Teresi, J. D., and Luck, J. M. (1952), *J. Biol. Chem. 194,* 823.
Tildon, J. T., and Ogilvie, J. W. (1969), 158th National Meeting of the American Chemical Society, New York, N.Y., Sept, Abstract BIOL-327. No. 327.
Tildon, J. T., and Ogilvie, J. W. (1972), *J. Biol. Chem. 247,* 1265.
Whitaker, J. R. (1962), *J. Am. Chem. Soc. 84,* 1900.

Purification and Properties of a Ribosomal Protein Methylase from *Escherichia coli* Q13†

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ABSTRACT: Ribosomal protein methylase has been purified from *Escherichia coli* strain Q13 using methyl-deficient 50S subunits as substrates. The purified enzyme (or enzyme complex) which is devoid of rRNA methylating activity is quite stable and has a pH optimum around 8.0. The K_m for *S*-adenosyl-L-methionine is 3.2 μM. The molecular weight of the enzyme is 3.1×10^4; minor methylating activity was also detected for protein peaks with molecular weights of 1.7×10^4 and 5.6×10^4. Protein L11 is the major protein methylated by the purified enzyme. Product analysis revealed the presence of N^ϵ-trimethyllysine, a methylated neutral amino acid(s) previously observed in protein L11 and N^ϵ-monomethyllysine. Free ribosomal proteins were much better substrates for the methylation, indicating that methylation of 50S ribosomal proteins can occur before the complete assembly of the 50S ribosomal subunit.

It has been shown previously that several 50S ribosomal proteins contain methylated amino acids (Alix and Hayes, 1974; Chang et al., 1974; Chang and Chang, 1975). Protein L11 is the most heavily methylated of all the 50S proteins and contains a total of approximately five methyl groups per molecule of protein (Chang and Chang, 1975; Alix and Hayes, 1974). Three of these methyl groups are located in a single N^ϵ-trimethyllysine molecule with the remainder in one or more neutral amino acid(s) (Alix and Hayes, 1974; Chang and Chang, 1975).

Undermethylated 50S subunits can be obtained from an *Escherichia coli rel*$^-$ *met*$^-$ strain after starvation for methionine. Such 50S subunits can be methylated in vitro using crude enzymes derived from a high salt wash of unstarved *E. coli* Q13 70S ribosomes (Chang and Chang, 1974). It has been observed that more than 80% of the methyl groups incorporated in vitro were located in protein L11, and that the product was predominately N^ϵ-trimethyllysine (Chang and Chang, 1974). Protein L11 has been implicated in both the peptidyl transferase reaction during protein biosynthesis (Nierhaus and Montejo, 1973; Hsiung et al., 1974) and the binding of 50S antibiotics chloramphenicol (Nierhaus, 1974) and thiostrepton (Highland et al., 1975). Although enzyme(s) responsible for the methylation of lysine residues of cellular proteins from different mammalian tissues have been purified and studied (for review see Paik and Kim, 1975), no similar studies on ribosomal protein methylases, to our knowledge, have been carried out. In an attempt to elucidate the function of the methylation of ribosomal proteins, we have partially purified and characterized some of the properties of the methylating enzyme (or enzyme complex).

Materials and Methods

Bacterial Strains. Escherichia coli strain 1500, a *rel*$^-$ mutant of a K-12 strain, was obtained from Coli Genetic Stock Center in New Haven, Conn. The *met B* marker was introduced (strain 1500 *met*$^-$) by P1 transduction from JC-355 which is a K-12 *met*$^-$ strain originating with A. J. Clark. *E. coli* Q13 and A19 cells were purchased from General Biochemicals, Inc.

Materials. [*methyl*-^{3}H]-*S*-Adenosyl-L-methionine ([^{3}H]Ado-Met),[1] specific activity 8.5 mCi/μmol, in 0.01 *N* H_2SO_4–ethanol (9:1) and [*methyl*-^{14}C]-*S*-adenosyl-L-methionine ([^{14}C]Ado-Met), specific activity 55 Ci/mol, were obtained from New England Nuclear and Amersham/Searle, respectively. In most experiments, [^{3}H]Ado-Met was diluted to a specific activity of 1.0 mCi/μmol before use. Bovine serum albumin, ovalbumin, myoglobin, β-lactoglobulin A, and unlabeled *S*-adenosyl-L-methionine were from Sigma Chemical Co. Ultra pure ammonium sulfate was obtained from Schwarz/Mann. Omniflour was purchased from New England Nuclear. Other reagents were obtained from Sigma or local sources.

