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Methylation of Ribosomal Proteins in *Escherichia coli*

F. N. CHANG, C. N. CHANG, AND WOON KI PAIK

Department of Biology, Temple University, Philadelphia, Pennsylvania 19122, and Fels Research Institute and Department of Biochemistry, Temple University School of Medicine, Philadelphia, Pennsylvania 19140

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Escherichia coli was grown in a medium containing [1-^{14}C]methionine and [*methyl*-^{3}H]methionine, and the ^{3}H/^{14}C ratio was determined for each of the ribosomal proteins derived from the 70*S* ribosome. Evidence indicates that six proteins from the 50*S* subunit were methylated: L7, L9, L11, L12, L18, and L33. Methylation of several other 50*S* proteins (such as L1, L3, L5, etc.) may also occur. The methylated amino acids in protein L11 have been characterized further and found to be predominately ϵ-trimethyllysine. A small amount of a compound tentatively identified as N^{G}, N'^{G}-dimethylarginine was also detected.

Ribosomal proteins have been reported to contain methylated amino acids (1, 14-17). Comb et al. (1) found that the ϵ-amino group of a lysine residue was methylated in vitro to yield ϵ-monomethyllysine in ribosomal proteins isolated from the aquatic fungus *Blastocladiella emersonii*. However, it was not determined whether the ϵ-monomethyllysine was contaminated with other methylated lysines such as ϵ-dimethyllysine and ϵ-trimethyllysine in these preparations. Terhorst et al. (16, 17) showed that 50% of one lysine residue in proteins L7 and L12 from *Escherichia coli* MRE 600 was methylated to ϵ-monomethyllysine. Proteins L7 and L12 are identical, except that in L7 the amino-terminal serine is acetylated. Recently, Reporter (15) observed the presence of methylated amino acids in the acid hydrolysates of ribosomal proteins from both *E. coli* and *Euglena gracilis*. It was of interest to determine whether proteins other than L7 and L12 from *E. coli* are also methylated. In this paper, we report that several proteins from the 50*S* ribosomal subunits are methylated. The methylated amino acids in one of these proteins (L11) were characterized further by two different methods. The possible function of the methylation reaction is discussed.

A preliminary account of this work was presented at the 74th Annual Meeting of the American Society for Microbiology, Chicago, Ill.

MATERIALS AND METHODS

Bacterial strain. *E. coli* JC-355, a K-12 (F$^-$ *met*$^-$) strain provided by A. J. Clark, was used.

Growth of cells. Cells were grown in 10 ml of a Tris-buffered medium [0.1 M tris(hydroxymethyl) aminomethane (Tris)-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 20 μg of each of 20 amino acids except methionine per ml. A 15-μCi amount of [1-^{14}C]methionine (specific activity 54 mCi/mmol; Amersham/Searle) and 150 μCi of [*methyl*-^{3}H]methionine (specific activity 2.5 Ci/mmol; Amersham/Searle) were then added. The final concentration of methionine was 6 μg/ml. After the cells were harvested at late log phase, 250 mg of carrier Q13 cells (purchased from General Biochemical Inc., Chagrin Falls, Ohio) was added.

Preparation of 70S ribosomes. The cells were disrupted with a French press in a buffer containing 0.01 M Tris-hydrochloride (pH 7.8), 10 mM $MgCl_2$, and 0.05 M KCl. 70*S* ribosomes were prepared by a modification (2) of the Nirenberg and Matthaei (11) procedure, and the final ribosomal pellet was resuspended in the above buffer. Sixty absorbancy units (260 nm) of the 70*S* ribosomes in 0.5 ml of buffer was then adjusted to 0.1 M $MgCl_2$, and the ribosomal ribonucleic acid was precipitated by the rapid addition of 2 volumes of glacial acetic acid according to the method of Hardy et al. (5). The supernatant solution was lyophilized to dryness, and the proteins were dissolved in 0.15 ml of the sample gel solution used for the first-dimension run of the two-dimensional polyacrylamide gel electrophoresis procedure of Kaltschmidt and Wittmann (7). After electrophoresis and staining of the gels, regions of the individual proteins were cut from the gels and counted by the procedure of Nashimoto et al. (9). Because of the high pH of the first-dimension run (pH 8.6), the alkali-labile methyl esters of glutamic and aspartic acids will not survive the two-dimensional polyacrylamide gel electrophoresis.

