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Characterization of Methylated Neutral Amino Acids from *Escherichia coli* Ribosomes

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The methylated neutral amino acids from both 30S and 50S ribosomal subunits of an *Escherichia coli* K strain were characterized. The 50S ribosomal subunit contains three methylated neutral amino acids: *N*-monomethylalanine, *N*-monomethylmethionine, and an as yet unidentified methylated amino acid found in protein L11. Both *N*-monomethylalanine and *N*-monomethylmethionine were found in protein L33. The amount of *N*-monomethylmethionine in this protein, however, is variable but not more than 0.25 molecules per protein. Thus protein L33 from this *E. coli* K strain has heterogeneity in its *N*-terminal amino acid and can start with either *N*-monomethylalanine or *N*-monomethylmethionine. The *N*-monomethylmethionine residue was not derived from the reduction of *N*-formylmethionine in the protein. The 30S ribosomal subunit contains only one methylated neutral amino acid: *N*-monomethylalanine.

Ribosomal proteins from both procaryotes and eucaryotes have been shown to be methylated (1, 5, 7-9, 11, 15, 17, 19). In *Escherichia coli*, methylation occurs predominately on the 50S subunit proteins (1, 7-9) with L11 as the major methylated protein. Previously, we have determined the stoichiometry of the methylated basic amino acids of the *E. coli* 50S subunit (7). Three unidentified methylated neutral amino acids were observed in the 50S subunit as well; two of these are located in protein L33 of an *E. coli* strain K (7). One of these two methylated amino acids in protein L33 has been shown independently by us (9) and by Wittmann-Liebold and Pennenbecker (19) to be *N*-monomethylalanine. The nature of the other methylated amino acid was unknown. A methylated neutral amino acid was also found to be present in the 30S subunit (11).

In this paper we have identified the other previously uncharacterized methylated amino acid in protein L33 of *E. coli* strain K as *N*-monomethylmethionine. Thus protein L33 from *E. coli* strain K has heterogeneity in its *N*-terminal amino acid and could start with either *N*-monomethylalanine (the predominant one) or *N*-monomethylmethionine. A possible reason for the heterogeneity of the methylated *N*-terminal amino acid in this protein is also discussed. The methylated neutral amino acid from the 30S subunit of *E. coli* was also characterized.

MATERIALS AND METHODS

Materials. [1-^{14}C]methionine (specific activity, 54 mCi/mmol) and [*methyl*-^{14}C]methionine (specific activity, 56 mCi/mmol) were obtained from New England Nuclear. *N*-monomethylalanine was obtained from Sigma Chemical Co. *N*-monomethylmethionine was synthesized according to the procedure of Means and Feeney (12). *N,N*-dimethylmethionine was synthesized according to the procedure of Bowman and Stroud (4) as recommended by Bailey (2). *N,N*-dimethylmethionine was detected by spraying the chromatogram with 0.1% orcinol in a mixture of *n*-butanol and ethanol (1:1), which was made 0.01 N with respect to H_2SO_4. Biosolve BBS-3 was a product of Beckman Instruments, Inc. All reagents were of analytical grade and obtained either from Sigma Chemical Co. or local sources.

Preparation of ribosomal subunits. *Escherichia coli* JC355, a K strain, was used for most of this work. *E. coli metE*$^-$ (strain 2549) was obtained from Coli Genetic Stock Center, New Haven, Conn. Cells were grown in 15 ml of a Tris-buffered medium [0.1 M tris(hydroxymethyl)aminomethane-hydrochloride, 1 mM $MgCl_2$, 0.01 mM $FeCl_3$, 0.1 mM $CaCl_2$, 1 mM KH_2PO_4, and 0.32 mM Na_2SO_4 adjusted to a final pH of 7.4] supplemented with 0.3% glucose, 2 μg of thiamine per ml and 15 μg of each of 20 amino acids except methionine per ml; 5 μg of either [*methyl*-^{14}C]methionine or [1-^{14}C]methionine per ml was then added. Cells were harvested at late log phase, and disrupted with a French press (together with 100 mg of carrier cells) in a buffer containing 0.01 M Tris-hydrochloride (pH 7.8), 10 mM $MgCl_2$, and 0.05 M KCl to obtain the 70S ribosome as described previously (8). The radioactive 70S ribosomes were then dissociated by dialyzing overnight against a buffer containing 0.01 M Tris-hydrochloride (pH 7.8), 0.1 mM $MgCl_2$, and 0.05 M KCl and layered on top of a 60-ml to 20% sucrose gradient in a SW 25.2 rotor. The ribosomal subunits were then concentrated by precipitation with ethanol (16). Electrophoresis of the ribosomal ribonucleic acid from both subunits by the procedure of Bishop et al.

