Specific ribosomal RNA recognition by a fragment of *E. coli* ribosomal protein S4 missing the C-terminal 36 amino acid residues

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Received 29 May 1985; Revised and Accepted 8 August 1985

SUMMARY

We have previously investigated the role of the N-terminal region of ribosomal protein S4 to participate in 30S ribosome assembly and function (1-3). In this report we extend these studies to the two fragments produced by the chemical cleavage of protein S4 at the tryptophan residue 167. We find that the carboxyl terminal fragment (168-203) does not bind 16S RNA nor does it participate in assembly with the other 20 proteins from the 30S ribosome. In contrast, the larger fragment (1-167), does bind 16S RNA specifically. If the S4-fragment (1-167) is used to replace protein S4 in the complete 30S assembly reaction, all 20 of the other 30S proteins are incorporated. We conclude that the carboxyl terminal section of the protein S4 is not directly involved in binding 16S RNA or in the assembly of any of the other 30S proteins.

INTRODUCTION

The ribosome can be viewed as an intricate network of intermolecular and intramolecular interactions involving some 60 component macromolecules. The non-covalent interactions can be subdivided into three obvious classifications: protein-protein, protein-RNA and RNA-RNA interactions. If the complete architecture of the ribosome is to be determined, techniques must be developed which permit the precise localization of all these macromolecular associations.

One experimental approach has been to use proteases and nucleases to identify sub-domains of a given macromolecular component responsible for interaction with a second component. Such sub-domains have been inferred either from direct protection against enzymatic attack or from the isolation of digestion products which retain activity for intermolecular binding. Thus, nuclease digestion studies have permitted the identification of specific sections of ribosomal RNA responsible

for recognition and binding of individual ribosomal proteins (4). Similarly, a trypsin digestion fragment of ribosomal protein S4 has been isolated which is missing 46 amino acid residues from the N-terminal region (1,5). The remaining fragment (47-203) retains 16S RNA binding activity, but can only partially replace intact protein S4 in the complete 30S ribosome assembly process.

Selective fragmentation of proteins can also be achieved through the use of chemical cleavage techniques. We have utilized the reagent 2-nitro-5-thiocyanobenzoic acid to cleave protein S4 at the position 31 cysteine residue. The fragments produced by this treatment were purified and analyzed for residual function (2, 3). It was demonstrated that the N-terminal section 1-30 is not important for any aspect of 30S ribosome assembly, but is essential for transfer RNA binding activity. It was also found that the fragment consisting of residues 31-203 retained specific 16S RNA binding function and supported complete ribosome assembly.

The use of chemical cleavage techniques has been extended in the investigation reported here. Using a mixture of dimethyl sulfoxide and hydrogen bromide (6), we have cleaved ribosomal protein S4 at the peptide bond between the tryptophan residue at position 167 and the leucine residue at 168. The purified C-terminal fragment, consisting of residues 168 to 203, was found to be completely inactive in the ribosome assembly reaction. The larger purified fragment (residues 1-167), on the other hand, retained specific 16S RNA binding activity and permitted the assembly of a complete ribosome containing all the other 20 ribosomal proteins. The ribosome reconstituted with S4 fragment 1-167, however, has a slightly slower sedimentation velocity (28S) when compared to the control ribosomes reconstituted with intact protein S4. We conclude from these results, and our previous investigations, that ribosomal protein S4 contains a sub-domain, somewhere within the region encompassed by residues 47 and 167, which is responsible for specific 16S RNA recognition. Furthermore, the C-terminal section of the protein appears to be important only for promoting a conformational

change in the complete particle during a late stage in the in vitro assembly process.

MATERIALS AND METHODS

Ribosomes and their subunits were isolated from E. coli MRE600 as previously described (7). Ribosomal proteins were extracted from the purified 30S subunits with 67% acetic acid and further fractionated by phosphocellulose column chromatography following the procedure described by Hardy, et al., (8).