Extraction of ribosomal protein. To analyze the methylated amino acids present in protein L11, the cells were grown in the presence of 25 μCi of [*methyl*-^{14}C]methionine (final concentration 7.5 μg/ml), and

the same procedures described for the preparation of 70S ribosome and polyacrylamide gel electrophoresis were followed. After electrophoresis and staining of the gel, protein L11 was cut from the gel and extracted overnight by gentle shaking with 6 ml of 0.01 M sodium phosphate buffer (pH 6.8) containing 0.1% sodium dodecyl sulfate. This was followed by dialysis against three changes of water to remove the excess dye and sodium dodecyl sulfate. The dialysate was filtered to remove the gel and then lyophilized to dryness. The protein was then hydrolyzed with 2 ml of redistilled 6 N HCl in a sealed tube at 110 C for 24 h. The hydrolysate was evaporated to dryness under reduced pressure and dissolved in 2 ml of water, followed by evaporation to dryness. The final pellet was dissolved in small amount of water for analysis of the methylated amino acids.

Analysis of the methylated amino acids in protein L11. The separation of methylated amino acids by high-voltage paper electrophoresis was carried out in a buffer containing pyridine-acetic acid-water (25:1:225 by volume; pH 6.5), using Whatman 3 MM paper (110 by 20 cm). This procedure causes separation of all of the arginine and histidine methylated derivatives. The three methylated lysines are not separated by the electrophoresis procedure but can be separated by the descending paper chromatography with the following solvent: pyridine-acetone-3 M ammonium hydroxide (50:30:25 by volume).

Identification of ε-trimethyllysine by an amino acid analyzer. ε-Trimethyllysine was also identified, using a Beckman automatic amino acid analyzer column (pH 5.84), by the procedure of Paik and Kim (13).

RESULTS

Localization of methylated ribosomal proteins. The amino acid compositions for most of the ribosomal proteins of *E. coli* have been determined (3, 6, 8). These data do not ordinarily reveal the presence of trace amounts of methylated amino acids, i.e., they must be specifically sought. So far, the primary amino acid sequences have been determined for only a few proteins. Among the proteins with known sequences, two acidic proteins from the 50S subunits (L7 and L12) have been found by Terhorst et al. (16, 17) to contain ε-monomethyllysine. Since there are at least 55 ribosomal proteins in *E. coli* (12), it would be of importance to determine whether other proteins are also methylated. Cells were grown in a medium containing [1-^{14}C]methionine and [*methyl*-^{3}H]methionine of very high specific activity. There is an equal probability that both isotopes will be incorporated into proteins and, in addition, if there is methylation, the radioactive donor will come from [*methyl*-^{3}H]methionine via *S*-adenosyl-methionine. Thus, if a protein is methylated, the ^{3}H/^{14}C ratio will be higher than in those that are unmethylated (14). Table 1 shows the ^{3}H/^{14}C ratios for all of the proteins from the 50S subunit of the 70S ribosome. There are five proteins (L7, L9, L11, L18, and L33) that have ^{3}H/^{14}C ratios significantly higher than that of the unfractionated total ribosomal proteins (which has a ratio of 4.12). The sensitivity of the double-labeling experiment is dependent upon the number of methionine residues in the protein: the lower the number, the higher the sensitivity. The reason for the high ^{3}H/^{14}C ratio in protein L33 is due to the fact that it contains no methionine residues (6); thus, any slight methylation would result in a high ^{3}H/^{14}C ratio. As expected from the results of Terhorst et al. (16, 17), protein L7 exhibits an elevated ^{3}H/^{14}C ratio. The lower-than-expected

TABLE 1. *Localization of ribosomal proteins, which are methylated, from the 50S subunit*[a]

