A cyanogen bromide fragment of S4 that specifically rebinds 16S RNA

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ABSTRACT

Escherichia coli ribosomal protein S4 was subjected to cyanogen bromide cleavage and was found to generate a complete cleavage product capable of rebinding 16S rRNA. This fragment, consisting of residues 1-103, was found to bind with an apparent association constant of $11 \mu\text{M}^{-1}$. This fragment was used in place of S4 in an *in vitro* reconstitution experiment. Although the particles formed had a protein composition not significantly different from reconstituted 30S ribosomal subunits, their sedimentation behavior was more like that of particles reconstituted without S4. These results indicate to us that, although residues 104-203 of S4 are involved in the assembly of the 30S ribosome, they are not necessary for the binding of S4 to 16S RNA. Taken with previous results, the domain of S4 involved in specific binding of 16S RNA can be confined to residues 47-103.

INTRODUCTION

The delineation of an assembly map for the 30S subunit of the Escherichia coli ribosome (1) was the first step in a series of experiments involved in probing the general question of how the bacterial ribosome self-assembles *in vitro*. The map elucidated some aspects of how the 21 ribosomal proteins and 16S rRNA interact with one another to form an integral unit. Primary binders, like S4, S7, S8, and S15, can bind 16S RNA independently of other proteins and in stoichiometric amounts. Many of the biochemical studies on the bacterial ribosome have relied on this property for probing specific protein-RNA interactions.

Early experiments with S8 (2), L18 (3) (a primary binder in the large subunit), and S4 (4,5) generated fragments of these proteins which remained capable of specifically binding rRNA. We investigated the S4 fragment further, and showed that its ability to function in an *in vitro* assembly reaction had been impaired

(5). This led to a series of experiments, working mostly with S4, in which the general scheme was to either enzymatically or chemically cleave a primary binding r-protein, and then determine if any fragment(s) retained rRNA binding ability. If such a fragment was found, it was purified and used in place of the intact protein in an in vitro reconstitution, and occasionally in subsequent functional assays.

We have determined that the N-terminal 46 amino acids of S4 are not required for S4 binding to 16S RNA, although residues 31-46 are involved in assembly (6). We have also shown that residues 168-203 are not involved in rRNA binding (7); they seem however to be involved in conformational changes in assembly and in the translational repression of the S4 operon (8, 9). The smallest active fragment of S4 that we have generated so far consisted of residues 1-124 and was produced by hydroxylamine cleavage (10).

We report here the production of a fragment of S4 consisting of residues 1-103, generated by cyanogen bromide (CNBr) cleavage, which rebinds 16S RNA with an affinity comparable to intact S4. However, this loss of almost half of the protein has severely affected its ability to participate in 30S assembly, not so much in terms of protein incorporation, but in a conformational contraction proposed to take place at a late stage in assembly (7).

MATERIALS AND METHODS

Ribosomes and their subunits were prepared from E. coli MRE600 as previously described (11). Ribosomal proteins were extracted from the purified 30S subunits with 67% acetic acid using the procedure of Hardy et al. (12) and further fractionated by cation exchange HPLC as described by Capel et al. (13). Protein was stored in a buffered urea solution (PSS) containing 50 mM Tris-HCl (pH 8.0), 6 M urea, 1 mM dithiothreitol (DTT). All urea solutions were stringently decolorized by treatment with Amberlite MB-1 (Sigma).

The 16S RNA used in all binding and reconstitution experiments was recovered from RNA precipitated by acetic acid during protein extraction, using the procedure described by Changchien and Craven (7).

Cleavage with cyanogen bromide (CNBr) was always done in 70% formic acid at room temperature. For initial studies, a freshly made stock solution of CNBr in 70% formic acid was diluted so that various precise amounts could be added to quantified amounts of protein in the same solvent. The time of reaction was varied as well. Each reaction was terminated by 6X dilution with water and immediate freezing in a dry-ice/ethanol bath. These frozen samples were then lyophilized directly. Lyophilized digestion mixtures were dissolved in 0.2M Tris-HCl (pH 8.2), 4M guanidine, 10mM DTT, and incubated with 5% beta-mercaptoethanol(v/v) at 37° for several hours to reduce methionine sulfoxides (14). The final conditions decided on for S4 were a 1,000-fold molar excess of CNBr over methionine groups, reacted for 3 hr.

