Chemical and functional characterization of an altered form of ribosomal protein S4 derived from a strain of *E. coli* defective in auto-regulation of the alpha operon

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ABSTRACT

We have isolated a mutant form of *Escherichia coli* ribosomal protein S4. This mutant is temperature sensitive and apparently fails to autogeneously regulate the gene products of the alpha operon, which consists of the genes for proteins S13, S11, S4, L17, and the alpha subunit of RNA polymerase (1). We have shown that this mutation results in the production of an S4 protein with a molecular weight approximately 4,000 daltons less than the wild-type protein. Our chemical analyses demonstrate that the mutant protein is missing its C-terminal section consisting of residues 170-203. However, our studies to determine the capacity of this mutant protein to bind 16S RNA show that this protein is unimpaired in RNA binding function. This observation suggests that the functional domain of protein S4 responsible for translational regulation of the S4 gene products requires more of the protein than the 16S RNA binding domain.

INTRODUCTION

Escherichia coli ribosomal protein S4 has been shown to act as a feedback regulator of an operon containing the structural genes for proteins S13, S11, L17, the alpha subunit of RNA polymerase, and S4 itself (1, 2). This regulation apparently takes place at the translational level by a direct binding of the S4 protein to the beginning section of the messenger RNA which encodes this operon (3, 4). It has been postulated that this mRNA binding function involves RNA structural features which are very similar to the 16S RNA region responsible for protein S4 binding (5). Corroborative evidence for the translational feedback regulatory capacity of S4 was obtained by the isolation of a temperature sensitive mutant strain of *E. coli* containing an altered form of S4. This mutation was shown to effectively inhibit protein S4's capacity to regulate the alpha operon (6).

We have purified this mutationally altered form of ribosomal protein S4 and attempted to determine the nature of the change in the protein's structure. Our chemical analysis of the protein strongly supports the conclusion that the C-terminal section (probably residues 170-203) is deleted by the mutation. This conclusion is consistent with that of other workers who have investigated mutational alterations of protein S4 with similar phenotypes (7, 8, 9).

We have also analyzed this mutant S4 protein for its capacity to bind 16S RNA. We find that it binds 16S RNA with the same specificity and similar strength as the wild-type protein. This is consistent with our previously published work (10) which demonstrated that a fragment of the protein consisting of amino acids 1-167 retained full 16S RNA binding function. On the basis of these experiments we suggest the possibility that the protein's two functional domains responsible for 16S RNA recognition and mRNA binding are not identical, though they may be overlapping.

MATERIALS AND METHODS

Ribosomes were isolated by previously described methods (11). *E. coli* strains used were MRE600 and a temperature-sensitive mutant K-12 strain, designated as NO2207 (6), obtained from S. Jinks-Robertson and M. Nomura. The 30S peak off the preparative sucrose gradient zonal centrifugation of the ts-mutant's ribosomes was collected with some 50S contamination to maximize the yield of the mutant protein S4. Ribosomal proteins were extracted from purified ribosomal subunits by the 67% acetic acid method (12). The extracted proteins were purified by high performance liquid chromatography (HPLC) using a Bio-Rad SP-5-PW preparative ion-exchange column (13). Proteins were identified by one- and two-dimensional polyacrylamide gel electrophoresis (PAGE) (14). Sodium dodecyl sulfate PAGE was used for molecular weight estimations (15).

Purified proteins were labeled in vitro by light reductive methylation of lysine residues using ^3H -formaldehyde (New England Nuclear) and sodium cyanoborohydrate (Sigma) (16). Protein concentrations were determined by the Lowry method (17) using

chicken egg white lysozyme (Sigma) as the standard.

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 (18). 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.* (19). 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 20 hours against 0.03 M Tris-HCl (pH 7.4), 0.02 M magnesium acetate, 0.5 M KCl, 1 mM DTT. Protein solution was centrifuged at 10,000xG for 15 min to remove any small amounts of precipitate which might have been present. Routinely 1.5 A₂₆₀ units of 16S RNA in 0.17 ml of 0.03 M Tris-HCl (pH 7.4), 0.02 M magnesium acetate, 1 mM DTT were mixed with 0.33 ml of the ³H-labeled protein. The mixture was incubated at 42°C for 40 minutes and then chilled to 4°C before isolation of complex. 16S RNA-protein complexes were purified from unbound protein by nitrocellulose membrane filtration at room temperature as described by Spicer *et al.* (20). The concentration of complex in each sample was measured spectrophotometrically at 260 nm. The radioactivity of each sample was determined by liquid scintillation spectrometry.