(3) showed less than 4% cross-contamination. The preparation of ribosomal proteins from each subunit and the separation of the ribosomal proteins by the two-dimensional polyacrylamide gel electrophoresis were described previously (7). The ribosomal proteins were then hydrolyzed with 2 ml of redistilled 5.7 N HCl in sealed tubes at 110°C for 24 h. The hydrolysates were evaporated to dryness under reduced pressure and redissolved in 2 ml of water. This was again followed by evaporation to dryness. The final pellets were dissolved in small amounts of water for the analysis of the methylated amino acids. Electrophoresis was carried out in 0.05 M sodium borate buffer (pH 9.3) at 2,000 V for 70 min. The analysis of methylated amino acids by ion-exchange column was run in a Beckman PA-28 resin (0.9 by 45 cm) according to the Beckman 121 manual (physiological program) as described previously (9).

RESULTS

Isolation and characterization of methylated neutral amino acids from the 50S subunits. Ribosomal proteins were isolated from purified *E. coli* 50S ribosomal subunits and were hydrolyzed. The methylated neutral amino acids were then separated from both the basic amino acids and methionine (and its oxidized derivatives) by high-voltage paper electrophoresis in sodium borate buffer, pH 9.3 (Fig. 1, left panel). It is evident from Fig. 1 that the 50S subunit contains methylated basic amino acids (which comigrated with the lysine marker), a radioactive peak (peak I) which migrated slightly faster (toward the cathode) than the proline marker and possibly other radioactive compounds that might be buried under the radioactive methionine peak. The methylated basic amino acids have previously been analyzed (7). In the present studies the methylated neutral amino acids were arbitrarily divided into three peaks (I, II, and III) and each peak was subjected to amino acid analysis. The results of the amino acid analyses are presented in Fig. 2 with the following conclusions. (i) Peak I contains a methylated neutral amino acid which migrated slightly faster than methionine sulfoxide in the ion-exchange column. This methylated amino acid was found in protein L11 (7; unpublished observations). (ii) The methylated amino acids in peak II were identified to be both *N*-monomethylalanine and *N*-monomethylmethionine. *N*-monomethylalanine which migrated around fraction 55 during the ion-exchange column chromatography (halfway between serine and glutamic acid) has previously been characterized as being present in protein L33 from all *E. coli* strains (K, B, and MRE600) analyzed (9). *N*-monomethylmethionine, which migrated around fraction 74 (between proline and glycine), was identified by comparison of its mobility with the authen-

