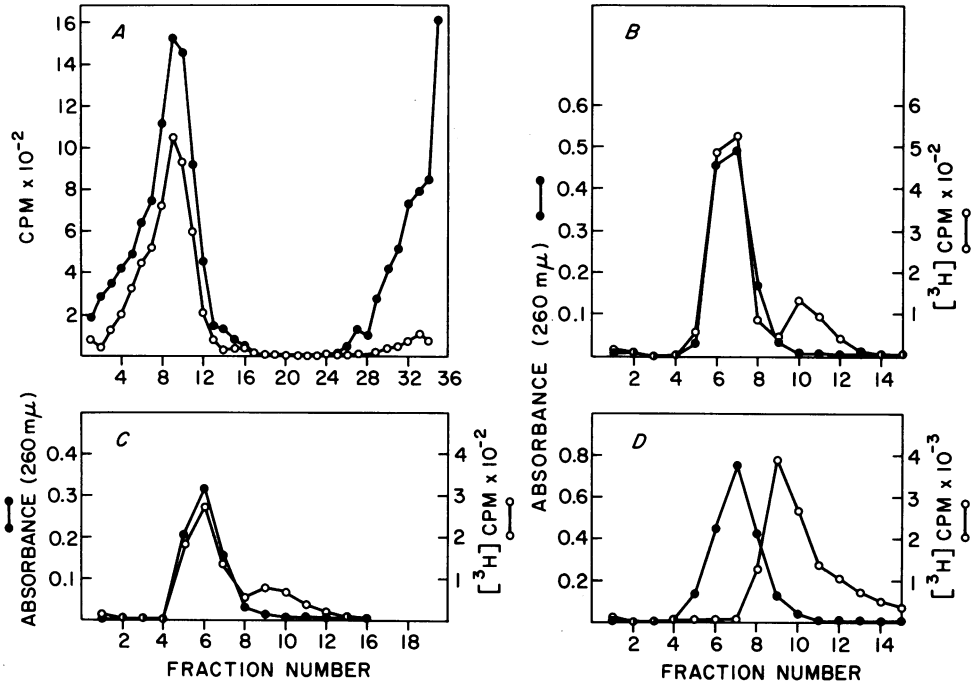

Figure 4. The reassociation of the K_{S8} fragment with 16S rRNA and competition experiments. A. The [^{3}H]-K_{S8} fragment (• •) was prepared as described in Materials and Methods, and incubated with [^{32}P]-16S rRNA (0 0 5 cpm/μgm) under ribosome reconstitution conditions. The sample was layered onto 15% to 30% sucrose gradients made up in reconstitution buffer and separation of the K_{S8}-16S rRNA complex was effected by zone ultracentrifugation in a Sorvall TV850 reorienting gradient rotor at 50,000 rpm for 1 hour. After centrifugation the bottom of the tube was punctured and .9 ml fractions were collected into scintillation vials and prepared for analysis as described in Materials and Methods. B. A [^{3}H]-K_{S8}-16S rRNA complex was prepared by ultracentrifugation and incubated with 2 molar excess of a mixture of unlabeled 30S proteins which did not contain intact S8. The sample was layered on a BioGel P150 column as described in Materials and Methods. Fraction size was 2 ml ([^{3}H]-K_{S8} 0 0, 16S rRNA • •). C. A [^{3}H]-K_{S8}-16S rRNA complex was prepared as in B and incubated with a 2 molar excess of non-radioactive intact protein S8. D. Non-radioactive S8 was used to generate a K_{S8}-16S rRNA complex and isolated as described above. This complex was incubated with 2 molar excess relative to the rRNA of [^{3}H]-S8 and layered on a BioGel column as described above [^{3}H-S8 0 0, 2120 cpm/μgm) 16S rRNA (• •).

the polypeptide as described by Hardy et al., 1969. This acid extractible material was then dialyzed into UT buffer and used in reassociation experiments. Figure 4A shows that approximately 80% of the recovered K_{S8} fragment will reassociate with 16S rRNA.

To test for binding specificity a K_{S8}-16S rRNA complex was prepared and separated from digestion products by ultracentrifugation. The complex was then incubated with a mixture of total 30S proteins which did not contain protein S8. There was approximately a 2 molar excess of each of the 30S proteins to rRNA in the incubation mixture. The mixture was layered on a biogel P150 column and the results shown in Figure 4B indicate that K_{S8} fragment is not removed from the 16S rRNA in the presence of other ribosomal proteins. Figure 4C shows the results obtained when the ^{3}H-K_{S8}-16S rRNA complex is incubated with a 2 molar excess relative to rRNA of unlabeled intact S8. The K_{S8} fragment does not exchange with intact S8. In another mixture nonradioactive S8 was used to generate the K_{S8}16S rRNA complex which was then incubated with intact ^{3}H-S8 present in two molar excess relative to the K_{S8}16S rRNA complex. The results are shown in Figure 4D and indicate that the binding of the K_{S8} fragment blocks the binding of intact S8. In control experiments 16S rRNA was treated with proteinase K followed by the addition of PMSF, a serine proteinase inhibitor. This rRNA was then incubated with ^{3}H-S8 and shown to retain competence in binding the protein.

