Volume 2 number 5 May 1975 Nucleic Acids Research

Maturation of a hypermodified nucleoside in transfer RNA

Paul F. Agris[1], Donald J. Armstrong[+], Klaus P. Schäfer[2] and Dieter Söll*

*Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06520 and [+]Institute of Plant Development, Birge Hall, University of Wisconsin, Madison, Wisconsin 53706, USA

Received 4 March 1975

ABSTRACT

E. coli C6 rel$^-$ met$^-$ cys$^-$ was cultured in a fully supplemented medium and in media lacking cysteine or methionine. tRNA isolated from the three cultures contained, respectively, a normal complement of modified nucleosides; a deficiency in thiolated nucleosides and a deficiency in methylated nucleosides. Both sulfur-deficient tRNA and methyl-deficient tRNA contained large amounts of N^6-(Δ^2-isopentenyl)adenosine and small amounts of the 2-methylthio derivative. Methyl-deficient tRNA contained, in addition a large amount of a cytokinin active, differently modified nucleoside that is believed to be a sulfur derivative of N^6-(Δ^2-isopentenyl)adenosine. The structure of this compound is unknown. When methyl-deficient tRNA and the precursor to $tRNA^{Tyr}$ su_3^+ A25 were enzymatically methylated *in vitro*, methyl groups were incorporated into derivatives of isopentenyladenosine. These results indicate that the biosynthesis of the 2-methylthio derivative of isopentenyladenosine may occur in a sequential manner, i.e., thiolation of isopentenyladenosine followed by methylation.

INTRODUCTION

The hypermodified nucleoside 2-methylthio-N^6-(Δ^2-isopentenyl)adenosine, ms^2i^6A (3), has been found adjacent to the anticodons of the *E. coli* tRNAs specific for phenylalanine, serine, tryptophan and tyrosine (4). It is believed that all tRNAs responding to codons beginning with U contain i^6A or one of its derivatives (4,5,6). The isopentenyl moiety is derived from mevalonic acid (7) and is attached to the tRNA in a reaction mediated by the enzyme Δ^2-isopentenyl pyrophosphate: tRNA-Δ^2-isopentenyl transferase (8). Gefter and Russell (9) have shown that adenosine adjacent to the anticodon in $tRNA^{Tyr}$ is isopentenylated prior to the production of the 2-methylthio derivative. Gefter (10) has reported that cysteine is the sulfur donor, and S-adenosyl-L-methionine the methyl donor in the biosynthesis of the 2-methylthio moiety. The work presented here elucidates some of the aspects of the *in vivo* biosynthesis of the 2-methylthio moiety and indicates that the modifying enzymes may interact with precursor tRNA (11) to produce ms^2i^6A.

MATERIALS AND METHODS

General. Benzoylated-DEAE cellulose was a product of Boehringer-Mannheim Co.; tlc plates of microgranular cellulose (250 μ thick) were obtained from Analtech. S-adenosyl-L-methionine-(methyl)-[^{14}C] (specific activity 50 mCi/mmole and carrier free [^{32}P]-phosphoric acid were purchased from New England Nuclear. The nucleoside standards, i^6A, mo^2i^6A, $(HO)^2i^6A$ and ms^2i^6A and the base s^2i^6Ade were kindly given by Dr. N. J. Leonard. Crude snake venom phosphodiesterase (Crotalus adamanteus) and alkaline phosphatase (calf intestinal mucosa, Type II) were purchased from Sigma. E. coli strain C6 rel$^-$ met$^-$ cys$^-$ (12) was the kind gift of Dr. E. B. Titchener.

Chromatography and Analytical Methods. Thin layer chromatography, autoradiography, and other analytical methods have been described previously (11, 13).

Growth and Harvesting of Bacteria. E. coli C6 rel$^-$ met$^-$ cys$^-$ was grown in minimal medium and harvested as previously described (13). A 100 liter culture was grown under conditions of methionine starvation (0.05 mM methionine) and another 100 liter culture was grown under conditions of cysteine and sulfate starvation (0.0015 mM cysteine, no sulfate). As a control, a third culture (10 liters) was grown in fully supplemented medium (1 mM methionine, 1 mM cysteine, 0.4 mM Na_2SO_4).

Extraction of tRNA. E. coli tRNA was isolated according to Zubay (14) with a final DEAE-cellulose chromatographic step.

Preparation and Analysis of [^{32}P]tRNA^{Tyr} and precursor tRNA^{Tyr}. The preparation and base analysis of tRNA^{Tyr} su_3^+ A25 and the precursor to tRNA^{Tyr} su_3^+ A25 were performed as described earlier (11).