Preparation of the Substrates. Methyl-deficient 50S subunits derived from *E. coli* strain 1500 *met*$^-$ were prepared as described previously (Chang and Chang, 1974), and used in most experiments. In some experiments, dissociated proteins from 50S subunits were used. Dissociated 50S proteins were obtained by treating 50S subunits with an equal volume of 4 *M* LiCl and 8 *M* urea for 16 hr at 0°, followed by centrifugation at 15,000 rpm for 20 min to remove rRNA which was pelleted.

Determination of Protein Concentration. Protein concentration was determined according to the procedure of Schaffner and Weissmann (1973) using bovine serum albu-

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[1] Abbreviations used are: EDTA, ethylenediaminetetraacetate; Ado-Met, *S*-adenosyl-L-methionine; rRNA, ribosomal RNA.

Table I: Purification of Protein Methylase from *E. coli*.

	Purification Steps	Volume (ml)	Protein		Enzyme Activity		Purification	Yield (%)
			mg/ml	Total (mg)	Specific Activity	Total Activity		
I	S-30	110	55	6×10^3	5.3	3.2×10^4	1.0	100
II	Ammonium sulfate precipitation	10.5	94	9.9×10^2	11.4	11.2×10^3	2.2	35
III	Ultracentrifugation supernatant	6.3	43	2.7×10^2	30	8.0×10^3	5.7	25
IV	DEAE-cellulose chromatography	25	0.13	3.2	2×10^3	6.4×10^3	380	20

min as a standard. This procedure is very sensitive and requires only microgram quantities of a protein.

Enzymatic Assay. The methylation of methyl-deficient 50S subunits was carried out in a final volume of 0.1 ml containing 0.05 *M* Tris-HCl (pH 7.8), 0.01 *M* $Mg(OAc)_2$, 0.01 *M* NaCN, 0.5 m*M* dithiothreitol, 0.02 *M* NH_4Cl, 0.3 m*M* EDTA, 0.75 A_{260} unit of *E. coli* strain 1500 *met*$^-$ 50S subunits, 4×10^{-6} *M* [^{3}H]Ado-Met, and 0.8 μg of purified enzyme (step IV). After incubation at 37° for 30 min, reactions were terminated by the addition of 2 ml of 10% cold Cl_3CCOOH. The reaction mixtures were then boiled at 100° for 20 min and chilled. This was followed by filtration onto Millipore filters, with the filters washed three times with 5 ml of 10% cold Cl_3CCOOH. The filters were dried and counted in 5 ml of scintillation cocktail (4 g of Omniflour/l. of toluene) with a scintillation counter having an efficiency of about 20%. The presence of rRNA methylases was also determined. The reaction mixtures were terminated by the addition of 10% cold Cl_3CCOOH and filtered as described above without the boiling step. The difference in counts between the cold and hot Cl_3CCOOH treatments was taken as rRNA methylase activity.

Estimation of Molecular Weight. Molecular weight of the purified protein methylase was estimated by the method of Andrews (1965) with a Sephadex G-100 column (1.0 × 65 cm). Blue Dextran was used to estimate the void volume of the column and myoglobin, ovalbumin, β-lactoglobulin A, and bovine serum albumin were used as standard proteins. Protein methylase activity was measured as described in the previous section.

Identification of the Methylated Ribosomal Proteins. The methylated ribosomal proteins were identified by both one-dimensional and two-dimensional polyacrylamide gel electrophoreses as described previously (Chang and Chang, 1974; Chang et al., 1974).

Characterization of the Methylated Amino Acids. Ribosomal proteins were prepared from methylated 50S subunits according to the procedure of Hardy et al. (1969). After hydrolysis of the proteins with redistilled 5.7 *N* HCl, the methylated amino acids were characterized by a combination of high-voltage paper electrophoresis at pH 9.3 and descending paper chromatography using a solvent system containing pyridine–acetone–3 *M* NH_4OH (10:6:5, v/v) as described previously (Chang et al., 1974; Chang and Chang, 1975).