Gel filtration was performed on a 2.5 X 120 cm Sephacryl S200 (Pharmacia) column in 6 M urea buffered by 50 mM Tris-HCl (pH 8.0). Reverse-phase HPLC was essentially as described by Capel et al. (13), but using a much shallower acetonitrile gradient.

Reconstitution of 16S RNA with r-proteins and/or their fragments was performed by a modification of the method of Held et al. (1). Before using in a reconstitution experiment, protein was dialyzed for 16 hours (2-3 changes) against 30 mM Tris-HCl (pH 7.4), 20 mM magnesium acetate, 500 mM KCl, 1 mM DTT. The protein in this buffer would then be combined with 1/2 volume of RNA in 30 mM Tris-HCl (pH 7.4), and the Mg concentration adjusted to 20 mM. The final buffer (30S reconstitution buffer, RB30) conditions are 30 mM Tris-HCl (pH 7.4), 20 mM Mg acetate, 333 mM KCl, 1 mM DTT. The reconstitution mixture was then incubated at 42° C for 40 minutes.

For quantitative binding studies, the protein moiety was tritiated by reductive methylation as described by Jentoft and Dearborn (15), and protein-RNA complexes were isolated by the nitrocellulose filter assay (16). Protein was quantified by the method of Sedmak and Grossberg (17), using lysozyme as a standard.

For qualitative binding studies, the reconstitution mixture (2.5:1 molar ratio of protein:RNA) was layered over a 12% sucrose cushion in RB30 and centrifuged at 100,000xG overnight. The

pellet was then resuspended in 6 M urea, the protein extracted with acetic acid, and the extract analyzed electrophoretically.

For total reconstitution particles, the pellet was resuspended in 40 μ l of resuspension buffer (10 mM Tris-HCl (pH 7.4), 0.5 mM magnesium acetate, 50 mM KCl, 1 mM DTT) and after it had completely dissolved, conditions were adjusted to those of reconstitution buffer. These were then subjected to zonal centrifugation through 10-25% sucrose gradients (240,000xG, 45 min, in VTi65 rotor at 4°C), and the location of each peak determined by scanning at 260 nm. Peaks were collected, pelleted, and the resulting ribonucleoprotein pellet redissolved in 6 M urea. Protein composition was determined by two-dimensional polyacrylamide gel electrophoresis as described previously (6).

Analysis of protein and protein fragments by polyacrylamide gel electrophoresis (PAGE) was performed using either the acid-urea procedure described by Hardy *et. al.* (12) or the SDS-PAGE system of Swank and Munkres (18).

RESULTS

CNBr Cleavage Yields Three Fragments That Bind 16S RNA

Cyanogen bromide cleaves on the carboxy-terminal side of methionine residues. The three methionine residues in S4 are at positions 103, 122, and 175. Although the reaction was never totally complete, conditions were experimentally determined to achieve a balance between the greatest complete cleavage and the least loss of activity. Initial qualitative binding studies were performed using this digest, unfractionated, in a reconstitution mixture with 16S RNA. The ribonucleoprotein complexes that formed were isolated by pelleting as described in Materials and Methods. Analysis of the protein content of the pellet by SDS PAGE (Figure 1) showed three bands in addition to intact S4. Compared to the control cleavage pattern, the bands appear to correspond to fragments consisting of residues 1-175 (indicated as F1 in the figure), 1-122 (F2), and 1-103 (F3), which should be the next three bands in the digest pattern.

These bands were compared to the products of the hydroxylamine cleavage of S4, fragments (1-124) and (125-203),

Figure 1. SDS Polyacrylamide gel electrophoresis (SDS-PAGE) analysis of r-protein S4 and its fragments. Lane A contains MW markers: 16.95, 14.40, 8.16, 6.21, and 2.51 kDa. Lane B is the S4 sample shown in lane C after hydroxylamine digestion. Lane D is the same S4 sample after CNBr digestion. Lane E is the banding pattern of the S4 CNBr digestion mixture which bound 16S RNA.

which we had identified previously (10). F1 and F2 can be unambiguously identified as fragments (1-175) and (1-122) by their pattern of migration in the gel, which correlates with their molecular weights. In addition, the F2 band comigrates approximately with the hydroxylamine cleavage product, fragment (1-124). The ability of these larger fragments to rebind 16S RNA is consistent with earlier work showing that fragments consisting of 1-167 and 1-124 both are capable of binding to 16S RNA (7, 10).