Digestion of proteins by *S. aureus* protease V8 (Miles Lab.) was carried out in 0.1 M K₂HPO₄ buffer (pH 7.8) at 37°C for 18 hr. The molar ratio of enzyme to protein was 1:10. Digestion with trypsin (Miles Lab.) was done at pH 8.6 for 18 hr in 0.2 M NH₄HCO₃. Chymotrypsin (Worthington) digestion was performed using the same conditions but for only 4 hr. After digestion the samples were lyophilized and the dried residue was re-dissolved in 1 ml of a buffer containing 0.03 M Tris-HCl (pH 8.0), 8 M urea, and 1 mM DTT. The sample was then applied directly to a Spherisorb S5-ODS (4.6 X 25 mm) column and eluted with a linear gradient of acetonitrile containing 0.1 M NaClO₄ (21).

Hydroxylamine cleavage was carried out using modified

conditions prescribed by Bornstein and Balian (22). The lyophilized protein was dissolved in 0.8 M Tris (pH 9.0), 4 M guanidine-HCl and 2 M hydroxylamine-HCl (Aldrich). The pH of the solution was maintained at 9.0 by the addition of LiOH. After a 2 hr incubation at 45°C, the cleavage reaction was terminated by the addition of 0.1 vol. of glacial acetic acid. The reaction mixture was dialyzed against 5% acetic acid overnight and subsequently lyophilized. The lyophilized protein was dissolved in 0.03 M Tris-HCl (pH 8.0), 8 M urea, 1 mM DTT.

Treatment of protein 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 (including DTT) groups was added to the protein in 4M guanidine-HCl, 0.2 M Tris acetate (pH 8.0), 0.5mM DTT from a 0.1 M stock solution in the same buffer minus DTT. 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 5% acetic acid and lyophilized.

Cleavage of the cyanylated protein was carried out by redissolving the lyophilized protein in 4M guanidine-HCl, 0.1 M sodium borate (pH 9.0) and incubating the solution at 37°C for 9 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 5% acetic acid and lyophilized.

RESULTS

Purification of the Mutant S4 Ribosomal Protein

We have purified the ribosomal protein S4 from the temperature sensitive mutant strain of *E. coli* designated NO2207 (6). The profile of the HPLC chromatographic separation is reproduced in Figure 1. The peaks were analyzed by one- and two-dimensional electrophoresis, primarily to determine the position of the mutant protein S4 (S4m), but also to tentatively identify most the other 30S proteins present. S4m comes off at a slightly higher salt concentration than wild-type S4, but still comes off before S15, the next protein to be eluted in the wild-type profile. Proteins identified as non-30S by this system were marked by

Figure 1. Separation of 30S ribosomal proteins from ts mutant strain N02207, using ion-exchange HPLC (see Materials and Methods). The insert is a photograph of one-dimensional PAGE analyses of TP-30 preparations from both the wild-type (lane 1) and mutant strains (lane 4) and the purified S4 proteins from these two strains (lane 2 wild-type; lane 3 mutant).

question marks on the profile -- these probably represent 50S proteins present due to the liberal cut taken from the zonal and ribosome-associated factors. The one-dimensional PAGE patterns of wild-type TP-30, mutant TP-30, wild-type purified protein S4 and the purified mutant protein S4 are shown in the insert in Figure 1.

Amino Acid Composition of the Mutant Protein, S4m

The amino acid compositions of S4m and wild-type S4 were determined and the results are summarized in Table 1. We attempted to fit the raw data of the S4m analysis to numerous possible fragments of the (wild-type) S4 sequence. Although an exact fit was not achieved with any, the only one that was even remotely consistent was the sequence 1-169.

Molecular Weights of the Mutant S4 Protein and Various Cleavage Fragments

Wild-type protein S4 contains a cysteine at position 31 which can be cleaved by NTCB (14). It also has a single Asn-Gly

Table 1. Amino Acid Compositions of Ribosomal Proteins S4 and a ts Mutant form of S4.