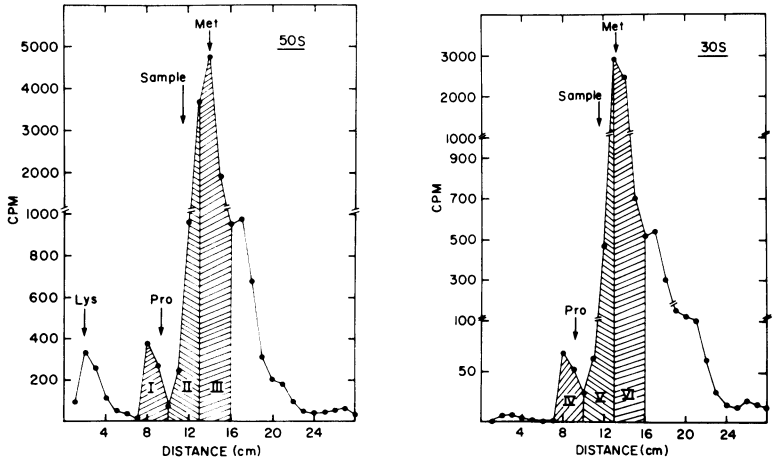

FIG. 1. *Analysis of methylated neutral amino acids derived from both 50S and 30S ribosomal subunit proteins by high-voltage paper electrophoresis in 0.05 M sodium borate buffer (pH 9.3). Four 10-μl samples of protein hydrolysates from each ribosomal subunit (described under Materials and Methods) were applied to a Whatman 3MM paper (20 by 57 cm) at 27 cm from one end, together with the following amino acids: methionine, proline, and lysine. Electrophoresis was carried out at 2,000 V for 60 min. After electrophoresis, the paper was dried and one of the four samples was sprayed with 0.4% ninhydrin in acetone and heated to 70°C for 5 min to locate the amino acids. The paper was then cut into 1-cm strips and counted in a toluene-based scintillator fluid (4 g of Omnifluor/liter of toluene). Peaks I through VI from the other three unsprayed samples derived from each subunit were then pooled individually, cut into small pieces, and extracted overnight at 30°C with water. This was followed by centrifugation, and the supernatant solutions were evaporated in vacuo to be analyzed by the ion-exchange column.*

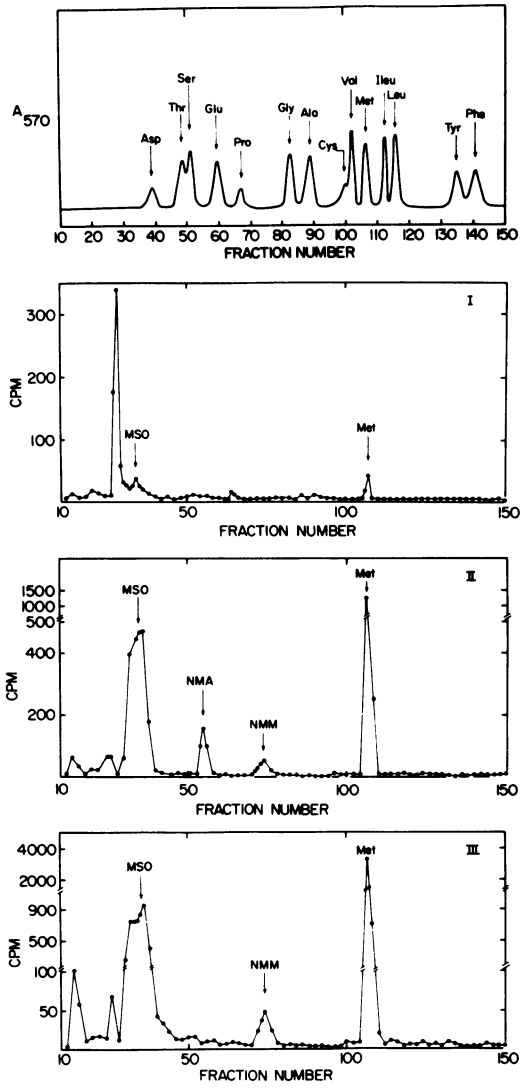

FIG. 2. *Ion-exchange column analyses of the methylated neutral amino acids from peaks I, II, and III. Each of the three peaks from Fig. 1 was dissolved in 0.2 ml of 0.2 M sodium citrate buffer (pH 2.0) together with 0.06 ml of the standard amino acids (2.5 nmol/ml). The above mixture was then applied to a Beckman automatic amino acid analyzer (model 121). The conditions for the ion-exchange chromatography were described previously (9). The radioactivity (0.7-ml fractions) was detected in a toluene-based scintillator fluid with 10% Biosolve BBS-3 solubilizer.*

tic compound and by other procedures described below. (iii) The methylated amino acid present in peak III was *N*-monomethylmethionine.

We have previously shown that protein L33 from *E. coli* strain K (but not from strains B or MRE600) contains a second methylated amino acid (9). This methylated amino acid has the same mobility as *N*-monomethylmethionine in the ion-exchange column. In order to further characterize this methylated amino acid, protein L33 was isolated from *E. coli* JC355 grown in a supplemented minimal medium containing [1-^{14}C]methionine. Figure 3 shows the amino acid profile of the protein L33 hydrolysate.