Results

Purification of Ribosomal Protein Methylase. All operations were carried out in the cold (0–4°); 25 g of *E. coli* strain Q13 or A19 cells was thawed in 25 ml of a buffer containing 0.01 *M* Tris-HCl (pH 7.4), 0.01 *M* $MgCl_2$, 0.05 *M* KCl, 0.5 m*M* dithiothreitol, 0.3 m*M* EDTA (buffer I), and 3 μg/ml of DNase. The cells were disrupted with a French press and the resulting homogenate was incubated at 30° for 10 min, followed by centrifugation at 30,000*g* for 30 min in the cold. The supernatant solution (S-30) was diluted with buffer I to 110 ml (step I). Solid ammonium sulfate was added slowly with stirring to 33% saturation (21.3 g of ammonium sulfate) and the precipitate was discarded. To the supernatant fluid ammonium sulfate was again added to 60% saturation (19.4 g of additional ammonium sulfate) and the precipitate was recovered by centrifugation. The precipitate was dissolved in 10 ml of buffer I (final volume, 18 ml), 2.4 g of solid ammonium sulfate was added, and the solution was gently stirred for 16 hr. The resulting solution was centrifuged at 150,000*g* for 5 hr to pellet the ribosomes. To the supernatant fluid (16 ml) ammonium sulfate was added to 80% saturation (8.8 g of ammonium sulfate) and the precipitate was dissolved in 6 ml of buffer I, followed by dialysis overnight against 1.5 l. of a buffer containing 0.01 *M* Tris-HCl (pH 7.4), 0.1 *M* KCl, 0.5 m*M* dithiothreitol, and 0.3 m*M* EDTA (buffer II; step III); 2.5 ml of the dialyzed solution was applied to a DEAE-cellulose column (1.6 × 30 cm) that had been previously equilibrated with the same buffer. Elution was carried out with buffer II at a flow rate of 20 ml/hr and 2.5-ml fractions were collected (Figure 1). After most of the A_{280} absorbing material was eluted, the KCl concentration was raised to 0.15 *M* and elution started with a linear concentration gradient to 0.6 *M* KCl in buffer II. Fractions 65–70 were pooled and stored frozen at −70° (step IV). The en-

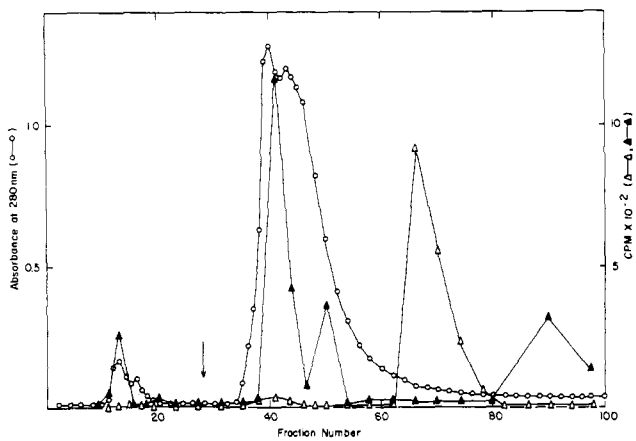

FIGURE 1: DEAE-cellulose chromatography of protein methylase. Detailed descriptions are given in the text; the arrow indicates the change of KCl concentration from 0.1 to 0.15 *M* and the start of a linear concentration gradient to 0.6 *M*. A total of 225 fractions (2.5 ml/fraction) were collected, and only the first 100 fractions were shown here. No activity was detected in the remaining fractions; 20 μl from each fraction was assayed for protein methylase activity according to the hot Cl_3CCOOH procedure described under Materials and Methods (△). rRNA methylating activity (▲) was also measured as described under Materials and Methods.

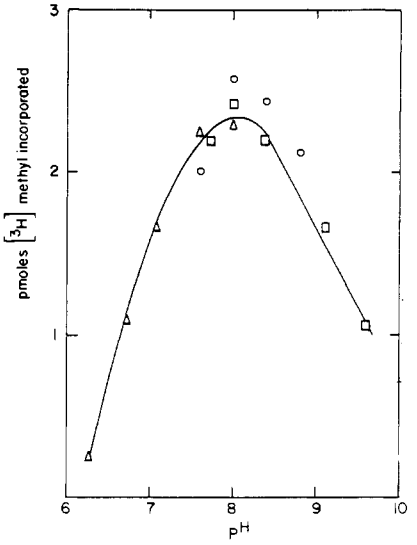

FIGURE 2: Effect of pH on the protein methylase activity. Potassium phosphate buffer (0.05 *M*) was used for the range of pH 6.2–8.0; 0.05 *M* Tris-HCl buffer, for the range pH 7.6–8.8, and 0.05 *M* borate buffer for pH 7.7–9.6. The assay was performed as described under Materials and Methods.