Amino Acid	Protein S4		Protein S4 ts Mutant	
	Observed	Predicted	Observed	Predicted
ASP	16.4	16	13.0	12
THR	7.3	6	6.1	5
SER	11.6	10	7.8	7
GLU	26.0	26	20.8	22
PRO	6.6	6	5.1	5
GLY	17.1	15	15.3	13
ALA	18.4	18	17.1	16
VAL	11.1	16	11.5	14
CYS	0.3	1	0.3	1
MET	1.6	3	1.3	2
ILE	7.6	8	6.1	6
LEU	21.5	20	17.4	17
TYR	7.3	8	5.1	7
PHE	4.3	4	3.6	3
LYS	20.2	20	15.6	16
HIS	3.4	3	2.3	2
ARG	22.3	22	19.7	20
TRP	0.0	1	0.0	1
TOTAL	203.0	203	168.1	169

Analyses were performed by classical techniques. All determinations were done in duplicate and the data presented here represents the average values. Predicted residue compositions were derived from the known sequence (25).

peptide bond at position 124-125 which can be chemically cleaved by NH_2OH (24). To help us determine the region of the mutant protein S4m altered by the mutation, we used these chemical fragmentation techniques to examine the primary structure of the protein. Figure 2 shows one-dimensional SDS PAGE analyses of protein S4, the mutant S4 and the fragments of these two proteins produced by NTCB and by NH_2OH . The molecular weights of all these fragments were estimated and the results are summarized in Table 2. Minor contaminants are present due to the fact that the shoulders of the S4m peak were used for these studies, so that the central portion of the peak could be used for the other studies requiring extremely pure protein. The results show that the S4m protein is approximately 4,000 daltons smaller than the

Figure 2. SDS PAGE analyses of mutant and wild-type S4 proteins before and after chemical cleavage. Lanes 1 & 8 = mixture of marker proteins; Lane 2 = wild-type protein S4; Lane 3 = ts-mutant S4 protein; Lane 4 = wild-type S4 protein cleaved by NTCB; Lane 5 = ts-mutant S4 protein cleaved by NTCB; Lane 6 = wild-type protein S4 cleaved by NH_2OH ; Lane 7 = ts-mutant S4 protein cleaved by NH_2OH . Marker proteins (with MW): ovalbumin (43kd); carbonic anhydrase (29kd); soybean trypsin inhibitor (21.5kd); myoglobin (17.2kd); lysozyme (14.3kd); pancreatic trypsin inhibitor (6.16kd); and glucagon (3.46kd). Arrows indicate the fragments produced by chemical cleavage.

wild-type S4 protein, which corresponds to about 36 amino acids. The molecular weights of the two NTCB cleavage products strongly suggest that the N-terminal 30 amino acids are unaltered by the mutation. As can be seen, the molecular weights of the large pieces, representing the C-terminal region, show large differences in estimated molecular weights, while the wild-type and the mutant small fragments are essentially identical within the precision of the SDS PAGE analysis. The analysis of the small pieces is somewhat obscured, as they tend to be affected by band curvature across the gel and the amount present stains poorly. However, several electrophoretic runs verified that they both migrated the same distance. A similar result was obtained with the NH_2OH produced fragments. The larger of the two fragments, derived from the N-terminal section of protein S4, has remained unchanged by the mutation, whereas the C-terminal region

Table 2. Summary of Molecular Weight Estimations for Protein S4, ts Mutant Protein S4, and Chemical Cleavage Fragments

Polypeptide Description	Observed	Predicted From Sequence
S4	25,000	23,137
S4 Fragment 31-203 (NTCB)	21,000	19,621
S4 Fragment 1-30 (NTCB)	3,520	3,471
S4 Fragment 1-124 (NH ₂ OH)	13,800	14,125
S4 Fragment 125-203 (NH ₂ OH)	86,00	8,967
S4 ts Mutant	21,000	18,539-19,066
S4 ts NTCB large	15,400	15,068-15,595
S4 ts NTCB small	3,520	3,471
S4 ts NH ₂ OH large	13,800	14,125
S4 ts NH ₂ OH small	4,920	4,871-5299

Molecular weights of the proteins and peptides as determined by SDS PAGE analysis.

appears to have lost approximately 4,000 daltons in the mutant protein. All of these data taken together strongly suggest that the ts-mutant protein is missing a substantial portion of its C-terminus.

UV Spectra of Proteins S4m and Wild-type S4

Ribosomal protein S4 contains only one tryptophan residue which is located at position 167 (25). Since the molecular weight analyses described above indicated that approximately 36 amino acids are deleted from the C-terminus of the S4m protein, we determined whether the tryptophan residue is deleted in the mutant protein. This was accomplished by conducting a UV spectral analysis on both proteins, which gave mass extinction coefficients of 1.67 OD₂₈₀/mg for the wild-type protein and 2.07 OD₂₈₀/mg for S4m. These factor to molar extinction coefficients at 280nm of 38,600 and 38,400-39,500 for the wild-type and mutant protein, respectively, using the MWs given in the last column of Table 2. Our conclusion is that the mutant protein contains a tryptophan.