Protein	^{14}C (counts/min)	^{3}H (counts/min)	^{3}H/^{14}C	Δ ^{3}H/^{14}C
L1	1,477	6,980	4.74	+0.62
L2	421	1,660	3.94	−0.18
L3	570	2,720	4.77	+0.65
L4	567	2,530	4.45	+0.33
L5	705	3,279	4.65	+0.53
L6	1,465	5,977	4.07	−0.05
L7	274	1,570	5.71	*+1.59*
L8	1,773	8,069	4.55	+0.43
L9	217	1,231	5.77	*+1.65*
L10	621	2,099	3.38	−0.74
L11	494	3,391	6.87	*+2.75*
L12 + S6	965	3,856	4.03	−0.09
L13	821	3,211	3.90	−0.22
L14	532	2,110	3.97	−0.15
L15	484	2,096	4.32	+0.20
L16	577	2,688	4.65	+0.53
L17	715	2,995	4.19	+0.07
L18	176	1,151	6.52	*+2.40*
L19	279	1,137	4.07	−0.05
L20	17	164		
L21	211	1,080	5.12	+1.00
L22	711	2,582	3.63	−0.49
L23	251	1,103	4.39	+0.27
L24	45	220		
L25	549	2,382	4.35	+0.23
L26 + S20	693	2,206	3.19	−0.93
L27	47	232		
L28	101	306	3.04	−1.08
L29	204	996	4.88	+0.76
L30	292	1,125	3.86	−0.26
L31	52	156		
L32	188	614	3.25	−0.87
L33	12	650	54?	*+50?*

[a] The ^{3}H/^{14}C ratio for the total unfractionated ribosomal protein was 4.12. Δ ^{3}H/^{14}C for any protein is ^{3}H/^{14}C − 4.12. Results for proteins in which methyl groups are present in excess of the methionine present are italicized.

$^3H/^{14}C$ value for protein L12, already known to be methylated (16, 17), was probably due to the fact that proteins L12 and S6 migrated together during two-dimensional polyacrylamide gel electrophoresis. Protein S6 contains approximately six methionine residues, whereas protein L12 contains three methionine residues (4, 6, 16, 17). Therefore, the contamination of protein L12 by protein S6 could significantly lower the $^3H/^{14}C$ ratio even though protein L12 may have a high $^3H/^{14}C$ ratio by itself. To clarify whether protein L12 is methylated, 50*S* ribosomal subunits were isolated from the 70*S* ribosome by sucrose gradient centrifugation in low-Mg^{2+} buffer (2) before the two-dimensional polyacrylamide gel electrophoresis. It was observed that both proteins L7 and L12 had nearly the same $^3H/^{14}C$ ratio, which was significantly higher than that of the unfractionated total 50*S* subunit proteins (F. N. Chang and C. N. Chang, unpublished data). This suggests that protein L12 is also methylated. Several other 50*S* proteins such as L1, L3, L5, L16, L21 and L29 also have significantly higher $^3H/^{14}C$ ratios, indicating they may also contain methylated amino acids.

Table 2 shows the $^3H/^{14}C$ ratios of proteins from the 30*S* subunit. With the possible exceptions of S10 and S16, none of these proteins has high $^3H/^{14}C$ ratio. It thus appears that methylated amino acids occur in several of the ribosomal proteins, being predominately (perhaps exclusively) localized in the 50*S* subunit proteins.

TABLE 2. *Localization of ribosomal proteins, which are methylated, from the 30S subunit*[a]

Protein	^{14}C (counts/min)	^3H (counts/min)	$^3H/^{14}C$	$\Delta\ ^3H/^{14}C$
S1	585	2,173	3.72	−0.40
S2	734	2,660	3.76	−0.36
S3	460	1,732	3.76	−0.36
S4	720	2,555	3.54	−0.58
S5	1,198	4,358	3.63	−0.49
S7	575	2,273	3.95	−0.17
S8	596	2,132	3.67	−0.45
S9	886	3,365	3.76	−0.36
S10	401	1,884	4.70	+0.58
S12	9	118		
S13	442	1,835	4.15	+0.03
S14	116	470	4.05	−0.07
S15	254	1,000	3.95	−0.17
S16	133	620	4.65	+0.53
S17	81	311	3.84	−0.28
S18	50	185		
S19	261	938	3.59	−0.53
S21	11	40		

[a] Conditions were the same as listed in Table 1, except that 30*S* proteins were used.