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 (1). 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 and 30S ribosomes was performed by a modification of the method of Held, et al. (9). The purified 30S ribosomal proteins were dialyzed overnight against 0.03 M Tris-HCl (pH 8.0), 8 M urea, 1 mM dithiothreitol, and then for 12 to 16 hours against 0.03 M Tris-HCl (pH 7.4), 0.02 M magnesium acetate, 0.5 M KCl, 1 mM dithiothreitol. Routinely 25 A₂₆₀ units of 16S RNA in 2 ml of 0.03 M Tris-HCl (pH 7.4), 0.02 M magnesium acetate, 1 mM dithiothreitol were mixed with a 2-fold molar excess of protein in 4 ml of 0.03 M Tris-HCl (pH 7.4), 0.5 M KCl, 0.02 M magnesium acetate, 1 mM dithiothreitol and the reconstitution mixture was incubated at 42°C for 40 minutes. The reconstituted particles were isolated by pelleting through 15 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 dithiothreitol) for 16 hours at 30,000 revs/min in a Beckman 30 rotor. The reconstituted particles were dissolved in 0.01 M-Tris-HCl (pH 7.4), 0.06 M-KCl, 0.5 mM magnesium acetate, and dialyzed overnight against the appropriate buffer at 4°C.

Cleavage of the tryptophanyl peptide bond of protein S4 was achieved using the method of Savige and Fontana (6). Lyophilized protein S4 (17 mg) was dissolved in a mixture of glacial acetic acid (4 ml) and 12 N HCl (2 ml) containing 20 mg of re-distilled phenol. To this mixture was added 50 μ l of dimethylsulfoxide (DMSO) (Fluka). The reaction mixture was allowed to stand for 30 minutes at room temperature, after which 200 μ l of 48% hydrogen bromide (HBr) (Fluka) and another 50 μ l of DMSO were added. The mixture was allowed to react for 30 minutes at room temperature. The cleavage reaction was terminated by the addition of 4 ml distilled H₂O. This solution was then dialyzed against 10% acetic acid and subsequently lyophilized.

Reduction of the DMSO-HBr cleaved protein was achieved by a combination of 4 M guanidine-HCl (Sigma), 0.03 M Tris HCl (pH 8.0), 0.002 M dithiothreitol (DTT) and 10% beta-mercaptoethanol. The mixture was incubated at 37°C for 48 hours in a sealed container. The product was dialyzed against a solution of 0.03 M Tris-HCl (pH 8.0), 0.001 M DTT and 6 M urea. This was followed by dialysis against a buffer containing 0.05 M sodium phosphate (pH 5.7), 6 M urea and 0.5 mM DTT.

Proteins were labeled in vitro by reductive methylation, using sodium borohydride (³H]hydride (320 mCi/mmol; Amersham/Searle) as described by Rice & Means (10). An average of .09 mol lysine/mol protein S4 were alkylated under this condition. It has been shown that the binding activity of 30S ribosomal proteins to 16S RNA is not impaired by reductive methylation (11, 12).

RESULTS

Ribosomal protein S4 contains a single tryptophan residue at position 167 (13). We have employed the recently developed procedure for tryptophanyl peptide bond cleavage (6), which utilizes a mixture of dimethyl sulfoxide (DMSO) and concentrated aqueous HBr to produce fragments of this protein. Figure 1 shows polyacrylamide gel electrophoresis analyses (8) of protein S4 before and after treatment with the DMSO-HBr mixture. Exposure of the protein to the reaction mixture for thirty

Figure 1. Polyacrylamide gel electrophoresis analysis of protein S4 before and after treatment with the mixture of DMSO-HBr. The treatment was for 30 minutes as described in Materials and Methods. The S4 fragment (168-203) does not appear on the gel under the conditions used here.

minutes gives optimal cleavage (40% to 60% in different experiments). The use of longer reaction times results in unwanted, non-specific cleavage as evidenced by the appearance of additional bands on the polyacrylamide gels.

The products of this cleavage reaction were tested for their capacity to bind 16S RNA and were found to be completely inactive. Other workers have shown that oxidation of the 3 methionines in protein S4 by performic acid treatment results in the loss of RNA binding activity (14). Since the known side reactions of the DMSO-HBr treatment include the oxidation of methionine and cysteine, we explored several different reduction conditions in an attempt to convert methionine sulfoxide back to methionine and restore RNA binding activity. We found that reduction for 48 hours at 37°C with a 10% solution of beta-mercaptoethanol and 2 mM dithiothreitol at pH 8.0 in 4M guanidine substantially restored the RNA binding activity of the DMSO-HBr reaction products.

