ENZYMATIC SYNTHESIS OF 3-(3-AMINO-3-CARBOXYPROPYL)URIDINE IN ESCHERICHIA COLI PHENYLALANINE TRANSFER RNA: TRANSFER OF THE 3-AMINO-3-CARBOXYPROPYL GROUP FROM S-ADENOSYLMETHIONINE

Susumu Nishimura, Yoichi Taya, Yoshiyuki Kuchino and Ziro Ohashi

Biology Division, National Cancer Center Research Institute,
Tsukiji 5-1, Chuo-ku, Tokyo (Japan)

Received February 12,1974

SUMMARY: A novel enzyme which catalyzes the transfer of the 3-amino-3-carboxypropyl group into tRNA to form 3-(3-amino-3-carboxypropyl)uridine was isolated from Escherichia coli. The enzyme required S-adenosylmethionine as donor molecule.

A modified nucleoside, designated as X, is known to be located in the extra-region of several Escherichia coli tRNAs [for examples see review by Zachau (1)]. This hitherto unknown modified nucleoside has recently been characterized as 3-(3-amino-3-carboxypropyl)uridine (2). This communication reports the enzymatic synthesis of this modified nucleoside in a cell-free system from E. coli using purified methyl-deficient $tRNA^{Phe}$ as acceptor and S-adenosylmethionine as donor molecule for the 3-amino-3-carboxypropyl group.

MATERIALS AND METHODS

Preparation of methyl-deficient $tRNA^{Phe}$. Unfractionated methyl-deficient tRNA was prepared from E. coli 58-161 (rel^-, met^-) as described by Biezunski et al. (3). It was first fractionated by DEAE-Sephadex A-50 column chromatography at pH 4.0 as shown in Fig. 1. Phenylalanine acceptor activity was separated into two peaks. The second major peak (fractions 122 to 135 of Fig. 1), which was not detected in the chromatogram of normal unfractionated E. coli tRNA was further subjected to benzoylated DEAE-cellulose column chromatography. As shown in Fig. 2, methyl-deficient $tRNA^{Phe}$ was eluted as the last peak and separated from other tRNAs. Fractions from 35 to 45 were pooled, and precipitated by adding 2.5 volumes of ethanol. This purified methyl-deficient $tRNA^{Phe}$ accepts 1.2 mμmoles of ^{14}C-phenylalanine per 1 A_{260} unit of tRNA when crude mixture of E. coli aminoacyl-tRNA synthetases was used (4). Analysis of an RNase T_2 digest of purified methyl-deficient $tRNA^{Phe}$ by two-dimensional thin-layer chromatography showed that it lacked 3-(3-amino-3-carboxypropyl)uridine as well as almost all the 7-methylguanosine and ribothymidine present in normal $tRNA^{Phe}$.

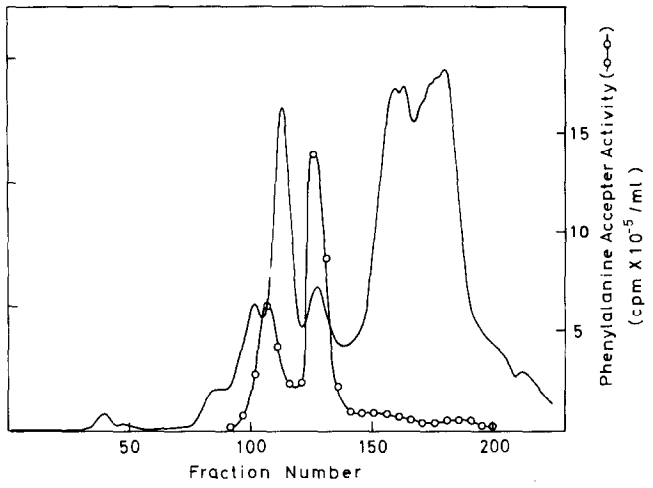

Fig. 1. Fractionation of methyl-deficient tRNA from E. coli by DEAE-Sephadex A-50 column chromatography at pH 4.0.
Methyl-deficient tRNA (30200 A_{260} units) was dissolved in 100 ml of 0.02 M sodium acetate buffer (pH 4.0) - 0.01 M MgCl - 0.4 M NaCl, and loaded on a column (diameter, 2.5 cm; length, 90 cm; DEAE-Sephadex A-50, product of Pharmacia Fine Chemicals) which was preequilibrated with the same buffer. The linear gradient elution was carried out using 1.6 l of 0.02 M sodium acetate buffer (pH 4.0) - 0.01 M $MgCl_2$ - 0.5 M NaCl in the mixing chamber, and 1.6 l of 0.02 M sodium acetate buffer (pH 4.0) - 0.01 M $MgCl_2$ - 0.7 M NaCl in the reservoir. The flow rate was 30 ml/hr. Each fraction contained 15 ml of the effluent.