Enzyme Preparations. Crude tRNA methylase from E. coli and from KB cells consisted of high speed supernatant fractions (100,000 x g) which had been passed over DEAE-cellulose and concentrated against polyethylene glycol (15).

Methylation. The incubation mixtures (0.1 ml) contained 0.05 M Tris-HCl (pH 7.9), 0.3-4.0 A_{260} units of tRNA, approximately 15 μg protein from a tRNA methylase preparation, 1 mM 2-mercaptoethanol, 0.25 M KCl, and 3 mM EDTA.

After incubation at 37° the samples were assayed for acid insoluble radioactivity by the filter paper technique.

Analysis of Cytokinin Activity. This was done in the tobacco callus assay as described by Armstrong et al. (16). tRNA (400 A_{260} units) was hydrolyzed to nucleosides with crude snake venom phosphodiesterase and alkaline phosphatase as described earlier (17).

The dry hydrolysate was extracted six times with water-saturated ethyl acetate and the combined extracts were evaporated to dryness in vacuo at room temperature. The ethyl acetate-soluble nucleosides were dissolved in 1.2 ml of 35% ethanol and chromatographed on a Sephadex LH-20 column (1.9 x 14 cm) in the same solvent. The UV absorbance (254 nm) of the column eluate was monitored and fractions were combined as indicated and taken to dryness in vacuo at 37°. Cytokinin standards i^6A, mo^2i^6A, $(HO)^2i^6A$ and ms^2i^6A were chromatographed on each column after the fractionation of tRNA nucleosides had been completed. After evaporation, the combined fractions from Sephadex LH-20 columns were acid hydrolyzed (0.1 N HCl, 100°, 45 minutes) and tested for cytokinin activity in the tobacco bioassay as described previously (18,19). Cytokinin activities are expressed as µg Kinetin Equivalents (µg KE); defined as the number of µg of kinetin required to give the same growth response as the test samples under the specified bioassay conditions.

Fractionation of i^6A-containing tRNA. tRNA (11,200 A_{260} units) isolated from E. coli C6 rel^- met^- cys^- grown under conditions of methionine starvation was chromatographed at room temperature on a column of BD-cellulose (2.2 x 50 cm) which had been equilibrated with 0.2 M NaCl in acetate buffer consisting of 0.05 M sodium acetate (pH 5.0) - 0.01 M $MgCl_2$ - 0.04% sodium azide (20). The column was then developed with acetate buffer containing first 0.2 M NaCl (190 ml), then 1.5 M NaCl (500 ml) and finally 1.5 M NaCl, 40% ethanol (500 ml). tRNA eluated in the 40% ethanol wash was precipitated by the addition of 0.8 volumes of ethanol, the precipitate collected, redissolved in glass distilled water and dialyzed extensively against glass distilled water.

RESULTS

E. coli C6 rel^- will produce modification deficient tRNA when the bacteria are auxotrophic for an amino acid precursor of a particular tRNA modification, and the cells are grown in medium deficient in that amino acid.

Transfer RNA was extracted from cultures of E. coli C6 rel⁻ met⁻ cys⁻ which had been grown in fully supplemented medium, methionine deficient medium, and cysteine and sulfate deficient medium. These tRNAs will be referred to as normal tRNA, methyl deficient tRNA, and sulfur deficient tRNA, respectively. All three tRNAs were used as substrates for methylation with a crude E. coli methylase preparation (Fig. 1). Only the methyl deficient tRNA incorporated [^{14}C]-methyl groups indicating that the growth conditions of the methionine starved culture were such that the tRNA synthesized was not fully methylated in vivo. Therefore this tRNA might be deduced to be deficient in ms^{2}i^{6}A as well as other methylated nucleosides.

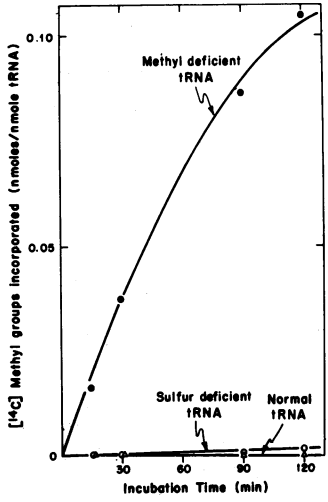

Fig. 1. Methylation of E. coli tRNAs. Transfer RNA extracted from cultures of E. coli C6 rel⁻ met⁻ cys⁻ which had been grown in fully supplemented medium, methionine deficient and cysteine deficient media were methylated in the presence of SAM-(methyl-^{14}C), 0.25 M KCl, 3 mM EDTA and a crude E. coli methylase preparation (see Materials and Methods). The drawing denotes the incorporation of ^{14}C-methyl groups per nmole (0.63 A_{260} units) of normal tRNA (-Δ-), methyl-deficient tRNA (-●-) and sulfur-deficient tRNA (-○-) during a 120 minute incubation period.