Figure 2. Phospho-cellulose ion-exchange chromatographic separation of DMSO-HBr cleavage products of protein S4. A 4 liter gradient of NaCl was used from 0 to 0.2 M. Other conditions were as described by Hardy, *et al.*, (8). (-----) NaCl concentration, M; (——) Absorbance at 230 nm.

Purification of DMSO-HBr Reaction Products

It was necessary to prepare sufficient amounts of the tryptophan cleavage products to permit characterization of their capacities to participate in ribosome assembly. Accordingly, 17 mg of protein S4 was treated with DMSO-HBr followed by extensive reduction (see Materials and Methods). The reaction products were purified by phosphocellulose ion exchange chromatography. The resultant profile is reproduced in Figure 2. Polyacrylamide gel electrophoresis analyses were performed on all regions of the profile and fractions were combined as indicated. The polyacrylamide gel analyses of the combined fractions are included in Figure 2. Because of the high electrophoretic mobility of fraction A, we identified this fraction as the C-terminal fragment containing residues 168-203. Fractions B and C are chromatographically separable forms of protein S4 and fractions E and F are separable forms of S4 fragment (1-167).

Figure 3. Binding isotherms for protein S4, S4 fragment (1-167) and S4 fragment (168-203) to 16S RNA. Increasing amounts of the ^3H -labeled protein were incubated with a fixed amount of 16S RNA under standard reconstitution conditions. Ribonucleoprotein complexes were isolated by sucrose gradient sedimentation and the molar ratio of protein bound to 16S RNA was calculated (2). (○—○) protein S4; (■—■) protein S4 fragment (1-167); (●—●) protein fragment (168-203); (▲—▲) protein S4 fragment (168-203) plus total 30S protein minus protein S4.

Identification of these fractions is corroborated by amino acid analysis. Table 1 gives the results of amino acid analyses for intact protein S4, fraction A and fraction F.

Molecular Weights of S4 Fragments

The molecular weights of S4-fragment (1-167) (fraction F) and S4 fragment (168-203) (fraction A) were determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The molecular weights observed for these two polypeptides are 20,500 and 4,800, respectively. Assuming that these estimated M.W.'s are high by the same factor that protein S4 is itself (25,500 observed compared to 23,137 from the sequence), the corrected figures are 18,500 and 4,300, respectively. These

values compare favorably with those predicted from the sequence, 19,000 and 4,100 respectively.

S4-fragment (1-167) Retains 16S RNA Binding Activity but S4-fragment (168-203) does not

Binding studies of the purified fragments were performed using [³H] labeled protein prepared by reductive methylation (10). Figure 3 shows the binding curves obtained by varying the ratio of radioactive protein to 16S RNA. As can be seen, S4 fragment (1-167) (fraction F) has retained a fully functional 16S RNA binding site when compared to protein S4. S4 fragment (168-203) (fraction A), on the other hand, fails to show any measurable binding up to a four-fold molar excess of protein to RNA. We also studied the binding of fragment (168-203) in the presence of a mixture of 30S proteins minus protein S4. In this case, some fragment became associated with the assembled complex; however, the amount of protein bound was 0.2 moles per mole of RNA at all ratios of protein to RNA. We interpret this to mean that the observed association of this fragment is due to some sort of non-specific entrapment into the complex.

We have also examined the 16S RNA binding properties of the other fractions isolated from the phosphocellulose purification. Fractions B and E were found to be inactive for 16S RNA binding, whereas Fraction C qualitatively appeared to bind RNA as well as untreated protein S4. Thus, a chemical modification of both protein S4 and its fragment (1-167) must have taken place during the DMSO-HBr treatment, which altered the charge of the proteins (more acidic), as judged by their chromatographic behavior, and caused a concomitant loss of 16S RNA binding function. Thus far, we have been unsuccessful in attempts to reactivate these polypeptides using more exhaustive reduction conditions. Furthermore, the amino acid analyses in Table 1 do not show any obvious changes in composition which might account for the observed chromatographic behavior.