Analysis of methylated amino acids in protein L11. Of the five proteins that have high $^3H/^{14}C$ ratios (L7, L9, L11, L18, and L33), L11 was chosen for further analysis because it has the highest ^3H counts, indicating it may be heavily methylated. The methylated amino acids in protein L11 were analyzed by two separate methods. The first method involved the high-voltage paper electrophoresis supplemented with descending paper chromatography, and the second method involved the amino acid analyzer. Figure 1 shows the presence of methylated lysine and a very small amount of a compound with the same mobility as N^G,N'^G-dimethylarginine, as determined by the paper electrophoresis procedure. Since methylated lysines cannot be separated by the paper electrophoresis procedure, the descending paper chromatography procedure was used to identify the nature of the methylated lysine. Protein L11 contains predominately ε-trimethyllysine and a trace amount of ε-monomethyllysine (Fig. 2). The amount of ε-monomethyllysine seemed to vary from experiment to experiment, and certain preparations were devoid of this methylated amino acid. No ε-dimethyllysine was found. The presence of ε-trimethyllysine was verified by an amino acid analyzer (Fig. 3). (The amino acid analyzer also revealed the presence of several radioactive peaks in the region of neutral amino acids. However, the exact identity of these radioactive compounds has not been determined. These radioactive peaks could be due to the following compounds: methionine, methionine sulfoxide, methionine sulfone, incomplete hydrolyzed peptides containing methionine, or other unidentified methylated amino acid[s].) The other two methylated amino acids (N^G,N'^G-dimethylarginine and ε-monomethyllysine) were present in such small amounts that we were unable to confirm their identify by use of an amino acid analyzer. Because of its sensitivity, the combined paper electrophoresis-paper chromatography procedure is preferable for the detection of small amounts of methylated derivatives of lysine, arginine, or histidine. We have also not completely eliminated the possibility that the minor methylated amino acids were due to contamination by other neighboring proteins during the cutting out of protein L11 from the two-dimensional gel. This possibility, however, seems to be unlikely because we have also analyzed proteins L6 and S5, which migrated very close to protein L11, and found no N^G,N'^G-dimethylarginine

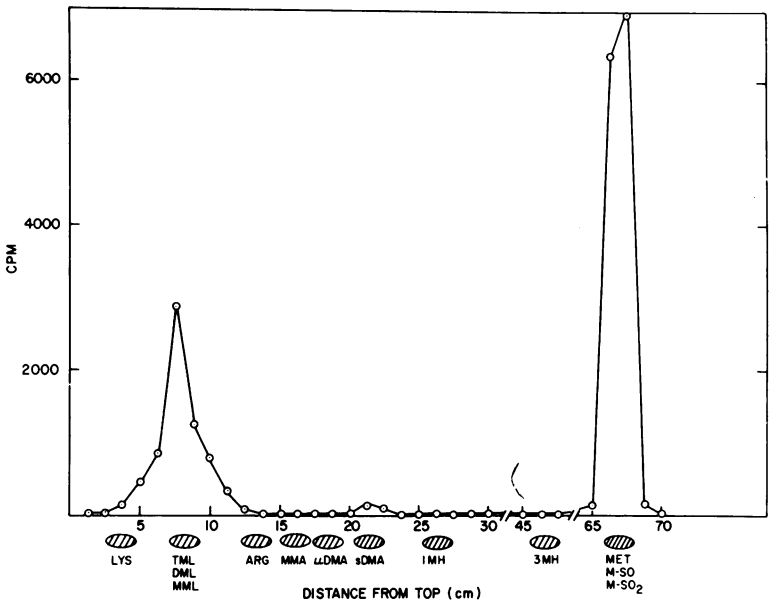

FIG. 1. *Analysis of methylated amino acids derived from E. coli protein L11 by paper electrophoresis. Protein L11 hydrolysate (20 μliters) was applied to Whatman 3 MM paper (20 by 110 cm), at 20 cm from one end, together with approximately 10 μg of each of the following methylated amino acids: ε-monomethyllysine (MML), ε-dimethyllysine (DML), ε-trimethyllysine (TML), monomethylarginine (MMA), N^G, N^G-dimethylarginine (uDMA), N^G, N'^G-dimethylarginine (sDMA), 1-methylhistidine (1-MH), and 3-methylhistidine (3-MH). The paper was subjected to electrophoresis for 3.5 h at 4,400 V in a buffer containing pyridine-acetic acid-water (25:1:225 by volume), pH 6.5. ε-Dinitrophenol-lysine was used as a marker for the electrophoresis. After electrophoresis, the paper was sprayed with 0.4% ninhydrin in acetone and heated to 70 C for 5 min to locate the methylated amino acids. The paper was then cut into 1.25-cm strips and counted in a toluene-based scintillation fluid (4 g of Omnifluor per liter of toluene). The two oxidized derivatives of methionine, methionine sulfoxide (M-SO) and methionine sulfone ($M\text{-}SO_2$), migrated together with methionine.*

was present (F. N. Chang and C. N. Chang, unpublished data).