The presence of s^{4}U in tRNA gives an ultraviolet absorption maximum at 335 nm which is usually 1.5 - 2.0% of that for the same tRNA at 260 nm (12). The unfractionated normal tRNA and the methyl deficient tRNA had absorbances at 335 nm which were 1.7 and 1.9% of that at 260 nm, respectively. The sulfur deficient tRNA had an absorbance at 335 nm which was only 0.7% of that at 260 nm. This indicates that the tRNA isolated from cells grown under conditions of sulfur starvation is lacking in s^{4}U (21) and may also be deficient in the other thiolated nucleosides such as s^{2}C, s^{2}U, and its derivatives and ms^{2}i^{6}A.

Since all derivatives of i^6A found in tRNA have cytokinin activity, a bioassay for this activity was used to determine the type and amounts of i^6A derivatives present in the normal, methyl-deficient and sulfur-deficient tRNAs. The tRNAs were hydrolyzed and the resulting nucleotides were dephosphorylated, extracted with water-saturated ethyl acetate and fractionated on Sephadex LH-20 columns. The UV absorption profiles of the fractionations of the nucleosides from the three tRNAs were very similar. Figure 2A depicts the UV absorption profile of the fractionation of the nucleosides from the normal tRNA and the positions of elution of the standards i^6A, $(HO)^2i^6A$, mo^2i^6A, and ms^2i^6A. The figure also denotes the pooled fractions (I-XIV) used for the cytokinin activity assays. Figures 2B, 2C, and 2D show the results of the cytokinin assays. Methyl-deficient tRNA (Fig. 2C) had approximately one-fourth the amount of ms^2i^6A and seven times the amount of i^6A as did the normal tRNA (Fig. 2B). In addition, the methyl-deficient tRNA contained a new

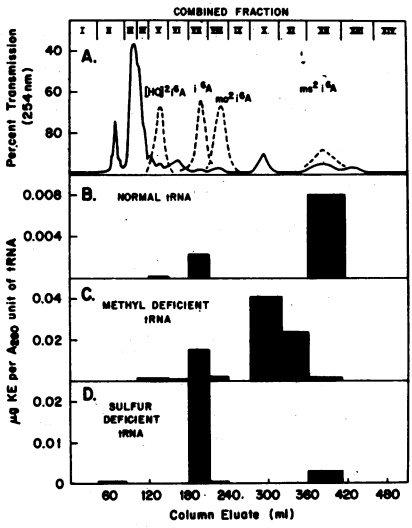

Fig. 2. Fractionation and Cytokinin Activities of Nucleosides in tRNA. Normal, methyl-deficient and sulfur-deficient tRNAs were hydrolyzed, the resulting nucleotides dephosphorylated, extracted with ethyl acetate and subjected to chromatography on Sephadex LH-20 columns as described in Materials and Methods. The UV absorption profiles from the chromatography of the nucleosides of the three tRNAs were similar. Fig. 2A illustrates the UV absorption profile (percent transmission at 254 nm) of the nucleosides from normal tRNA. The elution position of the standards i^6A, $(HO)^2i^6A$, mo^2i^6A and ms^2i^6A are also shown. Fractions from each column were combined into fourteen pools (I-XIV) and tested for cytokinin activity (Materials and Methods). The cytokinin activities of the nucleosides from the normal, methyl-deficient and sulfur-deficient tRNAs are shown as μg Kinetin Equivalents (KE) per A_{260} unit of tRNA in Fig. 2B, 2C, and 2D, respectively. Kinetin Equivalents are defined as the micrograms of kinetin required to give the same growth response as the test samples under specified bioassay conditions.

cytokinin active nucleoside which eluted in a new position (fractions X and XI). This nucleoside was present in large amounts compared to i^6A and ms^2i^6A. The sulfur deficient (Fig. 2D) had approximately one-third the amount of ms^2i^6A and over three times the amount of i^6A as did the normal tRNA. No cytokinin activity was detected in fractions X and XI of the nucleosides from the sulfur deficient tRNA. Therefore the *in vivo* modification of i^6A in tRNA of *E. coli* C6 rel^- met^- cys^- is inhibited by growth conditions of methionine and cysteine deprivation. Starvation for either of these two amino acids led to the production of larger amounts than normal of the incompletely modified i^6A in the tRNA. Starvation for methionine produced, in addition, a large amount of a new cytokinin active nucleoside.

