BIOSYNTHESIS OF 5-METHYLAMINOMETHYL-2-THIOURIDYLATE. I. ISOLATION OF A NEW tRNA-METHYLASE SPECIFIC FOR 5-METHYLAMINOMETHYL-2-THIOURIDYLATE

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Received March 2, 1973

SUMMARY: A new enzyme, which catalyzes the transfer of a methyl group to tRNA to form 5-methylaminomethyl-2-thiouridylate, was isolated from E. coli by a procedure including affinity chromatography. The purified enzyme was nearly homogeneous upon disc electrophoresis. Using methyl-deficient $tRNA^{Glu}$ of E. coli as substrate, the 5-methylaminomethyl-2-thiouridylate residue synthesized was mostly found in the anticodon loop, showing a coincidence of the modification site in vitro with that in vivo.

5-Methylaminomethyl-2-thiouridylate (hereafter referred to as mam^5s^2Up) is a minor component of tRNA and its structure was determined by Carbon et al. (1). It was found that this minor component is located in the first position of the anticodon of E. coli $tRNA_2^{Glu}$ (2,3). This minor component may be important not only in recognition of the codon but also in recognition of aminoacyl-tRNA-synthetase (4,5). We studied the biosynthesis of this minor component in order to elucidate its function in tRNA. This paper describes the isolation of a new tRNA-methylase that is specific for the terminal methyl group of mam^5s^2Up.

MATERIALS AND METHODS

Preparation of methyl-deficient $tRNA^{Glu}$. Methyl-deficient tRNA was prepared from E. coli 58-161 (rel^-, met^-) as described by Biezunski et al. (6). Methyl-deficient $tRNA^{Glu}$ was purified from this methyl-deficient tRNA by DEAE-Sephadex A-50 column chromatography at pH 4.0. The purified methyl-deficient $tRNA^{Glu}$ was at least 95 % pure judging from its acceptor activity for glutamic acid.

Assay of tRNA-methylase activity. The reaction mixture contained 5 μmoles of Tris-HCl (pH 8.3), 1 μmole of $MgCl_2$, 0.5 μmole of ATP, 0.6 μmole of 2-mercaptoethanol, 1 mμmole of ^{14}C-methyl-labeled S-adenosylmethionine (specific activity, 50 μCi/μmole), 0.09 OD unit of methyl deficient $tRNA^{Glu}$, and an appropriate amount of methylase in a final volume of 100 μl. After incubation at 37°C for 1hr, 50 μl of the reaction mixture was applied to a filter paper disc. Discs were washed and their radioactivity was counted as described previously (7).

Analysis of the reaction products. ^{14}C-Methylated tRNA was prepared from 200 µl of reaction mixture as described previously (8), and after complete digestion with RNase T_2, it was subjected to thin-layer chromatography using the method described previously (9). For analysis of the exact location of the methylated nucleotide in methyl deficient $tRNA^{Glu}$, 19 OD units of *E. coli* B $tRNA_2^{Glu}$, purified as described previously (3), were added to reaction mixture immediately before phenol treatment and completely digested with RNase T_1. The digest was fractionated by DEAE-Sephadex A-25 column chromatography.

Disc-electrophoresis. The purity of the tRNA-methylase was analyzed by polyacrylamide gel electrophoresis under the conditions described by Davis (10).

RESULTS

Methyl-deficient $tRNA^{Glu}$, the substrate for mam^5s^2U-methylase,* was purified as shown in Fig. 1. For isolation of mam^5s^2U-methylase, 40 g of wet

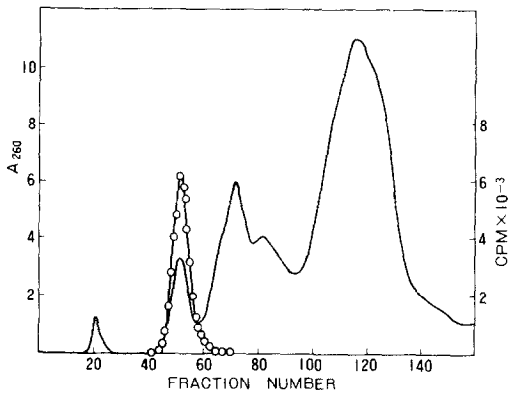

Fig. 1. DEAE-Sephadex A-50 column chromatography of methyl-deficient tRNA. The chromatographic conditions are as described in reference (13). ——, A_{260}; -o-o-, glutamic acid-acceptor activity.

E. coli B cells, harvested in the late log phase, were extracted with buffer A (0.05 M Tris-HCl, pH 8.0, 10 mM $Mg(CH_3COO)_2$, 1 mM EDTA, 6 mM 2-mercaptoethanol, 0.06 M KCl and 10 % glycerol). The extract (S-100) was subjected to DEAE-cellulose column chromatography, and four peaks of tRNA-methylase activity were obtained (Fig. 2). The products of the reactions of these tRNA-

*Abbreviation: mam^5s^2U-methylase, tRNA-methylase specific for 5-methylaminomethyl-2-thiouridylate.

