The use of hydroxylamine cleavage to produce a fragment of ribosomal protein S4 which retains the capacity to specifically bind 16S ribosomal RNA

Li-Ming Changchien and Gary R. Craven

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

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SUMMARY

In previous reports we have described the isolation of fragments of 30S ribosomal protein S4 using a number of different enzymatic and chemical cleavage techniques. These experiments were designed to determine the region of the protein responsible for 16S RNA recognition. We report here the isolation of two fragments produced by the hydroxylamine cleavage of the asparaginyl-glycyl peptide bond between positions 124 and 125. The purified fragments were chemically identified and tested for RNA binding capacity. The fragment consisting of residues 1-124 retains RNA binding activity and the fragment 125-203 is totally without RNA binding function. These results and previous results strongly suggest that the domain of protein S4 responsible for 16S RNA specific association is within the region consisting of residues 46-124.

INTRODUCTION

The ribosome isolated from *Escherichia coli* contains 55 polypeptides and three molecules of RNA (1). About half of these proteins have been shown to be capable of independent and specific recognition of different regions of the ribosomal RNA molecules (2). Many attempts have been made to identify the precise locations on the RNAs which the individual proteins bind. However, relatively little effort has been expended to establish the domains of the proteins responsible for RNA binding activity. We have been working to develop chemical cleavage and enzymatic cleavage techniques to yield fragments of ribosomal proteins which retain specific RNA recognition capacities. Our efforts thus far have centered on proteins S13 (3) and S4 (4, 5, 6, 7, 8). These investigations have shown that individual ribosomal proteins can be readily cleaved to produce polypeptides capable of specific RNA association. The experiments with fragments of protein S4 demonstrated that 16S

RNA binding activity resides within the region flanked by amino acids 46 and 167 (7). In this report we present evidence that this RNA binding domain can be narrowed to residues 46-124. This has been accomplished by the cleavage of protein S4 between positions 124 and 125 with hydroxylamine, the isolation of the resultant fragments and the demonstration that the fragment 1-124 retains the capacity to specifically bind 16S RNA whereas fragment 125-203 does not bind ribosomal RNA.

MATERIALS AND METHODS

Ribosomes and their subunits were isolated from *E. coli* MRE-600 as previously described (9). Ribosomal proteins were extracted from the purified 30S subunits by acetic acid (10), fractionated by carboxymethylcellulose column chromatography (5) or HPLC chromatography using a Bio-Rad SP-5-PW ion-exchange column (11).

Purified protein S4 was tritium labeled by the *in vitro* reductive methylation of lysine residues using ^3H -formaldehyde and sodium cyanoborohydride (12). Incorporation of the label was approximately 0.3 per protein molecule. Protein concentrations were determined by the Lowry method (13) using chicken egg white lysozyme (Sigma) as the standard.

Polyacrylamide gel electrophoresis (PAGE) analyses were performed as described in (10).

The 16S RNA used in all reconstitution experiments was recovered from that precipitated by acetic acid during protein extraction. The precipitate was washed several times with 0.03 M Tris-HCl (pH 8.0) and 0.02 M magnesium acetate until the pH of the suspension was 7.0. The RNA was dissolved in 0.03 M Tris-HCl (pH 7.8) and further de-proteinized with phenol (14). The RNA solution was dialyzed against 0.03 M Tris-HCl (pH 7.4) buffer overnight at 4°C.

Reconstitution of 16S RNA-protein complexes was performed by a modification of the method of Held, *et al.* (15). The purified protein S4 was dialyzed overnight against 0.03 M Tris-HCl (pH 8.0), 8 M urea, 1 mM dithiothreitol (DTT) and then for 16 hours against 0.03 M Tris-HCl (pH 7.4), 0.02 M magnesium acetate, 0.5 M KCl, 1 mM DTT. Routinely 2.5 A₂₆₀ units of 16S

RNA in 0.4 ml of 0.03 M Tris-HCl (pH 7.4), 0.02 M magnesium acetate, 1 mM DTT were mixed with the ^3H labeled protein. The mixture was then incubated at 42°C for 40 minutes. The reconstituted particles were isolated by pelleting through 7 ml of 10% sucrose in reconstitution buffer (0.03 M Tris-HCl, pH 7.4, 0.02 M magnesium acetate, 0.33 M KCl, 1 mM DTT) or 16 hours at 30,000 r.p.m. in a Beckman 50 Ti rotor. The reconstituted particles were dissolved in 0.01 M Tris-HCl (pH 7.4), 0.5 M NH_4Cl , 0.5 mM magnesium acetate. The concentration of each sample was measured spectrophotometrically at 260 nm. The radioactivity of each sample was determined by liquid scintillation.