If a non-methylated i^6A derivative is present in the methyl-deficient tRNA, then it should be possible to methylate this nucleoside using SAM-(methyl-^{14}C) and an enzyme preparation from *E. coli*. Approximately 4.0 A_{260} units of methyl-deficient tRNA and of sulfur deficient tRNA were reacted with radioactive SAM and a crude tRNA methylase preparation as described in Materials and Methods. After incubation at 37°C for two hours the reaction mixtures were extracted with phenol and the tRNAs precipitated with ethanol. The precipitated tRNAs were taken up in water and dialyzed against glass distilled water before being digested with ribonuclease T_2. The nucleotides resulting from the ribonuclease digestions were subjected to two dimensional tlc and subsequent autoradiography. The X-ray films showed that there was no methylation of the sulfur deficient tRNA but that many of the nucleotides of the methyl-deficient tRNA were methylated *in vitro*. A small amount of radioactivity, 2% of the total incorporated, was associated with the area of migration of i^6Ap and its derivatives. This finding supports the hypothesis that methyl-deficient tRNA contains s^2i^6Ap which could be further modified *in vitro* by methylation to ms^2i^6Ap. The major nucleotides formed in this *in vitro* tRNA methylation were m^5Up (Tp) and m^7Gp. When tRNA enriched in those species containing i^6A (see Materials and Methods) was used as substrate in the methylation reaction, 5% of all methyl groups incorporated into tRNA were associated with i^6Ap.

Transfer RNA^{Tyr} su_3^+ A25 and precursor $tRNA^{Tyr}$ su_3^+ A25 have been isolated and the amounts of i^6Ap and its derivatives in these molecules, reported by Schäfer *et al.* (11), indicate that pre-tRNA although a substrate *in vivo* for the isopentenyl modification is not fully modified. We have treated [^{32}P]-labeled precursor $tRNA^{Tyr}$ su_3^+ A25 with SAM-methyl-[^{14}C] and *E. coli* or KB tRNA methylase preparations. The RNA isolated from such reaction mixtures was digested with ribonucleases A, T_1 and T_2 and the resulting nucleotides analy-

zed as described previously (11). The amount of methyl groups incorporated (3-10 pmoles per 100 pmoles pre-tRNA with four different preparations) into a derivative of i^6A in pre-tRNA is comparable to the amount of *in vitro* isopentenyl modification (3 pmoles per 100 pmoles pre-tRNA) which has been reported by Schäfer *et al.* (11).

DISCUSSION

Transfer RNA isolated from *E. coli* C6 rel^- met^- cys^- grown under conditions of methionine starvation was found to contain a new cytokinin active nucleoside in addition to ms^2i^6A and i^6A. This nucleoside, which may be an incompletely modified derivative of i^6A, eluted from a Sephadex LH-20 column in a position different from that of the standards $(HO)^2i^6A$, i^6A, mo^2i^6A and ms^2i^6A. The unknown nucleoside could be s^2i^6A. All attempts to chemically synthesize s^2i^6A for use as a chromatographic standard have failed presumably because of instability (Agris, P.F., Leonard, N.J., and Söll, D., personal communication); whereas the corresponding base, s^2i^6Ade is stable and was chemically synthesized (22). Another indication of the instability of the unknown derivative of i^6A in methyl-deficient tRNA was the fact that the detection of the compound by Sephadex LH-20 chromatography deteriorated with age of the tRNA preparation.

An isopentenyladenosine in methyl-deficient tRNA was found to accept methyl groups in an *in vitro* assay supporting the indication that this tRNA contains an incompletely modified derivative of i^6A. Precursor $tRNA^{Tyr}$ su_3^+ A25 was found to accept methyl groups into a derivative of i^6A, indicating that this molecule may contain the same incompletely modified i^6A derivative. Sulfur-deficient tRNA contained only ms^2i^6A and i^6A. Thus, the 2-methylthio derivative of i^6A in tRNA may be biosynthesized in a sequential manner, isopentenylation of A, thiolation of i^6A, followed by methylation of the 2-thio group. These reactions proceed both at the tRNA and precursor tRNA level.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. N. J. Leonard for many helpful discussions and Ms. A. Körner and Ms. De LaMarche for their aid in the preparation of this manuscript.