Although the modified forms of protein S4 and S4 fragment (1-167) were inactive for 16S RNA binding using the established conditions for reconstitution, we have found that these polypeptides exhibit a weak, specific interaction with 16S RNA when the KCl concentration used is lowered to 0.1 M (data not

Table 1
Amino Acid Analysis of Protein S4 Cleavage Products

Protein S4			Fraction A		Fraction F	
Residues/203			Residues/36		Residues/167	
Amino Acid	Anal.	Seq.	Anal.	Seq.	Anal.	Seq.
Asp	15.6	16	4.2	4	11.9	12
Thr	7.0	6	1.6	1	6.1	5
Ser	10.8	10	2.8	3	7.8	7
Glu	23.9	26	5.0	5	19.0	21
Pro	6.2	6	1.2	1	5.3	5
Gly	16.2	15	2.5	2	14.2	13
Ala	19.4	18	2.6	2	16.1	16
Cys	0.3	1	0.01	0	0.3	1
Val	12.0	16	1.9	2	10.3	14
Met	2.4	3	0.2	1	1.4	2
Ile	7.2	8	1.4	2	6.0	6
Leu	24.8	20	3.7	4	18.7	16
Tyr	8.4	8	0.9	1	6.8	7
Phe	4.1	4	1.0	1	3.4	3
His	3.1	3	0.9	1	2.2	2
Lys	19.3	20	3.7	4	16.4	16
Arg	22.8	22	2.2	2	20.8	20
Trp	0.0	1	0.0	0	0.0	1
Total	203.5	203	35.8	36	167.0	167

Amino acid compositions were determined as described in Materials and Methods. Analyses were performed on fractions from the phosphocellulose separation of tryptophan cleavage products seen in Figure 2. All analyses were done in duplicate and the data presented here represents the average. Residue compositions were obtained from the published sequence (12).

shown). More interesting, the modified proteins show a substantial binding when included with a mixture of 30S proteins minus native S4. This would indicate that although the 16S RNA binding site has been damaged by the DMSO-HBr reaction, specific protein recognition sites remain. We are now in the process of identifying the 30S proteins responsible for these interdependent assembly interactions.

Assembly of All 30S Ribosomal Proteins is Unimpaired by the Loss of S4 Residues 168-203

The ability of S4 fragment (1-167) to participate in complete 30S ribosome assembly was investigated. Reconstituted

Figure 4. Two-dimensional polyacrylamide gel electrophoresis analysis of reconstituted particles. Particles were prepared by incubating a 2.0 fold molar excess of the protein mixture with 16S RNA and pelleting through a layer of 10% sucrose. The purified particle preparations were analyzed by the 2-dimensional gel system described in (2). (a) with protein S4; (b) with protein S4 fragment (1-167).

particles were prepared with a mixture of total 30S protein (minus protein S4) and the fragment (1-167). The purified particles were then analyzed by 2-dimensional gel electrophoresis (2) (Figure 4B). This is compared to a similar reconstitution with intact protein S4 as shown in Figure 4A.

Figure 5. Sedimentation velocity analyses of reconstituted particles. Radioactive 30S ribosomes were mixed with a 30 fold molar excess of non-radioactive particles, layered on a 10-25% sucrose gradient and centrifuged as described in (2). Panel (a) non-radioactive native 30S; (b) non-radioactive reconstituted particles containing protein S4 fragment (1-167); (c) non-radioactive particles containing intact protein S4.

All 30S ribosomal proteins appear to be present in qualitatively normal amounts in both samples of reconstituted ribosomes. We conclude that the S4 fragment (1-167) can carry out all necessary interdependent assembly interactions involving other 30S ribosomal proteins that are normally the function of intact protein S4. Thus, the C-terminal portion of protein S4, encompassing residues 168-203, is not required for the assembly of any ribosomal proteins.