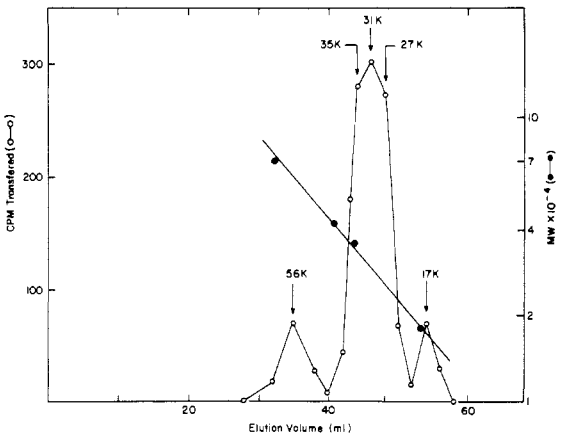

FIGURE 3: Determination of the molecular weight of the protein methylase by Sephadex G-100. The calibration curve for the determination of molecular weights (●) was described under Materials and Methods. Each fraction collected contained 1.0 ml. All proteins except myoglobin were assayed at A_{230}. Myoglobin was determined at A_{410}. After the calibration of the Sephadex G-100 column (1.0 × 65 cm), 0.4 ml of the purified enzyme (step IV) was applied to the column and 1.0-ml fractions were collected. The void volume was 27 ml; 40-μl fractions were used for assaying the enzyme activity following the procedure described under Materials and Methods (○).

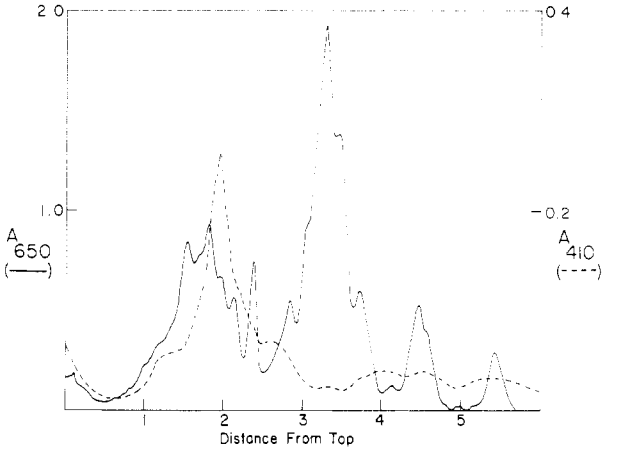

FIGURE 4: Localization of the methylated proteins by one-dimensional polyacrylamide gel electrophoresis. The methylation of 50S subunits was carried out in a final volume of 0.3 ml containing 0.05 *M* Tris-HCl (pH 7.8), 0.01 *M* $Mg(OAc)_2$, 0.01 *M* NaCN, 0.5 m*M* dithiothreitol, 0.02 *M* NH_4Cl, 0.3 m*M* EDTA, 5 A_{260} units of *E. coli 1500 met*⁻ 50S subunits, 1×10^{-5} *M* of [^{14}C]Ado-Met and 4.0 μg of purified enzymes. After incubating at 37° for 30 min the reaction mixture was terminated by dilution to 2 ml using the same buffer; 1.3 ml of cold ethanol was added to precipitate the 50S ribosomal subunits. Ribosomal proteins were extracted by the acetic acid procedure of Hardy et al. (1969). The procedures for the one-dimensional polyacrylamide gel electrophoresis and autoradiography of the sliced gel were the same as described previously (Chang and Chang, 1974).

zyme was purified 380-fold with a 20% yield (Table I). No detectable rRNA methylating activity was observed in the pooled fractions. There are at least four rRNA methylating peaks present and we have not carried out further studies for these rRNA methylases (Figure 1).

Properties of the Purified Protein Methylase. Protein methylase activity is proportional to the amount of purified enzyme used and the reaction rate with the enzyme is linear up to 30 min incubation at 37°. The purified enzyme has a pH optimum between 7.8 and 8.2 (Figure 2). A Lineweaver–Burk plot of the reaction kinetics for *S*-adenosyl-L-methionine gave a K_m value of 3.2 μ*M*, and a V_{max} of 2.7 pmol/30 min per 0.8 μg of enzyme protein.