Finally, since [*methyl*-^{14}C]methionine was used as a methyl donor, it was possible to determine the stoichiometry of ε-trimethyllysine in protein L11 from the published data on the mole percent methionine and the molecular weight of protein L11. Kaltschmidt et al. (6) have determined the mole percent of L11 and L6 to be 3.1%, and the molecular weight of L11 has been shown to be approximately 19,600 (4). Assuming that the methionine mole percent in protein L11 is 3.1%, L11 contains approximately five methionine residues. Since the ε-trimethyllysine peak accounts for approximately ⅓ of the total radioactivity (Fig. 1 and 2), L11 contains about 2.5 methyl groups (or 0.83 molecules of ε-trimethyllysine) per molecule of protein. The quantities of the other two minor methylated amino acids, if present, are much lower and amount to less than 0.04 molecules per L11.

DISCUSSIONS

Terhorst et al. (16, 17) have shown previously that two 50S ribosomal proteins (L7 and L12) from *E. coli* contain about 0.5 molecules of ε-monomethyllysine per molecule of protein. Based on the fact that most contractile proteins contain methylated amino acids (14) and the involvement of proteins L7 and L12 in the translocation step of peptide elongation, Terhorst et al. suggested that the presence of ε-monomethyllysine in L7 and L12 may be required for the translocation process (17). Our present data demonstrated that, in addition to proteins L7 and L12, several other 50S ribosomal proteins, such as L9, L11, L18 and L33, are also methylated (Table 1). Of these proteins, L11 is most heavily methylated. The methylated amino acids in protein L11 have been characterized and shown to contain approximately 0.83 molecules of ε-trimethyllysine and 0.04 molecules of a compound tentatively identified as N^G,N'^G-dimethylarginine per molecule of protein. It remains to be established whether the ε-trimethyl group is located only in one lysine residue or more than one lysine residue in protein L11 is trimethylated.

The specific trimethylation of protein L11 is of great interest. It has been suggested that

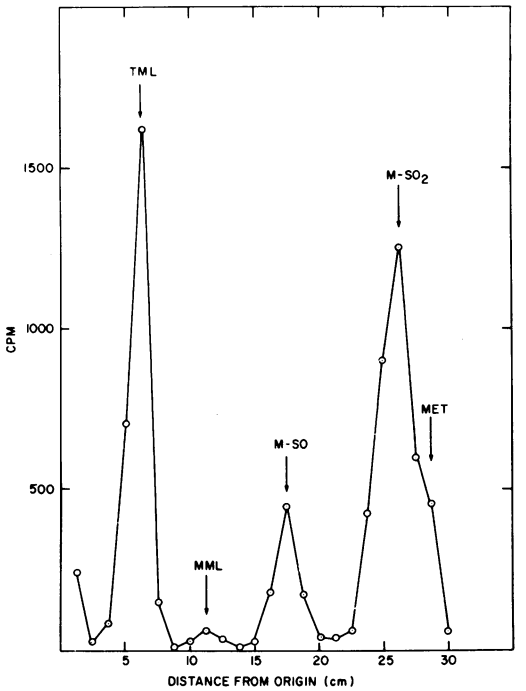

FIG. 2. *Analysis of methylated lysines derived from protein L11 by descending paper chromatography. Protein L11 hydrolysate (15 μliters) was applied to Whatman no. 1 paper, together with approximately 8 μg of each of the following amino acids (see the legend): MML, DML, TML, sDMA, Met, M-SO, and M-SO₂. The solvent system was pyridine-acetone-3M NH₄OH (50:30:25). Time of chromatography was 13 h. ε-Dinitrophenol-lysine was used as a marker. After chromatography, the paper was sprayed with ninhydrin and counted as described in the legend to Fig. 1.*