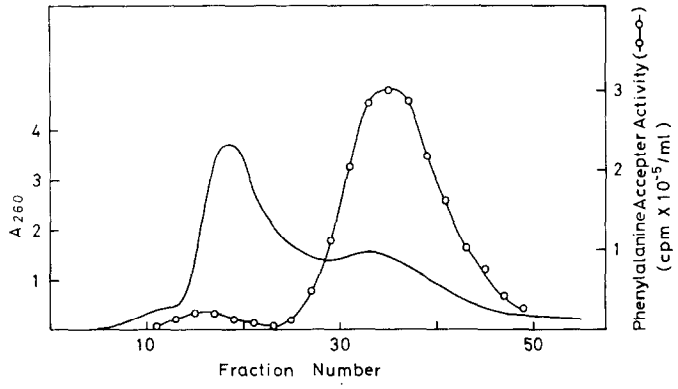

Fig. 2. Isolation of methyl-deficient $tRNA^{Phe}$ by benzoylated DEAE-cellulose column chromatography.
The tRNA fractions (750 A_{260} units) dissolved in 4 ml of water was mixed with 1 ml of 0.02 M sodium acetate buffer (pH 6.0) - 0.4 M NaCl, and loaded on a column (diameter, 1 cm; length, 80 cm; BD-cellulose, product of Boehringer Mannheim). The linear gradient elution was performed using 500 ml of 0.02 M sodium acetate buffer (pH 6.0) - 0.5 M NaCl in the mixing chamber, and 500 ml of 0.02 M sodium acetate buffer (pH 6.0) - 1.5 M NaCl in the reservoir. The flow rate was 15 ml/hr. Each fraction contained 12 ml of the effluent.

Isolation of the enzyme extract from E. coli. Thirty grams of wet E. coli B cells, harvested in the late log phase of growth, were ground with 60 g of alumina 305, and extracted with 100 ml of buffer consisting of 0.01 M Tris-HCl (pH 7.5), 0.01 M $MgCl_2$, 0.001 M EDTA, 0.1 mM dithiothreitol and 10 % glycerol. The extract was centrifuged at 13,000 x g for 30 min and the resulting supernatant was recentrifuged under the same conditions. The supernatant thus obtained (70 ml) was mixed with 12 ml of 1 M potassium phosphate buffer (pH 7.5), 9.8 ml of 20 % dextran T-500 and 26.2 ml of 30 % polyethyleneglycol 6000. The mixture was stirred for 2 hr at 4°C, and centrifuged at 8,000 x g for 10 min. The upper layer (90 ml) containing 504 mg of protein was used as the enzyme source.

Assay of enzyme activity. The standard reaction mixture contained 50 μmoles of Tris-HCl (pH 8.4 - 8.7), 10 μmoles of $MgCl_2$, 2.5 μmoles of ATP, 18 mμmoles of S-adenosyl-L-[carboxyl-^{14}C]methionine (specific activity, 55 μCi/μmole) or 12 mμmoles of S-adenosyl-L-[2-^{3}H]methionine (specific activity, 2.1 mCi/μmole), 0.1 - 0.2 ml of enzyme extract and methyl-deficient $tRNA^{Phe}$ as specified in a final volume of 1 ml. The reaction mixture was incubated at 37°C. Aliquots (0.02 - 0.05 ml) were taken at interval, and applied to filter paper discs. The discs were washed successively with cold, 5 % trichloroacetic acid, ether-ethanol (1:1, v/v), and ether, as described previously (4). Radioactivity was measured in a liquid scintillation counter.

Analysis of the ^{14}C-labeled reaction product. One ml of the reaction mixture containing 2.1 A_{260} units of methyl-deficient $tRNA^{Phe}$ without ATP was incubated at 37°C for 30 min. The reaction mixture was treated with 1 ml of 88 % phenol. The aqueous layer was extensively dialyzed against distilled water, and then lyophilized. The residue was dissolved in 0.1 ml of 0.05 M potassium acetate buffer (pH 4.7) and an aliquot of the solution was mixed with 2 A_{260} units of E. coli $tRNA^{Phe}$ and completely hydrolyzed with RNase T_2. The hydrolyzate was subjected to thin-layer chromatography as described previously (5).

Materials. S-Adenosyl-L-[carboxyl-^{14}C]methionine and S-adenosyl-L-[2-^{3}H]methionine were purchased from the Radiochemical Centre, Amersham. E. coli $tRNA^{Phe}$ was prepared by successive DEAE-Sephadex A-50 column chromatography at pH 4.0 and 7.5, and reverse phase partition column chromatography, following a procedure similar to that described previously (6).