The F3 band is inferred to be fragment (1-103), since its staining intensity is consistent with complete rather than partial cleavage; and since its molecular weight would be next in the cleavage hierarchy after (1-122). One other fragment, (104-203), would have a molecular weight close to this.

Figure 2. Reverse-phase HPLC separation of Sephadryl S200 column fractions 84 to 88. A Synchropak RP-P analytical column was used, with a gradient of acetonitrile in 0.1% TFA as indicated. Eluate was scanned at 230nm and fractions were collected and read at the same wavelength.

Purification of F3

The CNBr cleavage products of S4 were partially separated by size on a Sephadryl S200 column. The major peak from this column was enriched for material corresponding to the F3 band.

We pooled the fractions from this peak, and produced a further separation on RP-HPLC. Figure 2 shows that the protein mixture was resolved into two peaks. We presumed that the second peak, containing the majority of protein, was (1-103), with some F2 showing in the tail end of the peak; and that the first peak

Figure 3. A schematic diagram of S4. Cleavage sites for NTCB, hydroxylamine, and CNBr are indicated along the length of the protein. The lone tryptophan residue is also indicated.

was fragment (104-203). This is consistent with the position of other C-terminal fragments of S4 in the elution profile of this column (7, 10).

Identification of F3

S4 has a tryptophan residue at position 167, which is the major source of absorbance at 280 nm. Absorbance at 280 nm versus that at 230 nm in PSS was used to determine extinction coefficients at 280 nm (9). The material in RP-HPLC peak 1 had a molar extinction coefficient ($42 \text{ OD}_{280}/\text{mM}$) approximately 6 times higher than the material in peak 2 ($7.2 \text{ OD}_{280}/\text{mM}$; each based on a MW of 12 kDa). The value of S4 (MW 23,131 Da) using this system is $39 \text{ OD}_{280}/\text{mM}$. This implied that the material in the second peak was (1-103).

This identification was further substantiated by the use of secondary chemical cleavage at sites indicated in Figure 3. Material from RP-HPLC peak 2 followed the expected behavior of F3(1-103), in that it: (a) was unable to be further cleaved by CNBr; (b) was unable to be cleaved by hydroxylamine (which cleaves between residues asn-124 and gly-125, see ref. 10); and (c) was cleaved at cys-31 (data not shown) by the cysteine-specific reagent NTCB (19). In a qualitative binding assay, only the material in RP-HPLC peak 2, i.e., fragment (1-103), bound 16S RNA.

Figure 4. RNA binding properties of F3(1-103). The top figure shows the binding isotherms for F3(1-103) to 16S RNA (o) and 23S RNA (Δ). The lower figure shows a double reciprocal plot of the 16S RNA binding data.

Residues 1-103 are Sufficient for the Binding of S4 to 16S RNA

Purified fragment (1-103) was tritiated and used in quantitative binding assays. The binding isotherms for both 16S and 23S rRNA are shown in Figure 4. It can be seen that the fragment binds specifically only to 16S RNA. The data in the 16S isotherm was analyzed using a double reciprocal plot (also shown in Figure 4) and gave values of $n = 0.81$ (binding sites per RNA molecule) and an apparent association constant $K = 10.6 \text{ } \mu\text{M}^{-1}$. Repeats of this experiment gave n -values from 0.8 to 1.2, and K -values from 8.8 to $12.7 \text{ } \mu\text{M}^{-1}$.

Figure 5. Sedimentation behavior of reconstituted particles. Particles were reconstituted using either a normal 30S reaction mixture (+S4), a normal mixture without S4 (-S4), or a mixture with F3(1-103) in place of S4 (+CBf). All conditions are described in the text.