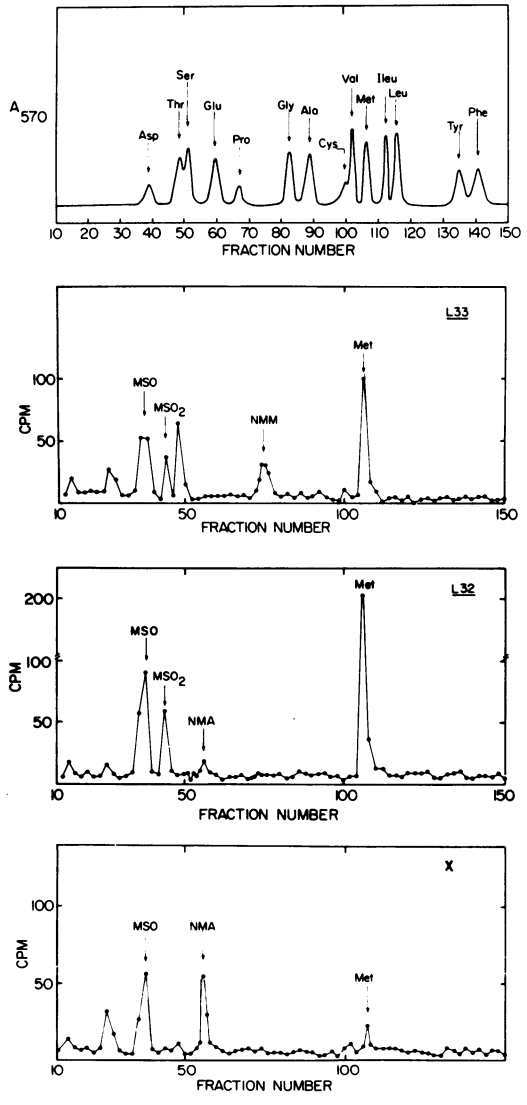

FIG. 3. *Ion-exchange column analyses of the methylated neutral amino acid from proteins L33, L32, and X. The conditions were the same as described in the legend of Fig. 2, except that protein L33 hydrolysate from [1-^{14}C]methionine-labeled cells and protein L32 and X (spot above proteins L32 and L33 as described in the text) hydrolysates from [methyl-^{14}C]methionine-labeled cells were analyzed.*

Again, a radioactive peak migrating around fraction 74 was obtained. Since this peak was obtained from both [1-^{14}C]-methionine-labeled and [*methyl*-^{14}C]methionine-labeled cells and had the same mobility as authentic *N*-monomethylmethionine, it is concluded that protein L33 from *E. coli* strain K contains this methylated amino acid. The presence of *N*-monomethylmethionine in protein L33 from strain JC355 was also confirmed by subjecting a [1-^{14}C]methionine-labeled protein L33 hydrolysate to high-voltage paper electrophoresis in sodium borate buffer, which also separates *N*-monomethylmethionine from *N*,*N*-dimethylmethionine (figure not shown). It should be noted that the amount of *N*-monomethylmethionine in protein L33 derived from either *E. coli* JC355 or Q13 is variable. In some preparations the amount of *N*-monomethylmethionine was as high as 0.25 molecules per protein (9), but in other preparations it was only barely detectable (approximately 0.05 molecules per protein; our unpublished data). The reason for this will be discussed later. Figure 3 also shows the results of the analysis of protein L32, which migrated very close to protein L33 during the two-dimensional polyacrylamide gel electrophoresis procedure (10). Protein L32 from *E. coli* JC355 does not contain either *N*-monomethylalanine or *N*-monomethylmethionine. Sequence analysis of this protein by Wittmann-Liebold et al. (18) showed the same result. We have frequently found that there is a protein spot (protein X) just above proteins L33 and L32 during the two-dimensional polyacrylamide gel electrophoresis run (see Fig. 8 of reference 10). In order to determine whether this protein spot is related to either protein L32 or L33 we have also analyzed this protein. It is clear from Fig. 3 again that this protein (protein X) contains *N*-monomethylalanine and could be derived from protein L33.