RESULTS

Fig. 3 shows the incorporation of radioactivity from ^{14}C-carboxyl labeled S-adenosylmethionine into E. coli tRNAs with increasing time of incubation. Incorporation of the radioactivity was greatest, when purified methyl-deficient $tRNA^{Phe}$ was used as acceptor. Unfractionated methyl-deficient tRNA was also effective for incorporation of ^{14}C-carboxyl-labeled S-adenosylmethionine. Incorporation of radioactivity was proportional to the time of incubation for up to 20 min. Neither normal E. coli $tRNA^{Phe}$ nor unfractionated E. coli tRNA was utilized as acceptor.

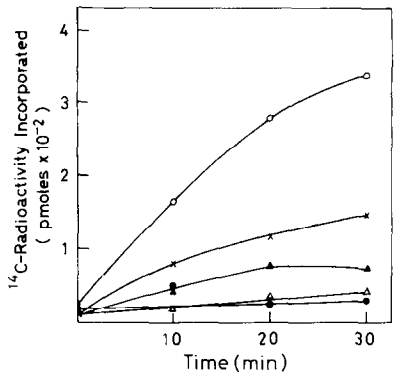

Fig. 3. Incorporation of radioactivity from S-adenosyl-L-[carboxyl-^{14}C]-methionine into tRNA. ○, methyl-deficient tRNA^{Phe} (4.3 A_{260} units in a final volume of 1 ml); X, unfractionated methyl-deficient tRNA (4.6 A_{260} units); ▲, normal unfractionated tRNA (5.0 A_{260} units); △, normal tRNA^{Phe} (1.0 A_{260} unit); ●, minus tRNA.

TABLE I. Effect of pH and requirement of magnesium ion on incorporation of radioactivity from ^{14}C-carboxyl-labeled S-adenosylmethionine into methyl-deficient tRNA^{Phe}.

Conditions	Incorporation of ^{14}C-radioactivity into methyl-deficient tRNA^{Phe} (pmoles/ml)
pH 7.0	14.9
pH 7.8	120
pH 8.4	145
pH 8.7	185
pH 8.4, minus Mg^{2+}	0

Time of incubation was 20 min.

Table I shows results on the requirements of the reaction. Magnesium ion was necessary for the incorporation. The pH optimum of the reaction was found to be higher than 8.4. Incorporation of radioactivity into methyl-deficient tRNAs was also observed when S-adenosyl-L-[2-^{3}H]methionine was used instead of S-adenosyl-L-[carboxyl-^{14}C]methionine (Table II). This suggests

TABLE II. Incorporation of radioactivity from ^{3}H-2-labeled S-adenosylmethionine into methyl-deficient tRNA.

Conditions	Incorporation of ^{3}H-radioactivity into acid insoluble fraction (pmoles/ml)
None	18.7
Unfractionated normal tRNA	51.7
Normal tRNA^{Phe}	34.1
Unfractionated methyl-deficient tRNA	193
Methyl-deficient tRNA^{Phe}	574

Amounts of tRNA added were the same as described in Fig. 1. The incubation was carried out for 30 min.

that incorporation of radioactivity from S-adenosyl-L-[carboxyl-^{14}C]methionine was due not only to transfer of the carboxyl group, but also to transfer of the 3-amino-3-carboxypropyl moiety of S-adenosylmethionine. When the ^{14}C-labeled product was treated with alkali (pH 10.5, overnight at 4^{o}C), the incorporated radioactivity was still acid insoluble (results not shown), indicating that the methionine moiety was not attached to the CCA-terminal through an ester-linkage.

To analyze the ^{14}C-labeled product, the ^{14}C-labeled tRNA was completely hydrolyzed with RNase T_2, and the digest was subjected to two-dimensional thin-layer chromatography. As shown in Fig. 4, the radioactive spot completely coincided with the position of the 3'-phosphate of 3-(3-amino-3-carboxypropyl)-uridine, indicating that the product synthesized was in fact 3-(3-amino-3-carboxypropyl)uridine.

DISCUSSION

In the biosynthesis of spermidine and spermine in bacteria, yeast, rat prostate and rat regenerating liver, S-adenosyl-L-methionine is first de-

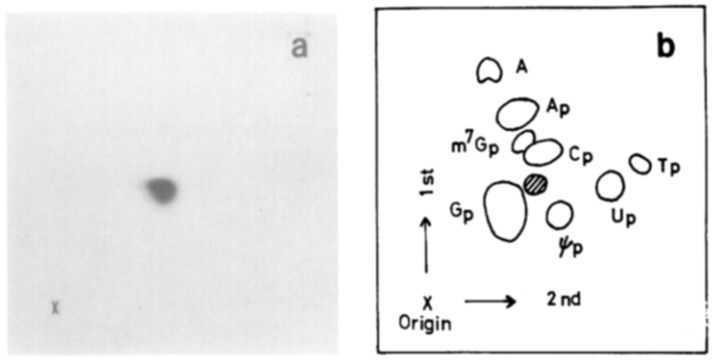

Fig. 4. Autoradiogram of a thin-layer chromatogram of the RNase T_2 digest. (a) Autoradiogram of the chromatogram. (b) Composite tracing of (a) in relation to the locations of spots with UV absorption.