The molecular weight of the enzyme was determined by Sephadex G-100 chromatography. A calibration curve for the Sephadex G-100 column was obtained by plotting the elution volume of the reference proteins against the logarithms of molecular weights (Figure 3). The elution volume of the major protein methylase peak corresponded to a molecular weight of 3.1×10^4. Minor methylating activity was also observed with molecular weights of 1.7×10^4 and 5.6×10^4. The purified enzyme is stable when stored at −70° for at least 1 year.

Specificity of the Reaction. The methylation reaction is dependent upon 50S subunits which had previously been starved for methionine. Fully methylated 50S subunits from unstarved *E. coli* 1500 *met*⁻ show less than 10% methylation. A very small amount of methylation (12% of control) was also observed with starved *E. coli* 1500 *met*⁻ 30S subunit. This is most likely due to the contamination by the 50S subunit.

Free ribosomal proteins derived from the 1500 *met*⁻ 50S subunit were much better substrates for the purified methylase (4.1 and 1.8 pmol of methyl groups incorporated for free ribosomal proteins and 50S subunits, respectively, under the standard assay condition). This indicates that the methylation of ribosomal proteins can take place before the final assembly of the 50S subunit.

Nature of the Methylated Products. The major methylated protein when 1500 *met*⁻ 50S subunits were used as substrates was identified by one-dimensional polyacrylamide gel electrophoresis (Figure 4). This protein is the same one which was previously identified to be protein L11 when the unfractionated ribosome wash was used as an enzyme source (Chang and Chang, 1974). Two-dimensional polyacrylamide gel electrophoresis revealed that 85% of the total radioactivity was in protein L11 with the remainder distributed primarily in proteins L5, L3, and L1 (data not shown).

The methylated amino acids, using as enzyme sources both unfractionated ribosome wash (step III, Table I) and DEAE-cellulose fractionated enzymes (step IV), were compared by descending paper chromatography using a solvent

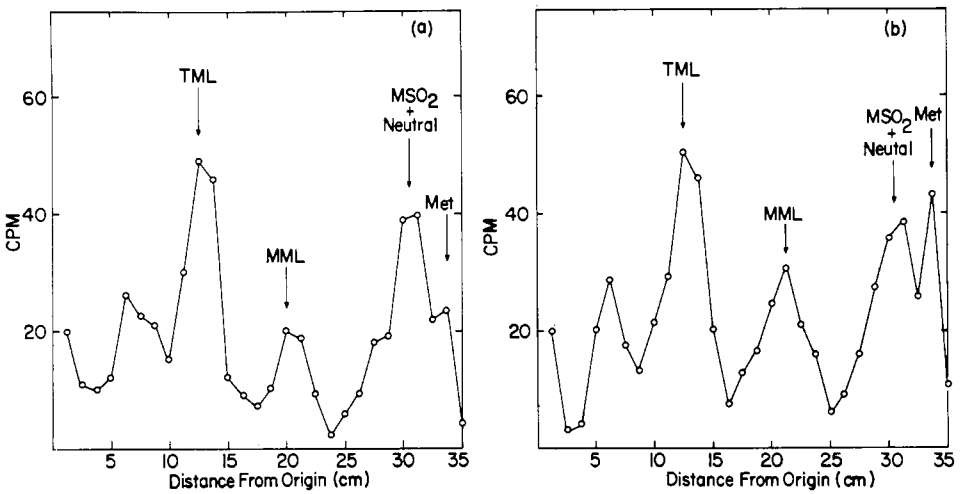

FIGURE 5: Comparison of the methylated amino acids from enzymes derived from steps III and IV. The procedures were the same as described in the legend of Figure 4 except 2 A_{260} units of 1500 *met*$^-$ 50S subunits was used as substrates and 4 × 10^{-6} *M* of undiluted [^{3}H]-Ado-Met and 3.0 μg of either step III enzyme (a) or step IV enzyme (b) was used. After isolation of the ribosomal proteins, they were hydrolyzed with redistilled 5.7 *N* HCl in sealed test tubes at 110° for 22 hr as described under Materials and Methods. Descending paper chromatography of the methylated amino acids was carried out in a solvent system containing pyridine–acetone–3 *M* NH_4OH (10:6:5, v/v) for 16 hr as described before (Chang et al., 1974). After chromatography, the paper was cut into 1-cm strips and counted as described under Materials and Methods. Neutral indicates the unidentified methylated neutral amino acid found in protein L11 (see Figure 6).