Recently the primary structure of protein L16 from an *E. coli* strain K was published by Brosius and Chen (5) and shown to contain *N*-monomethylmethionine. We have confirmed this by an ion-exchange column analysis of this protein. Thus *N*-monomethylmethionine is not restricted to protein L33.

The radioactive peaks migrating beyond peak III (distance 16 to 24 cm) in Fig. 1 were also analyzed, and no methylated neutral amino acids were detected (data not shown).

N-monomethylmethionine is not derived from the reduction of N-formylmethionine residue of the protein. Since bacterial proteins are known to start with *N*-formylmethionine, the identification of *N*-monomethylmethionine in both proteins L33 and L16 from *E. coli* strain K has prompted us to investigate the possibility that *N*-monomethylmethionine may be derived from the reduction of the *N*-formylmethionine residue in the protein. An *E. coli metE*$^-$ strain was used to prevent resynthesis of the methionine from homocysteine and the one carbon pool. When such a strain was labeled with [^{14}C]formic acid and the ribosomal protein hydrolysates were analyzed either by ion-exchange column or by high-voltage paper electrophoresis in sodium borate buffer, pH 9.3, no *N*-monomethylmethionine was detected (most of the incorporated radioactivity was in glutamic acid and aspartic acid; data not shown). Thus, it is concluded that *N*-monomethylmethionine is not formed by the reduction of the *N*-monomethylmethionine residue of the protein.

Characterization of the methylated amino acids from the 30S subunit. Since Klagsburn and Furano (11) reported that one of the three unidentified methylated neutral amino acids in *E. coli* 50S subunit was also present in the 30S subunit, we have performed a similar analysis with the protein hydrolysates from purified *E. coli* 30S subunits. Figure 1 (right panel) shows the fractions of the 30S protein hydrolysate that were analyzed. The absence of radioactivity in the basic region (around 2 cm) indicates that the 30S preparation is devoid of 50S subunit contamination. Figure 4 shows the following. (i) No discernible methylated amino acid was present in peak IV of Fig. 1. (ii) The radioactive peak V of Fig. 1 contains *N*-monomethylalanine as the only methylated amino acid. *N*-monomethylmethionine is present in protein S11 (unpublished observations). (iii) No methylated amino acid was found in peak VI of Fig. 1. Thus it is concluded that the unidentified methylated amino acid which was detected by Klagsburn and Furano (11) in the *E. coli* 30S subunit is *N*-monomethylalanine.

DISCUSSION

The *E. coli* 50S ribosomal subunit contains three methylated neutral amino acids. Two of these have been characterized and found to be *N*-monomethylalanine and *N*-monomethylmethionine. Interestingly, protein L33 from *E. coli* strain JC355 contains these two methylated amino acids, although the amount of *N*-monomethylmethionine is variable and never more than 0.25 molecule per molecule of protein. This may explain why *N*-monomethylalanine rather than *N*-monomethylmethionine was recently reported to be the *N*-terminal amino acid of protein L33 (19). The presence of *N*-monomethylmethionine in protein L33 in our preparation is presumably due to the incomplete removal of methionine residue (derived originally