In parallel experiments the K_{S8}-16S RNA complex was prepared for analysis on SDS polyacrylamide gels. The results are shown in Figure 5 for a K_{S8} fragment prepared from unlabeled protein S8. The preparation method and digestion conditions were as previously described. The results indicated that the fragment is not intact S8 which escapes digestion and the size of the K_{S8} fragment is less than that of intact S8. In SDS gels it is difficult to estimate the size of polypeptides with molecular weights below 10,000 daltons. The K_{S8} fragment, on the basis of digestion data, would be approximately 6000 daltons and displays an electrophoretic mobility comparable to insulin subunits (5,733 daltons). We estimate that the pro-

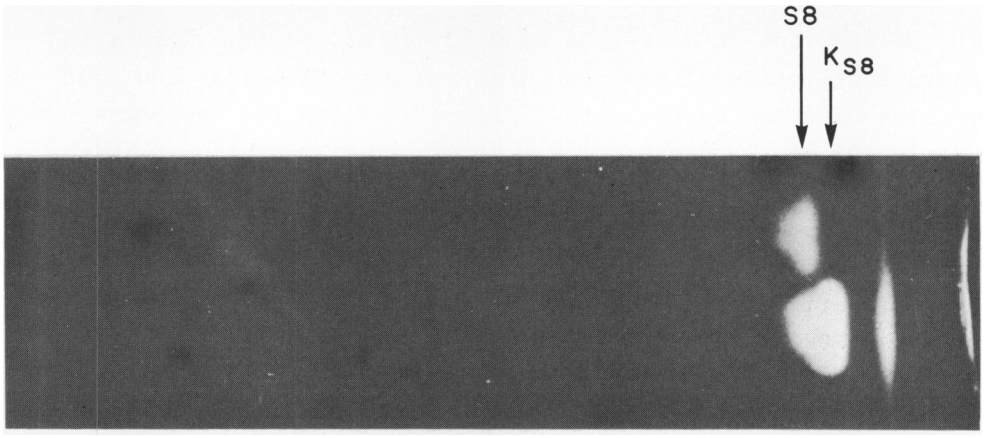

Figure 5. 15% SDS polyacrylamide gel separation of intact S8 and K_{S8} found associated with 16S rRNA as described in Materials and Methods. The proteins migrate toward the anode at the left of the photograph.

tected polypeptide is about 40 to 50 amino acid residues in length. The lower band in gels which is in the region of the tracking dye is unreacted dansylchloride which shows a fluorescence distinct from that observed for reacted dye. The presence of the unreacted dye is a consequence of poor separation of the material from small molecular weight polypeptides in the sephadex G-25 column prior to electrophoresis of the samples.

Aliquots of the sample shown on polyacrylamide gels in Figure 5 were prepared for amino acid analysis and the results are shown in Table 1. To minimize sample loses the complex was prepared as described above and hydrolysis of the polypeptide (K_{S8}) was effected in the presence of the 16S rRNA with which it was associated. In control experiments the 16S rRNA alone was treated and analyzed for contaminating amino acids. The rRNA appears to be contaminated with Serine, Glutamic acid and significant quantities of Glycine. The data reported in Table I are corrected for this contamination. Without correction the respective values for Serine, Glutamic acid and Glycine were 7.6, 11.2, and 15.9 residues/100 residues. These data indicate that the recovered polypeptide K_{S8} has an amino acid composition distinct from intact protein S8. When these

Table I. Amino Acid Composition

Amino Acid	PROTEIN: Residues/100 Residues K_{S8}	S8
Aspartic Acid	15.1	9.4
Threonine	4.7	6.2
Serine	4.9	5.1
Glutamic Acid	7.8	10.0
Glycine	6.3	11.4
Alanine	13.1	12.9
Valine	7.7	10.2
Isoleucine	3.4	5.8
Leucine	8.3	6.7
Tyrosine	-	1.6
Phenylalanine	1.5	3.3
Lysine	4.3	7.3
Histidine	2.3	1.7
Arginine	4.3	6.0

data are compared to the reported primary structure of protein S8 the presence and level of Phenylalanine place a constraint on the regions which can be included in the binding site. These would be either residues 1 through 47 or 49 through 109. If these data are normalized to that which would be expected for a polypeptide which is 47 residues in length and compared to the reported amino acid sequence for protein S8 the best fit of the data suggest that the binding site contains residues 1 through approximately 47. This region contains the N-terminal portion of the large CNBr fragment which appears to bind independently to 16S rRNA.