carboxylated, and then this decarboxylated S-adenosyl-L-methionine serves as a propylamine donor (7-10). Feldman et al. reported that the two reactions could not be separated from each other in rat liver, suggesting that one enzyme participates in both reactions (11). However, it is thought that the two reactions occur successively. On the contrary, in the synthesis of 3-(3-amino-3-carboxypropyl)uridine at the tRNA level described here, S-adenosylmethionine serves directly as the donor molecule without prior decarboxylation. In this sense, synthesis of 3-(3-amino-3-carboxypropyl)uridine is a novel transfer reaction involving S-adenosylmethionine. S-Adenosylmethionine is probably cleaved to form methylthioadenosine in the reaction resulting in formation of 3-(3-amino-3-carboxypropyl)uridine. The methyl-deficient $tRNA^{Phe}$ used in this study lacked almost all the ribothymidine and 7-methylguanosine present in normal $tRNA^{Phe}$, in addition to 3-(3-amino-3-carboxypropyl)uridine. Nucleotide sequence analysis of methyl-deficient $tRNA^{Phe}$ suggested that its extra-region contained G-U-C instead of m^7G-X-C (K. Yamaizumi and S. Nishimura, unpublished results). We have no data yet as to the location of ^{14}C-labeled 3-(3-amino-3-carboxypropyl)uridine in methyl-deficient $tRNA^{Phe}$ synthesized in vitro. However, it is very likely that the enzyme retains the same strict specificity to modify the nucleotide residue in the tRNA. If so, the uridylate

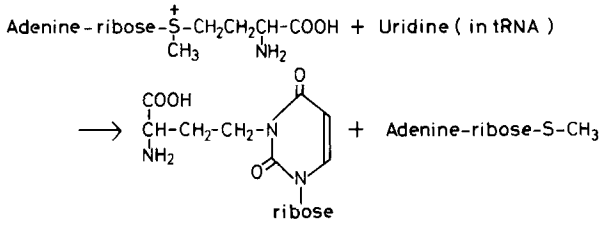

Fig. 5. Proposed reaction scheme for the synthesis of 3-(3-amino-3-carboxypropyl)uridine.

residue should be a precursor of 3-(3-amino-3-carboxypropyl)uridine. The proposed reaction scheme is shown in Fig. 5. The question arises of whether the syntheses of 3-(3-amino-3-carboxypropyl)uridine and 7-methylguanosine adjacent to each other can proceed simultaneously or whether one of the two reactions must occur first. Although ATP was added in the standard reaction mixture, its omission had no effect on the synthesis of 3-(3-amino-3-carboxypropyl)uridine if crude enzyme extract was used. However, ATP strongly stimulated the synthesis of 3-(3-amino-3-carboxypropyl)uridine when enzyme preparation purified by DEAE-cellulose chromatography was used (N. Okada and S. Nishimura, unpublished results). We are now purifying the enzyme to study these problems.

REFERENCES

(1) H. G. Zachau, in "The Mechanism of Protein Synthesis and Its Regulation" ed. by L. Bosch, p.173, North-Holland (1972).
(2) Z. Ohashi, M. Maeda, J. A. McCloskey and S. Nishimura, submitted to Biochemistry.
(3) N. Biezunski, D. Giveon and U. Z. Littauer, Biochim. Biophys. Acta, 199, 382 (1970).
(4) S. Nishimura, F. Harada, U. Narushima and T. Seno, Biochim. Biophys. Acta, 142, 133 (1967).
(5) F. Harada, F. Kimura and S. Nishimura, Biochemistry, 10, 3269 (1971).
(6) S. Nishimura, in "Procedures in Nucleic Acid Research" ed. by G. L. Cantoni and D. R. Davies, Vol. 2, p.542, Harper and Row, New York (1971).
(7) C. W. Taber, in Methods in Enzymology, ed. by S. P. Colowick and N. O. Kaplan, Vol. V, p.761, Academic Press (1962).
(8) J. Jänne and H. G. Williams-Ashman, Biochem. Biophys. Res. Commun., 42, 222 (1971).
(9) J. Jänne, H. G. William-Ashman and A. Schenone, Biochem. Biophys. Res. Commun., 43, 1362 (1971).
(10) A. Raina and P. Hannonen, Acta Chem. Scand., 24, 3061 (1970).
(11) M. J. Feldman, C. C. Levy and D. H. Russell, Biochemistry, 11, 671 (1972).