Hydroxylamine cleavage was carried out using modified conditions perscribed by Bornstein and Balian (16). The lyophilized protein was dissolved in 0.8 M Tris, 4 M guanidine and 2 M hydroxylamine (Aldrich). The pH of the solution was maintained at 9.0 by the addition of LiOH. The cleavage reaction was carried out at 45° for 2 hr and terminated by the addition of 0.1 volume of acetic acid. After completion of cleavage the reaction mixture was dialyzed against 5% acetic acid overnight and subsequently lyophilized. The lyophilized protein was dissolved in 0.03 M Tris (pH 8.0), 8 M urea, 1 mM DTT.

RESULTS

Cleavage of ribosomal protein S4 yields two fragments

Hydroxylamine has been shown to cleave asparaginyl-glycyl (asn-gly) peptide bonds with relatively high efficiency (16). Ribosomal protein S4 has one asn-gly bond between residue positions 124-125. We treated protein S4 with NH_2OH and found two cleavage products detected by SDS polyacrylamide gel electrophoresis (see Figure 1). We then purified all of the cleavage reaction products using a combination of reverse phase HPLC and size exclusion chromatography. Figure 2 shows the separation achieved by reverse phase HPLC. This separation technique yielded three major components, as predicted from the PAGE separation seen in figure 1. One of the fragments purified as a homogeneous fraction, denoted peak A in figure 2. The other components, consisting of intact protein S4 and one of the

Figure 1. SDS Polyacrylamide gel electrophoresis analysis of protein S4 before (lane A) and after (lane B) treatment with hydroxylamine. The PAGE conditions and the conditions of the reaction are described in Materials and Methods.

fragments, chromatographed as two overlapping peaks. We combined all fractions labelled B and C in figure 2, concentrated and separated these polypeptides on a Sephacryl-S200 size exclusion column. The profile of the separation is

Figure 2. C-18 reverse phase separation of NH_2OH protein S4 cleavage products. A Synchropak RP-P preparative column was used. A gradient of increasing acetonitrile was used. The collected fractions were analyzed for absorbance at 230 nm.

Figure 3. Separation of protein S4 cleavage products B and C as indicated in fig. 2 using a Sephacryl S-200. The column dimensions were 2.5 X 105 cm. The flow rate was 19 ml/hr and the volume of each fraction was 3.0 ml. The volume of the applied sample was 5.5 ml.

reproduced in figure 3. This chromatographic technique yielded two homogeneous components. Peak A proved to be uncleaved protein S4 and peak B was shown to be the fragment consisting of

Figure 4. PAGE analyses of purified NH_2OH cleavage products. The PAGE separation technique is outlined in Materials and Methods. Lane A is intact protein S4. Lane B is the NH_2OH cleavage product. Lane C is the fraction identified as A in fig. 2. Lane D is the fraction identified as B in fig. 3.

Table 1

Amino Acid Analyses of Hydroxylamine Cleavage Products of Protein S4				
Fragment 1-124			Fragment 125-203	
Amino Acid	Analysis	Predicted	Analysis	Predicted
Asp	9.8	9	7.0	7
Thr	4.6	4	2.1	2
Ser	5.2	4	6.1	6
Glu	15.0	14	12.3	12
Pro	3.6	3	3.0	3
Gly	11.7	12	3.0	3
Ala	11.9	11	7.3	7
Cys	0.1	1	0.04	0
Val	8.3	8	5.8	8
Met	1.5	2	1.0	1
Ile	5.3	4	3.7	4
Leu	13.3	14	6.4	6
Tyr	1.4	6	2.0	2
Phe	2.8	3	1.2	1
His	3.5	2	1.2	1
Lys	11.4	11	11.8	9
Arg	14.4	16	8.3	6
Total	124.5	124	77.8	78

Amino acid compositions were determined using classical techniques. All analyses were done in duplicate and the values presented here represents the average.

residues 1-124. PAGE analyses of all these separated components of the NH_2OH digestion product are shown in figure 4. From the amounts of the proteins recovered from the chromatographic separations, we estimate that the cleavage efficiency is between 50 and 55 percent, which is comparable to that found in other studies (16).