The C-terminal Region of Protein S4 is Critical for a 28S to 30S Conformation Change in Ribosome Assembly

The sedimentation properties of the particles reconstituted with protein S4 fragment (1-167) were compared with that for particles containing intact protein S4. We consistently observed the particles containing S4 fragment (1-167) to sediment slightly more slowly than either native 30S particles or reconstituted particles containing intact protein S4. To

demonstrate this altered sedimentation behavior, a sample of native 30S particles was lightly, reductively methylated using ^3H sodium borohydride. The radioactive particles were mixed with an approximately 30-fold excess of non-radioactive particles and sedimented through a sucrose gradient (see Materials and Methods). The resultant sedimentation profiles are seen in Figure 5. Panel (a) is the mixture of radioactive native 30S with an excess non-radioactive native 30S. No apparent difference in sedimentation is observed indicating that the reductive methylation did not appreciably perturb the ribosome's conformation. Panel (b), however, represents the mixture of non-radioactive reconstituted particles containing S4 fragment (1-167) in excess over the radioactive 30S ribosomes. These particles are significantly retarded in sedimentation having a relative sedimentation constant of approximately 28S. Finally, panel (c) represents the control experiment with reconstituted particles containing intact protein S4. In this case, the sedimentation of the reconstituted particles is indistinguishable from that of the native 30S ribosomes. We conclude that the missing segment of protein S4, residues 168-203, must be critically important for some sort of final conformational change during ribosome assembly that converts the particles from a 28S form to the native-like 30S form.

DISCUSSION

Several independent lines of evidence have led to the suggestion that the carboxyl-terminal region of protein S4 plays an important role in site specific recognition of 16S RNA. The most convincing investigations have involved a set of mutant strains of *E. coli* which contain altered forms of protein S4. These strains, which have been independently isolated in several different laboratories, have generally been selected as revertants to streptomycin dependence mutants containing an altered form of protein S12 (15-19). Several of these mutationally altered proteins have been isolated and shown to have altered 16S RNA binding activities (12, 20, and 21). These proteins have been characterized chemically (21, 22, and 23). In two cases, the proteins have been shown to be shortened by 20

to 30 amino acids at the C-terminus.

A different approach has been taken by Daya-Grosjean, et al.(14). These workers investigated the susceptibility of protein S4 to digestion by carboxypeptidase. They found that free S4 was readily digested but protein bound to 16S RNA was completely inaccessible to the enzyme. Furthermore, carboxypeptidase digested protein was claimed to be completely inactive in binding RNA. From the analysis done on the amino acids released by the carboxypeptidase digestion, it could be concluded that the loss of as little as 7 of the C-terminal amino acid residues results in complete loss of RNA binding function.

These two different investigations taken together form a reasonable argument that the carboxy-terminal region of protein S4 provides an essential component to the RNA recognition domain. Our work seemingly contradicts this conclusion. We have shown that removal of the C-terminal 36 amino acid residues does not measurably impair the specific binding of the remaining fragment (1-167) to 16S RNA. There are several arguments one can develop to rationalize our results with the results of the other workers cited above. For example, it can be argued that the binding we observe for the S4 fragment (1-167) is completely artifactual representing some sort of non-specific chelation with the RNA. We can suggest three lines of evidence which oppose this argument. First, the binding isotherm we observe for the fragment is almost indistinguishable from that found for intact S4. In other work, we have found that double reciprocal and Scatchard analysis demonstrates that the binding of S4 behaves as a classical single site association with a binding constant of $1.8 \times 10^7 \text{ M}^{-1}$ (24). Secondly, protein S4 and its fragments fail to bind 23S RNA. Finally, it should be remembered that the S4 fragment (1-167) chromatographs as two distinct forms, one of which binds RNA and the other form, which must retain some sort of chemical modification due to the DMSO-HBr treatment, fails to bind RNA under the identical conditions. On the basis of these considerations we are confident that the binding of S4 fragment (1-167) to 16S RNA is to a single specific site.

The experiments with carboxypeptidase can possibly be rationalized if the preparations of enzyme used in these investigations contained some endopeptidase activity. Indeed, carboxypeptidase preparations have occasionally been found to produce internal cleavages of proteins containing highly susceptible sites (25).

The experiments involving mutationally altered forms of protein S4 are more readily rationalized. Indeed, several mutants have been isolated which produce an S4 protein altered in the C-terminal region, however, the chemistry performed on these proteins has not been very complete. Funatsu and co-workers (23) studied six mutants which produced proteins that exhibited altered 16S RNA binding characteristics. Three of these mutant proteins were demonstrated to have no appreciable change in molecular weight and retained the wild type C-terminal amino acids as released by carboxypeptidase A plus B treatment. In addition, it was observed that under the conditions studied, these proteins retained RNA binding activity.