Peptide Separations of Protein S4 and Mutant Protein S4

In an effort to more precisely identify the section of protein S4 deleted by the mutation, we digested the two proteins with three different proteases and developed peptide maps using reverse phase HPLC (see Materials and Methods). Figure 3 consists of reproductions of the trypsin digests (frames A & B) and the chymotrypsin digests (frames C & D). Similar separations were also done with peptides derived from S. aureus V8 protease digestions (data not shown). The most important conclusion to be drawn from these peptide separation experiments is that the mutant protein differs from the wild-type protein by having fewer peptides (indicated by unmarked arrows). If one examines the known sequence of protein S4, this is exactly what would be predicted if the C-terminal region of the protein is deleted by the mutation.

We have also done peptide separations of protease digests and analyzed the peptides for the presence of tryptophan by monitoring the column effluent at 280 nm. The reason we wanted to conduct this analysis is that protein S4 contains a chymotrypsin sensitive leucine at position 168, a S. aureus sensitive glutamic acid residue at position 169 and a trypsin sensitive lysine at position 174. Our peptide separations show that no significant changes in the retention times for the tryptophan containing peptides appear for either the chymotrypsin digest or the S. aureus protease digest. However, the trypsin digest showed a marked alteration in the retention time of the tryptophan containing peptide. The analogous peaks in the scans at 230nm shown in Figure 3 were determined and are marked by asterisked arrows. We conclude that the deletion must be in a region between residues 169 and 174. Because of our molecular weight estimates, the amino acid composition determinations, and the magnitude of the shift in the retention time for the tryptophan-containing trypsin fragment, we suggest that the deletion is closer to position 169.

The Binding of the Mutant Protein S4 to 16S RNA is not Altered when Compared to Wild-type Protein S4

The mutant protein S4 and the wild-type protein were tritium labeled by light reductive methylation. Binding to 16S RNA was

Figure 3. HPLC C-18 reverse phase separations of protease digests of protein S4 and mutant protein S4m. The elution system used was that designed by Meek (21). Frame A is the chromatographic pattern of a trypsin digest of S4 and frame B is a trypsin digest of S4m. Frame C is a chymotrypsin digest of S4 and frame D is a similar digest of S4m. Non-asterisked arrows indicate peptides in the wild-type profile missing from that of the S4m protein for each digest. The asterisked arrows in the trypsin digests indicate the tryptophan-containing peptide in each profile.

then determined as previously described (20). Figure 4 shows the binding isotherms for the mutant protein (frame A) and for the wild-type protein (frame B). Inserts in this figure represent reciprocal plots of the same data. Using a double reciprocal analysis of the binding data, the actual apparent binding constant of the mutant protein is estimated to be $7.96 \times 10^6 \text{ M}^{-1}$, which is very close to that for intact wild-type protein S4 ($8.93 \times 10^6 \text{ M}^{-1}$). These experiments demonstrate that the purified mutant protein and wild-type protein have similar affinity for specific binding to 16S RNA.

Figure 4. Binding isotherms of mutant protein S4m (A) and wild-type protein S4 (B) to 16S RNA (o) and to 23S RNA (Δ). Increasing amounts of tritium labeled protein were incubated with a constant concentration of r-RNA. The protein-RNA complexes were separated from unbound protein by nitrocellulose membrane filtration. The inserts in A and B are the double reciprocal plots of the binding data to 16S RNA.

DISCUSSION

The regulation of ribosomal protein synthesis has been shown to take place at the level of translation (for review see 26). It appears that an auto-regulatory mechanism, involving specific ribosomal proteins, exists for every operon containing r-protein genes. The mechanism postulated for this auto-regulation is that one protein specified by the structural gene