Amino Acid Analyses and Molecular Weight Estimations Permit Identification of the Fragments

Amino acid compositions of the three products of digestion were done and the results are tabulated in table 1. The results show that peak A isolated from the reverse phase HPLC separation is the C-terminal region of the protein consisting of residues 125-203. Component A from the size exclusion purification is intact protein S4 and component B, as seen in figure 3, is a homogeneous fragment consisting of residues 1-124. These conclusions are completely consistent with molecular weight estimations done by SDS PAGE as seen in figure 5. These studies

Figure 5. Molecular weight estimations of NH_2OH cleavage products using sodium dodecyl sulfate polyacrylamide gel electrophoresis (17). 1=ribosomal S4; 2=soybean trypsin inhibitor; 3=myoglobin; 4=protein S4 fragment 1-124; 5=cytochrome C; 6=protein S4 fragment 125-203; 7=pancreatic trypsin inhibitor; 8=glucagon.

show that peak A from figure 2 has a molecular weight of 8,800, consistent with the predicted molecular weight of the C-terminal region of the protein which is 8,967 daltons. Peak B of the size exclusion chromatography has a molecular weight estimated at 13,800, which is reasonably close to the predicted M.W. of 14,125 for the N-terminal section. Therefore, we conclude that we have managed to purify the two fragments of ribosomal protein S4 produced by chemical cleavage using NH_2OH .

The N-terminal fragment 1-124 can specifically bind 16S RNA

We lightly labeled the two purified fragments of protein S4 by reductive methylation (see Materials and Methods). Binding isotherms were then determined for the interaction of these fragments with 16S and 23S ribosomal RNA. The results are reproduced in figures 6A and 6B. We conclude from these experiments (isotherm in fig. 6B) that fragment 125-203 has virtually no affinity for 16S RNA. On the other hand, fragment 1-124 binds 16S RNA extremely well, but fails to have any

Figure 6. Binding isotherms of cleavage products to 16S RNA and 23S RNA. The binding studies of protein S4 fragment 1-124 to 16S RNA (o) and to 23S RNA (Δ) are illustrated in A. The insert in A is a double reciprocal plot of the binding data to 16S RNA. B shows the binding data for protein S4 fragment 125-203.

significant association with 23S RNA (fig. 6A). The actual apparent binding constant of fragment 1-124 is estimated to be $3.5 \times 10^6 \text{ M}^{-1}$ using a double reciprocal analysis of the binding data (see insert in figure 6A). This number is identical to that found for intact protein S4 as previously published (18), although we have conducted more recent experiments which indicate an apparent association constant of roughly twice this value. On the basis of these results we suggest that the fragment 1-124 retains most of the 16S RNA recognition domain.

DISCUSSION

Hydroxylamine has been demonstrated to be an extremely useful reagent for the specific cleavage of the rare peptide bond Asn-Gly (16). We have found conditions for the use of this chemical, which not only cleaves the single Asn-Gly peptide bond of ribosomal protein S4 with good efficiency, but also yields a fragment retaining RNA binding activity. Previous studies by

other workers were not concerned with the cleavage of proteins to produce fragments consisting of functional domains. Instead, their experiments were designed to determine reaction conditions which were optimal for the efficiency of cleavage. In our experiments, we found that slightly altered solution conditions and shortened reaction times yielded biologically functional fragments. This result means that hydroxylamine potentially could be used for the isolation of functional domains in many proteins.

Our use of hydroxylamine cleavage of ribosomal protein S4 has led to the isolation of two fragments, one consisting of residues 1-124 and the other composed of residues 125-203. The experiments we report here demonstrate that the 1-124 segment contains a substantial section of the 16S RNA recognition domain of the protein.

Other techniques have been used to determine the RNA binding region of protein S4. Bi-functional chemical crosslinking has shown that amino acid residue 59 is closely involved with 16S RNA interaction (19). Additionally, lysine residue 119 has been reported to be chemically unreactive when the protein is bound to 16S RNA (20). These results are totally consistent with our observations and give us confidence in concluding that the RNA binding region is within the region spanned by residues 46-124. Although this work represents an important step forward in the understanding of the mechanism of protein recognition of ribosomal RNA, it falls short in allowing us to suggest any viable models. We are presently exploring new techniques which may permit more precise identification of the RNA binding domain.

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