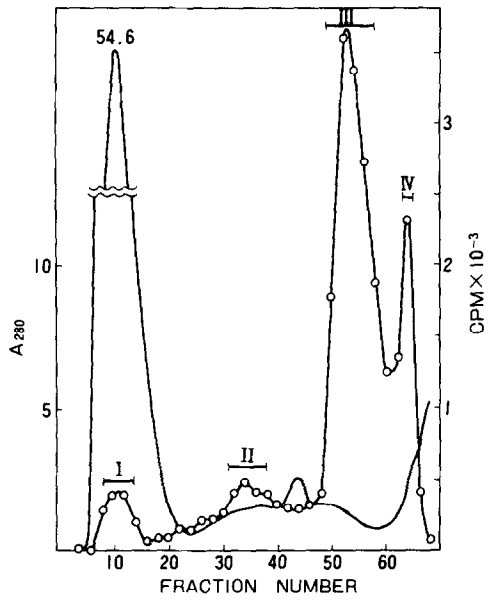

Fig. 2. DEAE-Cellulose column chromatography of crude extracts of E. coli. Fraction S-100 (70 ml) was applied to a column equilibrated with buffer A, and washed with 130 ml of buffer A. Then elution was performed with a linear NaCl gradient obtained with 200 ml of buffer A and 200 ml of buffer B (buffer A plus 0.3 M NaCl). Column size, 1.9 x 20 cm; flow rate, 30 ml/hr; fraction size, 10 ml; ———, A_{280}; —o—o—, methylase activity.

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methylases were digested completely with RNase T_2 and analyzed. Only one spot was detected in the case of Peak II (Fig. 3). This radioactive spot was located in the same area as authentic mam^5s^2Up (2), indicating that the final methylated product of the methylase in Peak II is mam^5s^2Up. Peak III and IV both yielded mainly ribothymidylate (not shown). After precipitation with ammonium sulfate the methylase in Peak II was dissolved in 4 ml of buffer C (0.02 M potassium phosphate, pH 6.5, 10 mM $MgCl_2$, 1 mM EDTA, 6 mM 2-mercapto-ethanol and 10 % glycerol), and dialyzed against the same buffer. Half the dialyzate was subjected to affinity chromatography on a column of Sepharose 4B bound with E. coli normal $tRNA^{Glu}$ (purified by the method described for methyl-deficient $tRNA^{Glu}$). $tRNA^{Glu}$-Sepharose was prepared as described by Remy et al. (11). As shown in Fig. 4, most of the protein, containing only a little methylase activity, emerged as a single peak with buffer C.

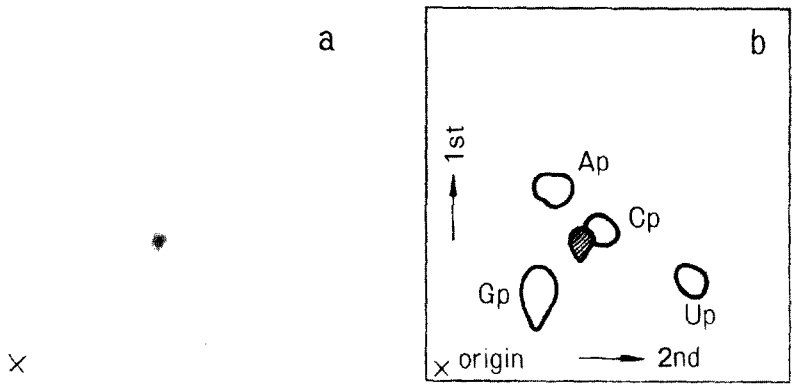

Fig. 3. Autoradiogram of a thin-layer chromatogram of the RNase T_2 digest. (a) Autoradiogram of the chromatogram. (b) Composite tracing of (a) in relation to the locations of the 4 normal nucleotides.

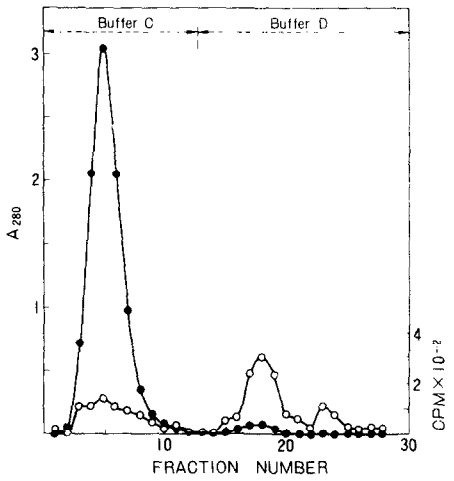

Fig. 4. Elution pattern from a $tRNA^{Glu}$-Sepharose column. 200 OD units of *E. coli* $tRNA^{Glu}$ were bound to the column; column size, 1.8 x 5 cm; fraction size, 3 ml; —●—●—, A_{280}; —o—o—, methylase activity.

On changing to buffer D (0.1 M Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 1 mM EDTA, 6 mM 2-mercaptoethanol, 10 % glycerol and 0.2 M KCl), a small peak of protein was eluted with most of the methylase activity. The peak fractions 17-19 were pooled and concentrated. The protein recovered was analyzed by polyacrylamide gel electrophoresis (Fig. 5). As can be seen, it gave a single strong band, with a few faint bands.