in the operon has the capacity to recognize and bind a region of the messenger RNA preceding the mRNA region which encodes the protein primary structures. The proteins which so far have been identified as having the capacity to regulate their own operons are L1 (2, 27), L10 (28, 29, 30), L4 (31), S4 (2, 32), S7 (33), S8 (2, 33) and S20 (34). All of these proteins have been shown to be capable of independent, strong, and specific binding to their cognate ribosomal RNAs (for review see 35). This correlation led to the postulation that the basis of the auto-regulation is due to a direct recognition of mRNA by the regulatory protein and that this recognition involves mRNA sequences homologous with the rRNA binding sites. In support of this idea, the leader sequences of several r-protein operons have been determined and found to have considerable homology with the rRNA binding sites of the respective regulatory proteins (5, 36, 37, 38, 39, 40). In all cases, including r-proteins S4, S7, S8, S15, L1, L4, and L10, these homologous sequences can be arranged into secondary structures which appear to have many similar features. This observation has generated the speculation that the feedback control of ribosomal protein biosynthesis involves a direct competition between mRNA and rRNA binding sites.

If feedback regulation is due to certain ribosomal proteins having the capacity to bind specifically to both mRNA and to rRNA, this might mean that a mutant strain of the organism, defective in the feedback control mechanism for a r-protein operon, would also contain a regulatory protein defective in its capacity to bind rRNA. We have examined this concept by isolating the protein S4 from the mutant strain of E. coli denoted NO2207, which fails to successfully regulate the alpha operon at 37° (6). Using a variety of chemical analyses, we have determined that the C-terminal region of the protein is deleted by the mutation. This result is completely consistent with findings by workers who have determined the primary structure of S4 proteins derived from other mutant strains of E. coli, which were isolated as streptomycin independent (8, 9). These reports indicate that in some mutant strains the S4 protein is deleted at the C-terminal region.

Our studies have so far dealt with chemical modification

(cleavage) of proteins that can bind 16S RNA directly, to determine the domains of these proteins involved in the actual protein-RNA interaction. Previous work in our lab on S4 has demonstrated that this ability is retained after the loss of the N-terminal 46 amino acid residues (18) and that the loss of the C-terminal 79 amino acid residues has little to no effect on the thermodynamics of the S4-16S rRNA interaction (24). One experiment demonstrated the functional activity lost with the scission of the C-terminal 36 amino acid residues was predominantly of a conformational nature -- it assembled into a fully protein-complemented 30S-like particle, but that this particle sedimented at a slightly lesser S-value than the normal 30S subunit (10). This present work demonstrates further the viability of an almost identical fragment in an in vivo system. However, the mutant strain does not grow at 42°, and this lethality could be due to either a perturbation in 16S RNA binding and assembly, or to a destabilization of the post-translational control exerted by S4 (2, 32). The first would be consistent with our characterization of a conformational stability being lost with this section of the protein; if this domain of S4 stabilized the final subunit (and its position within it as well), causing a 27S to 30S "contraction", its loss should tend to destabilize this conformation. This temperature-sensitivity can be seen as reflection of this destabilization.

Previous work with this mutation in a different (wild-type rpsL) genetic background demonstrated that the defect in post-translational repression was strongly expressed at 37° (6), even though growth at this temperature exceeded that at both 30° and 42°. It is tempting to speculate that, since our 16S RNA binding studies done at room temperature showed no more difference from intact S4 than those done at 0°, the post-translational control (mRNA binding) ability of the protein is being affected to a much greater degree than the 16S RNA binding ability, which would further imply that the domains involved in binding these two RNA species are not identical (though they could be overlapping). This is an unresolvable conclusion at this point, since we have found that the growth rates for the mutant and the parental strains are very close at permissive temperature (data not

shown). This emphasizes that no distinct phenotypic differences are readily apparent between these two variants of protein S4 at (and presumably below) this temperature. Comparative binding studies between 16S RNA and alpha-operon mRNA, using both wild-type and mutant S4, possibly could demonstrate a difference and prove this point. Unfortunately, isolation of the required amount of pure mRNA is not technically feasible and our binding assays cannot be performed at 37^o. If a higher temperature binding assay could be designed, it would provide more insight into this question.

In conclusion, the viability of a mutant possessing an S4 protein similar to one generated chemically by us confirms the veracity of our previous experiments demonstrating RNA-binding (and assembly) domains. Our research has shown that excisions of peripheral sections of a binding protein can still give a polypeptide that binds 16S rRNA in a specific fashion. By the in vivo studies done by Nomura and his group and shown by us to be the result of a similarly truncated polypeptide, it has been demonstrated that an organism can survive with a truncated protein similar to one shown by us to possess the minimal functional regions needed for RNA binding. This implies that our in vitro results have ramifications extending into actual biologically functional organisms.

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