This work was supported by grants from the National Institutes of Health and the National Science Foundation.

REFERENCES

1. The work reported here was undertaken initially during the tenure of a Damon Runyon Memorial Fund for Cancer Research Postdoctoral Fellowship and completed during the tenure of an American Cancer Society Postdoctoral Fellowship. Present address: Division of Biological Sciences, University of Missouri, Columbia, Missouri 65201.

2. Postdoctoral Fellow of the Deutsche Forschungsgemeinshaft. Present address: Labor für Genetik, Universität Konstanz, BRD-7750 Konstanz, West Germany.

3. Abbreviations used: BD-cellulose, benzoylated DEAE-cellulose; tlc, thin layer chromatography; SAM, S-adenosyl-L-methionine; tRNA methylase, S-adenosyl-L-methionine; tRNA methyl-transferase (E.C. 2.1.1.); A_{260} unit, that amount of material having an absorbance of 1.0 at 260 nm when dissolved in 1 ml of water measured with a 1 cm light path. Nucleotides and nucleosides are abbreviated as recommended by the IUPAC-IUB Commission on Biochemical Nomenclature (*Biochemistry 9*, 4022 (1970); ms^2i^6A, 2-methio-N^6-(Δ^2-isopentenyl)adenosine; i^6A, N^6-(Δ^2-isopentenyl)adenosine; $(HO)^2i^6A$, 2-hydroxy-N^6-(Δ^2-isopentenyl)adenosine; mo^2i^6A, 2-methoxy-N^6-(Δ^2-isopentenyl)adenosine; s^2i^6A, 2-thio-N^6-(Δ^2-isopentenyl)adenosine.

4. Nishimura, S. (1972) in *Progress in Nucleic Acid Research and Molecular Biology* (Davidson, J.N. and Cohen, W.E., eds.) *Vol. 12*, pp. 49-85, Academic Press, New York.

5. Söll, D. (1971) *Science 173*, 293-299.

6. Bartz, J., Söll, D., Burrows, W.J., and Skoog, F. (1970) *Proc. Natl. Acad. Sci. U.S.A. 67*, 1448-1451.

7. Peterkofsky, A. (1968) *Biochemistry 7*, 472-482.

8. Bartz, J., Kline, L. and Söll, D. (1970) *Biochem. Biophys. Res. Commun. 40*, 1481-1487.

9. Gefter, M.L. and Russell, R.L. (1969) *J. Mol. Biol. 39*, 145-157.

10. Gefter, M.L. (1969) *Biochem. Biophys. Res. Commun. 36*, 435-441.

11. Schäfer, K.P., Altman, S., and Söll, D. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 3626-3630.

12. Harris, C.L., Titchener, E.V. and Cline, A.L. (1969) *J. Bact. 100*, 1322-1327.

13. Agris, P.F., Söll, D., and Seno, T. (1973) *Biochemistry 12*, 4331-4337.

14. Zubay, G. (1962) *J. Mol. Biol. 4*, 347-356.

15. Agris, P.F., Spremulli, L.L. and Brown, G.M. (1974) *Arch. Biochem. Biophys. 162*, 38-47.

16. Armstrong, D.J., Burrows, W.J., Evans, P.K. and Skoog, F. (1969) *Biochem. Biophys. Res. Commun. 37*, 451-456.

17. Burrows, W.J., Armstrong, D.J., Skoog, F., Hecht, S.M., Boyle, J.T.A., Leonard, N. and Occulowitz, J. (1969) *Biochemistry 8*, 3071-3076.

18. Armstrong, D.J., Skoog, F., Kirkegaard, L.H., Hampel, A.E., Bock, R.M., Gillam, I. and Tener, G.M. (1969) *Proc. Natl. Acad. Sci. U.S.A. 63*, 504-511.

19. Linsmaier, E. and Skoog, F. (1965) *Physiol. Planatarum 18*, 100-127.

20. Roy, K.L., Bloom, A. and Söll, D. (1971) in *Procedures in Nucleic Acid Research* (Cantoni, G.L. and Davies, D.R., eds.) Harper and Row, New York, *Vol. 2*, pp. 524-541.

21. Lipsett, M.N. (1965) *Biol. Chem. 240*, 3975-3978.

22. Burrows, W.J., Armstrong, D.J., Skoog, F., Hecht, S.M., Boyle, J.T.A., Leonard, N.J., Occolowitz, J. (1969) *Biochemistry 8*, 3071-3076.