DISCUSSION

The specificity of binding of the CNBr fragment from protein S8 is supported by the observed stoichiometric binding of it to 16S rRNA. If nonspecific interaction were occurring, either excessive or below optimal binding might be expected. The binding curve for the fragment to the rRNA is essentially that which would be observed for intact protein. The specificity of binding for the K_{S8} fragment is supported by the competition experiments with 30S proteins. If the fragment were bound nonspecifically, we would expect it to be displaced

by other proteins which have a higher affinity for their respective binding sites within the 30S subunit. Additional support for specificity is the observation that the presence of the K_{S8} fragment blocks the binding of intact S8 to 16S rRNA. We conclude that the fragment is binding specifically to the rRNA and to the same rRNA region which is normally occupied by intact protein.

The possibility that the results reported above were a consequence of intact protein S8 which escaped digestion with either CNBr or proteinase K is not consistent with the data. The binding of CNBr fragments involved approximately 60 to 70% of the protein incubated with the rRNA. Analysis of the digested material on polyacrylamide gels prior to use indicated that no more than 20% of the material escaped complete digestion. Subsequent analysis of the material which binds to the 16S rRNA demonstrated that the bulk of the material was localized in a component which is smaller than intact S8 and in the gels there was very little material in the region where intact S8 would be found. The amount of radioactivity found in the large CNBr digestion product is consistent with that polypeptide representing residues 27 through 94 in the intact protein. In these gels other higher and lower molecular weight material was observed. This could represent specific binding polypeptides which would include incomplete digestion products on the high molecular weight side which contain the large CNBr fragment as well as regions associated with the N terminal portion of the protein. The binding of such products however should not alter the conclusion that residues 27 through 94 represent the major component in the mixture. The lower molecular weight components may reflect independent specific binding of one or more of the smaller CNBr fragments or fortuitous binding of these components.

Proteinase K is a nonspecific endopeptidase which is approximately 6 times more active than pronase (Ebeling et al, 1974). The digestion product associated with 16S rRNA in sucrose gradients is smaller than intact S8 and has an amino acid composition which is distinct from intact S8. The results obtained with the K_{S8} fragment are clearly not a result of

intact protein S8 escaping digestion. On the basis of the amino acid composition it appears that the protein binding site for 16S rRNA is contained approximately within residues 1 through 47 (Table II). This result indicates an overlap between the K_{S8} fragment and the large CNBr fragment which binds to 16S rRNA. The overlap is between residues 25 through 47 suggesting that this region is of some importance in the binding of the protein to the rRNA. This region represents approximately 20% of the residues in the intact protein.

Analysis of the probable secondary structure of the region 1 through 47 using Fasman and Chou (1976) rules indicate that the polypeptide would have a high probability of forming α-helices. Similar analysis of the region 25 through 47 also suggest a high probability for α-helical formation. However, a complete assessment of the mode of interaction between protein S8 and 16S rRNA will have to wait further physical and biochemical studies of the complex as well as the construc-

Table II. Amino Acid Composition

	Protein K_{S8}	S8	S8
Amino Acid	Residues/47 residues	Residues 1 to 47	Residues 50 to 109
Aspartic Acid	7.0	6	3
Threonine	2.2	2	3
Serine	2.3	3	2
Glutamic Acid	3.7	5	8
Glycine	3.0	2	4
Alanine	6.1	7	2
Valine	3.6	3	6
Isoleucine	1.6	4	3
Leucine	3.9	3	6
Tyrosine	0	0	3
Phenylalanine	.70	1	1
Lysine	2.0	4	8
Histidine	1.1	-	-
Arginine	2.0	2	5

tion of appropriate molecular models. We are currently preparing sufficient quantities of the K_{S8} fragment to pursue such work.

ACKNOWLEDGMENTS

This work was supported by grants from the National Institute of General Medical Sciences (GM 21380) and an NIH Biomedical Science Support Grant GM 5 S07 RR07079-11. H.W.S. is a recipient of a PHS Research Career Development Award (GM 00073). We thank Dr. R.R. Becker for providing the amino acid analyses described in this work and for his helpful criticisms in the preparation of this manuscript.

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