Three other mutants yielded proteins which have altered molecular weights (two were shorter and one longer) and reveal different C-terminals when digested with carboxypeptidases. Similarly, Schmitt and co-workers (21) have studied a single mutant protein which is apparently shorter than wild type by about 20 amino acids and which has a C-terminal region that is distinct from wild type as demonstrated by carboxypeptidase analysis and 2-dimensional separation of tryptic peptides. However, in all these cases, amino acid analysis and fingerprint analysis of tryptic peptides reveals that these proteins have alterations far more extensive than merely a deleted C-terminal region. The most plausible interpretation of these mutations as a class is that they represent small insertions or deletions which change the reading frame and ultimately produce a new termination point. This has been suggested earlier by Schiltz, as quoted by Wittmann & Wittmann-Liebold, (26). Since the chemistry on these proteins is incomplete, it is not possible to know even in general terms what regions of the proteins have been changed by the mutations and what regions remain the same as wild type. It is not unlikely that the changes involve amino

Figure 6. Schematic summary of the structure-function relationships for protein S4. The upper line summarizes the information described in the text concerning regions of the protein suggested to be involved in specific functions. The lower line illustrates potential secondary structure as predicted by Allen and Wong (30). Alpha-helix is represented as and beta sheet as .

acids within the boundaries of residues 1 through 167. Thus, we suggest that these studies with mutationally altered forms of protein S4 are not inconsistent with our conclusion that the C-terminal 36 amino acids are not responsible for 16S RNA recognition.

Our present work with the fragments of protein S4 produced by cleavage of the tryptophanyl peptide bond at residue position 167 complements earlier work by ourselves (1-3), and by other workers (5, 27). In these older experiments the role of the N-terminal region of protein S4 was investigated. It was found that the N-terminal amino acid residues 1-46 could be removed by trypsin digestion without any detectable impairment of the 16S RNA binding function. Ribosome assembly studies with the fragment 47-203 and with a fragment produced by cleavage at the position 31 cysteine residue, demonstrated that the 15 amino acid section 32-46 is somehow responsible for the assembly of proteins S1, S2, S10, S18 and S21. Additionally, it was inferred that the N-terminal region 1-31 is important for the binding of transfer RNA to the ribosome. These observations, together with these from the present investigation are summarized in Figure 6. In addition, we have included notations which summarize data from other laboratories relevant to a delineation of the RNA binding domain. Specifically, Amos, *et al.*, (28) have shown that lysines 119 and 147 of protein S4 are protected against reductive methylation when the protein is pre-bound to 16S RNA and Ehresmann, *et al.*, (29) have presented

indirect evidence that peptides 56-59, 144-151 and 181-185 may be crosslinked to the 16S RNA by ultraviolet irradiation. Also included in this figure is a representation of the predicted secondary structure of the protein (30). It is interesting to note that the region within the boundaries of the protein containing the RNA binding function also contains the greatest degree of secondary structure.

We have also demonstrated that not only does the C-terminal section of protein S4 fail to participate significantly in 16S RNA recognition, it also apparently is not involved in any substantial protein-protein interactions during 30S ribosome assembly. This can be inferred from the observation that the fragment (1-167) is fully capable of replacing the intact S4 protein during reconstitution to yield a particle containing all the other 30S ribosomal proteins.

The C-terminal region of the protein is not without any function, however. We find that the particle obtained by reconstitution with the fragment (1-167) does not display a normal sedimentation behavior. Instead of having a relative sedimentation velocity of 30S, the particle containing the fragment sediments at approximately 28S (Fig 7), an assembly intermediate which has been previously observed (31, 32). We conclude that the C-terminal region of S4 is somehow important for a critical rearrangement in the conformation of the ribosome which must take place as one of the final steps in self-assembly in vitro.

ACKNOWLEDGEMENTS

We would like to acknowledge the Amino Acid Analyzer Facility of the Biophysics Laboratory and the assistance of Dr. Daniel Omilianowski for performing the analyses. This work was supported by the College of Agricultural and Life Sciences as well as the Graduate School, University of Wisconsin-Madison. Primary support was derived from research grant GM-24019 from the National Institutes of Health.

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