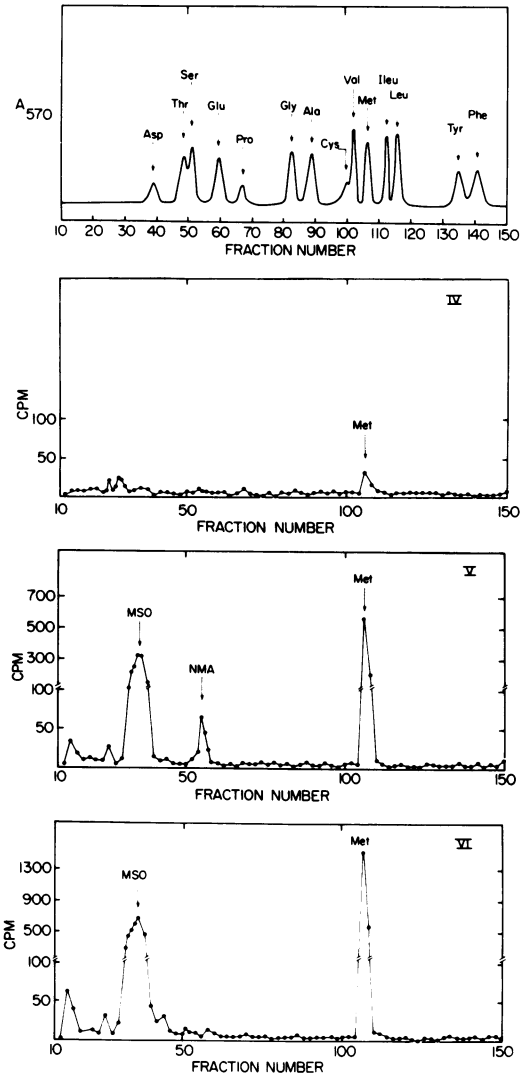

FIG. 4. *Ion-exchange column analyses of the methylated neutral amino acids from peaks IV, V, and VI. The conditions were the same as described in the legend of Fig. 2 except that protein hydrolysates from peaks IV, V, and VI of Fig. 1 were used.*

from *N*-formylmethionine) of the protein. This suggests that the methionine-removing enzyme may be rate-limiting in the processing of protein L33 during the biosynthesis of the 50S subunit. Any delay in the removal of the methionine residue will result in the formation of *N*-monomethylmethionine. This could also explain the fact that the amount of *N*-monomethylmethionine is variable in this protein and was not found to be present in proteins L33 obtained from either *E. coli* strains B or MRE600 (9). Presumably the latter two strains have an efficient methionine (or formylmethionine)-removing enzyme. The possibility that the *N*-monomethylmethionine residue is derived from the reduction of *N*-formylmethionine was also ruled out. Since there appears to be only one copy of protein L33 in the ribosome, this represents the first case where a protein shows heterogeneity in its modified *N*-terminal amino acid residue. It will be interesting to see if other proteins show such a heterogeneity.

The other methylated neutral amino acid from the 50S subunit is present in protein L11 (1, 7). The nature of this methylated amino acid is not known, although it appears to contain two methyl groups (7; our unpublished data). It has a mobility slightly faster than methionine sulfoxide during the ion-exchange column chromatography. We have ruled out the possibility that this methylated amino acid is a derivative of methionine since protein L11 from [1-^{14}C]methionine-labeled cells does not contain radioactively labeled compound. Our amino acid labeling studies appear to rule out that it is a derivative of any of the following amino acids: glycine, alanine, leucine, isoleucine, valine, serine, proline, arginine, histidine, lysine, aspartic acid, and glutamic acid. Further studies are underway to identify this compound.

Protein L33 has been implicated to be near the peptidyl transferase center by affinity labeling using *N*-bromoacetyl-phenylalanyl-transfer ribonucleic acid (tRNA) (6). Cantor et al. also showed that protein L33 reacts specifically with peptidyl-tRNA's containing longer peptide chains thus indicating that it is located slightly away from the 3′ end of the peptidyl-tRNA (the P site). Protein L16, on the other hand, is located at the A site and protein L11 is situated nearby. Both proteins L11 and L16 are implicated in the peptidyl transferase reaction (13, 14). Although it may be quite a coincidence that these three proteins, which play such an important role in the peptidyl transferase center, are methylated, it remains to be seen whether the methylation of these proteins is essential for the formation of the peptide bond.

Klagsburn and Furano (11) showed that a methylated amino acid was present in the *E. coli* 30S subunit. Our present analysis indicates that this methylated amino acid is *N*-monomethylalanine and is present in S11. No other methylated neutral amino acid appears to be present in the 30S subunit.

Our data also showed that a protein that migrated above proteins L32 and L33 (10; our unpublished data) contains *N*-monomethylalanine. This protein may be derived from protein L33, although sequence analysis has to be

performed on it before any definite conclusion can be reached.

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