Residues 104-204 are Involved in the Formation of a Stable 30S Particle During Reconstitution

Reconstitutions were performed with 16S RNA and three different protein mixtures. In the first case, the mixture consisted of 30S subunit proteins without S4 ("-S4"); in the second case, the (1-103) fragment was added, but not S4 ("CBf"); and in the third case, intact S4 was added ("S4"). The sucrose gradients of the reconstituted particles were scanned at 260 nm and the peaks collected directly for two-dimensional gel electrophoresis. The sucrose gradient profiles are shown in Figure 5 and the 2D gels for the "CBf" and "S4" particles in Figure 6. Note that although the sedimentation behavior of the "CBf" is closer to that of the "-S4" particle, its protein composition is indistinguishable from that of the "S4" particle. The repetitive centrifugation of the particles tends to cause the

Figure 6. Two-dimensional PAGE analysis of the (A)" +S4" and (B)" +CBf" particles sedimented in figure 6.

loss of a majority of certain proteins such as S2, S12 and S21, which are barely visible in either the "+CBf" or "+S4" 2D gels. Although S2 shows extremely lightly in Figure 6B, it was found present at slightly higher levels in other trials of the same experiment (data not shown). Of the three proteins, S2, S12 and S21, two (S2, S21) have previously been reported as missing or present in greatly reduced amounts in reconstituted "-S4" particles isolated after a single centrifugation; along with S1,

S13, and S18 (6). This finding is consistent with our results (data not shown).

DISCUSSION

Various cleavage techniques, both chemical and enzymatic, have been used extensively both by us and by other groups to study ribosomal proteins (for review, see 20). CNBr has been used successfully on S1 (21) and S8 (2) to yield RNA-binding fragments. An inherent problem with using CNBr is that unless the fragment of interest is a complete cleavage product, its purification in sufficient quantity for biochemical studies is not usually practical since its yield is often low and, in our experience, attempts to purify these types of fragments have invariably led to copurification of several related fragments. We used various cleavage conditions on S4, S7, S8, and S15; all but S15 produced fragments capable of binding 16S RNA in qualitative tests of the type shown for S4 in Figure 1. S7 showed several partial cleavage products, which we were unable to purify; while S8 showed one partial cleavage product, which was present in such low yield that it was impractical to pursue further. Our results with S8 contradict the earlier conclusions of Bruce *et al.* (2). The preparation of the S4 fragment described here was aimed at maximizing the yield of the complete cleavage fragments, while keeping the reaction time short to minimize the inactivation we observed with longer incubation times, presumably due to side reactions.

We have previously shown that residues 1-46 (5) and 168-203 (7) are not necessary for the binding of S4 to 16S RNA. In addition, we recently reported that an S4 fragment generated by hydroxylamine cleavage, consisting of residues 1-124, could bind 16S RNA (10). This fragment had an apparent association constant of $3.5 \mu\text{M}^{-1}$. The higher binding constant found for the (1-103) fragment described here could be due to some interfering effect from the polypeptide chain consisting of residues 104-124 or due to side effects from the hydroxylamine cleavage. The latter explanation seems more likely, especially since we found that allowing hydroxylamine cleavage to proceed beyond two hours resulted in a progressive loss of RNA-binding activity in both

the (1-124) fragment and intact S4. This is consistent with our experience that chemical methods of cleavage tend to damage protein. Because of this, we do not consider significant the difference between the apparent K_d for fragment (1-103), $11 \mu\text{M}^{-1}$, and that for intact S4, $18 \mu\text{M}^{-1}$ (22). We conclude that residues 104-203 are not necessary for S4 to specifically bind 16S RNA, and that residues 47-103 encompass the core RNA-binding region of S4.

Other researchers have presented evidence for the involvement of the C-terminal half of S4 in the interaction of this protein with 16S RNA. Amons *et al.* (23) showed lysines at positions 119 and 147 to be specifically protected from reductive methylation by 16S RNA, and Ehresmann *et al.* (24) inferred the site of three UV-induced crosslinks to 16S RNA in regions 56-59, 144-151, and 181-185. These observations can be explained if one allows for interactions with RNA that are not directly involved in the initial binding reaction. The function of these "secondary interactions" could be to further arrange the structure of the RNA. This would be consistent with our results produced by substituting fragment (1-103) for S4 in a total 30S subunit reconstitution reaction. Although the protein content of these particles is not noticeably different from that of 30S particles reconstituted under identical conditions (see Fig.6), it sediments in a broader, more slowly migrating peak. In fact, the broad shape of the peak mimics that of the particle reconstituted without S4 (see Fig.5). This interpretation of the data is completely consistent with our previous findings that the C-terminus of S4 is involved in a conformational shift of the small ribosomal subunit during its assembly *in vitro*.

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