protein L11 is involved in the peptidyl transferase reaction during protein biosynthesis (10). It is possible that the ε-trimethyllysine residue is necessary for the peptidyl transferase reaction. The substitution of a trimethyl group into lysine(s) would result in the formation of a strongly basic quaternary ammonium ion $[-N(CH_3)_3]^+$, thus increasing the basicity of the lysine residue(s) (14). This increase in basicity may play an important role in the interaction of protein L11 with either the 23*S* ribosomal ribonucleic acid or other ribosomal protein(s) (or both). It also suggests that the lysine residue(s) that is trimethylated must be located on or near the surface of the protein so that this residue(s) is accessible to the methylation enzyme. In addition to an increase in basicity, the trimethylation of a lysine residue could also result in an increase in stereochemical disturbance of the protein molecule. This may lead to a change in the functional properties of the protein.

Since several other 50*S* proteins (e.g., L9, L18, L33, L1, L3, L5, etc.) are also methylated (Table 1), we are presently investigating the nature and stoichiometry of the methylated amino acids in each ribosomal protein. Our preliminary results indicate that these proteins contain considerably fewer methylated amino acids than does protein L11 (F. N. Chang and

N^G, N'^G-dimethylarginine migrated with methionine sulfoxide (M-SO) under the experimental conditions. ε-Dimethyllysine, if present, would migrate at 21 cm from origin.

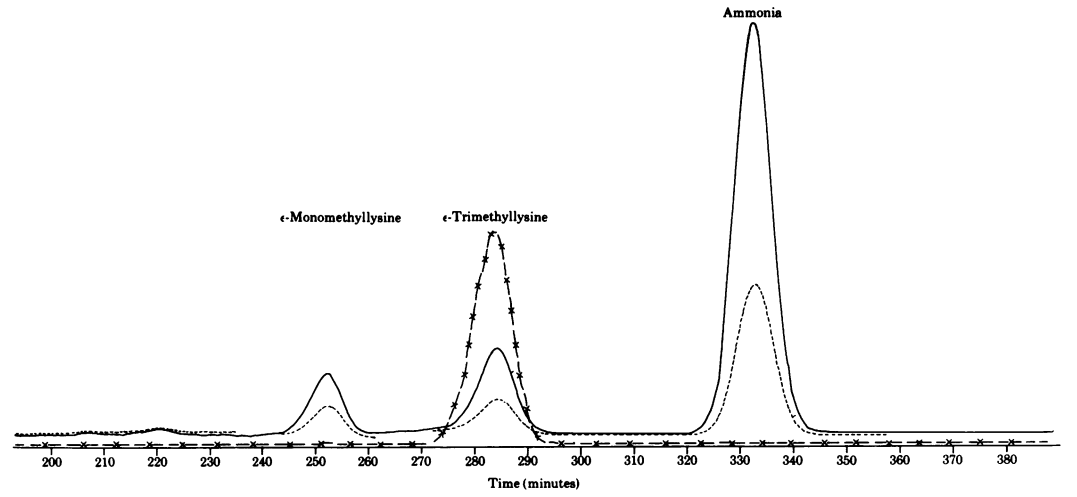

FIG. 3. *Identification of the presence of ε-trimethyllysine in protein L11 by the amino acid analyzer. The amino acid compositions of the hydrolysates were determined with a Perkin-Elmer automatic amino acid analyzer by the method of Paik and Kim (13). The eluate from the analyzer column was fed through a solid-state flow scintillation cell (Packard Tri-Carb liquid scintillation spectrometer, model 2002), and radioactivity was monitored (×). ε-Dimethyllysine, if present, would migrate at about 264 min.*

C. N. Chang, unpublished data). Although the biological significance of protein methylation is unclear at present, the post-translational modification of ribosomal proteins such as methylation may play an important role in both regulation of biosynthesis and function of ribosomes.

ACKNOWLEDGMENTS

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ADDENDUM IN PROOF

Alix and Hayes (J. Mol. Biol., **86**:139, 1974) have also observed the presence of nearly one molecule of ϵ-trimethyllysine in protein L11. Furthermore, they have detected an unidentified methylated neutral amino acid. We have also found this radioactive compound (except its stoichiometry is less than that observed by Alix and Hayes). The identity of this compound has to await further investigations.

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