system containing pyridine–acetone–3 *M* NH_4OH (10:6:5, v/v) which separates all three methylated lysines (Chang et al., 1974; Chang and Chang, 1975). Figure 5 shows that both enzyme preparations gave similar product profiles with N^ϵ-trimethyllysine as the predominant product. The purified enzyme (Figure 5b) forms slightly more N^ϵ-monomethyllysine when compared to that with the unfractionated enzyme (Figure 5a). A methylated neutral amino acid(s) which had previously been detected in protein L11 was also observed with the purified enzyme by electrophoresis in sodium borate buffer at pH 9.3 (Figure 6; Alix and Hayes, 1974; Chang and Chang, 1975).

Both one-dimensional and two-dimensional polyacrylamide gel electrophoreses were also used to identify the methylated proteins when dissociated 50S proteins (obtained from LiCl–urea treatment of 50S subunits) were used as substrates. The results indicated that 88% of the total radioactivity was in protein L11 with the remainder again distributed primarily in proteins L5, L3, and L1 (data not shown).

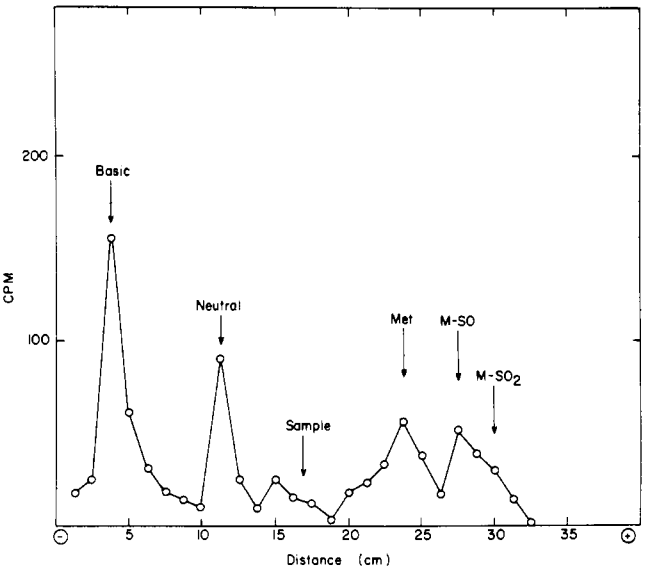

FIGURE 6: Identification of the methylated neutral amino acid(s) using purified enzyme. The procedure was the same as described in the legend of Figure 5 except 2 A_{260} units of 1500 *met*$^-$ 50S subunits, 5 × 10^{-6} *M* [^{14}C]Ado-Met and 3.0 μg of the purified enzyme were used. After isolation of the ribosomal proteins, they were hydrolyzed with redistilled 5.7 *N* HCl in sealed test tubes at 110° for 24 hr as described under Materials and Methods. Methylated amino acids were separated by high voltage paper electrophoresis in 0.05 *M* sodium borate buffer (pH 9.3) at 2000 V for 65 min as described previously (Chang and Chang, 1975). After electrophoresis, the paper was cut into 1-cm strips and counted.

Discussion

Ribosomal protein methylase has been purified from *E. coli* Q13 using methyl-deficient 50S particles from an *rel*$^-$ *met*$^-$ strain as substrates. Product analysis of the methylated proteins revealed the presence of N^ϵ-trimethyllysine, the methylated neutral amino acid(s), and N^ϵ-monomethyllysine (Figures 5 and 6). The relative amount of N^ϵ-monomethyllysine formed seemed to increase with the enzyme purification (compare Figure 5a and b). This may indicate that the formation of N^ϵ-trimethyllysine requires an enzyme complex composed of more than one enzyme (e.g., trimethylation of a lysine residue could conceivably involve three enzymes in a complex with each enzyme involved in the sequential addition of one of the methyl groups. Purification of the enzyme may cause a partial inactivation or loss of one (or more) of the enzymes in the complex resulting in the formation of more N^ϵ-monomethyllysine.