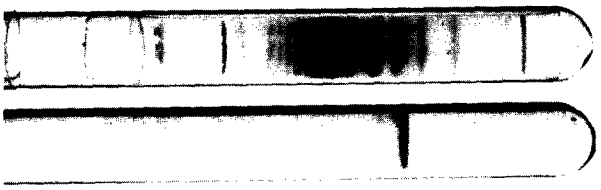

Fig. 5. Enzyme purity at the purification steps of tRNA-methylase as monitored by polyacrylamide gel electrophoresis. Upper: Peak II from the DEAE-cellulose column. Lower: Fraction No. 17-19 from the $tRNA^{Glu}$-Sepharose column.

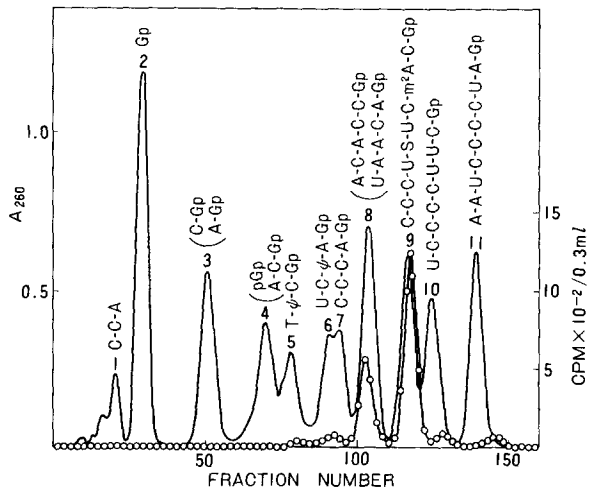

Fig. 6. DEAE-Sephadex A-25 column chromatography of a RNase T_1 digest of $tRNA^{Glu}$. Column size, 0.3 x 50 cm; the mixing chamber contained 70 ml of 0.02 M Tris-HCl (pH 7.5)-0.14 M NaCl-7 M urea and the reservoir 70 ml of 0.02 M Tris-HCl (pH 7.5)-0.7 M NaCl-7 M urea; fraction size, 0.5 ml; ———, A_{260}; -o-o-, radioactivity. "S" represents mam^5s^2U.

The product of the reaction of this enzyme was identified in two ways. First, on complete digestion with RNase T_2, mam^5s^2Up was obtained as the only ^{14}C-methyl labeled product. Second, on complete digestion with RNase T_1 (Fig. 6), two-thirds of the radioactivity was found in the region of

Peak 9, derived from the anticodon region of E. coli $tRNA_2^{Glu}$ and containing mam^5s^2Up (3 and Z. Ohashi, F. Harada and S. Nishimura, unpublished results). However, about one-third was eluted in the region of Peak 8. This result is discussed later.

DISCUSSION

Our results show the existence of a new tRNA-methylase which specifically synthesizes mam^5s^2Up. The precursor of mam^5s^2U is provably 5-aminomethyl-2-thiouridine lacking the terminal methyl group, although there is still no direct evidence for this.

Hurwitz et al. (12) reported the partial purification of several tRNA-methylases from E. coli, including a "cytosine-methylating enzyme". Their characterization of the "cytosine-methylating enzyme", however, was based on rather incomplete identification of the methylated product as 5-methylcytosine. In addition, no 5-methylcytosine has been detected in the sequences of about 20 E. coli tRNAs studied so far, or in unfractionated E. coli tRNA. It is possible that their "cytosine-methylating enzyme" is in fact the same enzyme as our mam^5s^2U-methylase. The presence of two oligonucleotides containing radioactive mam^5s^2U shown in Fig. 6 is provably due to the occurrence of two kinds of methyl-deficient $tRNA^{Glu}$. It is known that there are two species in E. coli B $tRNA^{Glu}$ (13) and both seem to contain mam^5s^2Up (Z. Ohashi and S. Nishimura, unpublished results). Therefore, it seems that the radioactivity in Peak 8 was from the minor component of methyl-deficient $tRNA^{Glu}$. The slight difference between the elution positions of Peak 9 and the major radioactive peak may be due to the absence of the 2-methyl group of 2-methyl adenylate in methyl-deficient $tRNA^{Glu}$.

Our results indicate that affinity chromatography using a tRNA-Sepharose column is very satisfactory for purification of enzymes participating in the biosynthesis of other minor bases in tRNA as well as for the purification of aminoacyl-tRNA synthetase as reported by Remy et al. (11).

Studies are in progress to characterize mam^5s^2U-methylase and elucidate the overall mechanism of biosynthesis of mam^5s^2Up.

ACKNOWLEDGEMENTS

We would like to thank the members of the Laboratories of Kaken Chemicals for large scale culture of E. coli B. Thanks are also due to Drs. Y. Kaziro and Y. Sokawa of the University of Tokyo for a gift of E. coli 58-161.

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