The purified enzyme (or enzyme complex) which is devoid of any rRNA methylating activity has a molecular weight of 3.1 × 10^4. Minor methylating activity was also observed with molecular weights of 1.7 × 10^4 and 5.6 × 10^4 (Figure 3). It is uncertain whether they are different methylating activities. Since more than one amino acid is methylated (lysine, a neutral amino acid, and maybe other amino acid(s) as well), it is also not clear whether the 3.1 × 10^4 protein peak contains more than one protein. Further purifications will be necessary. A rough estimation of the

molecular weight of the methylating enzyme has also been carried out by Alix and Hayes (1974). They used sucrose gradient and found the molecular weight of the enzyme to be approximately 4×10^4.

The observation by Alix and Hayes (1974) that ethionine-treated free 50S ribosomal proteins can be methylated in vitro by a crude enzyme preparation has been confirmed and extended to the purified enzyme in our present study using methyl-deficient 50S ribosomal proteins. Furthermore, free ribosomal proteins were shown to be far better substrates for the methylation than the 50S particles. Protein L11 is the major methylated protein when either methyl-deficient 50S subunits or free ribosomal proteins derived from them were used as substrates. This suggests that at least some methylation can take place after (or during) the synthesis of these ribosomal proteins in vivo, but before their assembly into the 50S subunit. In this regard, it should be pointed out that another type of post-translational modification in *E. coli* ribosomal protein, namely, acetylation of protein L12 (to form L7), can occur in the free protein state (Brot and Weissbach, 1972).

As indicated in the introduction, protein L11 has been implicated in the peptidyl transferase reaction during protein biosynthesis (Nierhaus and Montejo, 1973; Hsiung et al., 1974). This has recently been challenged by other workers (Ballesta and Vazquez, 1974; Howard and Gordon, 1974; Moore et al., 1975). The function of N^{ϵ}-trimethyllysine in protein L11 is not clear at present. It may either be involved in the assembly of late proteins or in the functioning of the fully assembled 50S subunit (or both). It would be interesting to isolate mutants which are deficient in the methylation of ribosomal proteins. Since protein L11 has been shown to be involved in the binding of 50S antibiotics chloramphenicol (Nierhaus, 1974) and thiostrepton (Highland et al., 1975), mutants resistant to either antibiotic might provide good candidates for investigation. The successful in vitro assembly of 50S ribosomal subunit has also been reported (for most recent reconstitution see Nierhaus and Dohme, 1974). The purified enzyme should aid the elucidation of the function of protein methylations.

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References

Alix, J. H., and Hayes, D. (1974), *J. Mol. Biol. 86,* 139–159.

Andrews, P. (1965), *Biochem. J. 96,* 595–605.

Ballesta, J. P. G., and Vazquez, D. (1974), *FEBS Lett. 48,* 266–270.

Brot, N., and Weissbach, H. (1972), *Biochem. Biophys. Res. Commun. 49,* 673–679.

Chang, C. N., and Chang, F. N. (1974), *Nature (London) 251,* 731–733.

Chang, C. N., and Chang, F. N. (1975), *Biochemistry 14,* 468–477.

Chang, F. N., Chang, C. N., and Paik, W. K. (1974), *J. Bacteriol. 120,* 651–656.

Hardy, S. J. S., Kurland, C. G., Voynow, P., and Mora, G. (1969), *Biochemistry 8,* 2897–2905.

Highland, J. H., Howard, G. A., Ochsner, E., Stöffler, G., Hasenbank, R., and Gordon, J. (1975), *J. Biol. Chem. 250,* 1141–1145.

Howard, G. A., and Gordon, J. (1974), *FEBS Lett. 48,* 271–274.

Hsiung, N., Reines, S. A., and Cantor, C. R. (1974), *J. Mol. Biol. 88,* 841–855.

Moore, V. G., Atchison, R. E., Thomas, G., Moran, M., and Noller, H. F. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 844–848.

Nierhaus, K. H. (1974), *Nature (London) 250,* 379–380.

Nierhaus, K. H., and Dohme, F. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 4713–4717.

Nierhaus, K. H., and Montejo, V. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 1931–1935.

Paik, W. K., and Kim, S. (1975), *Adv. Enzymol. 42,* 227–286.

Schaffner, W., and Weissmann, C. (1973), *Anal. Biochem. 56,* 502–514.