THE IN VITRO SYNTHESIS OF 2'-OMETHYLGUANOSINE AND 2-METHYLTHIO ^{6}N (γ,γ, DIMETHYLALLYL) ADENOSINE IN TRANSFER RNA OF ESCHERICHIA COLI

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Summary

A cell-free extract of E. coli catalyzes the transfer of the methyl group from S-adenosylmethionine[1] to the 2'OH group of a guanosine residue present in a GG sequence in transfer RNA$_{tyr}$. SAM is also shown to serve as a methyl donor in vitro for the synthesis of 2-methylthio ^{6}N (γ,γ, dimethylallyl) adenosine in transfer RNA.

Introduction

The occurrence of modified bases in transfer RNA as well as their biosynthesis has been well studied (Borek and Srinivasan, 1966; Borek, 1966), however the function of these minor constituents in tRNA is poorly understood. Recently, we have demonstrated (Gefter and Russell, 1969) that complete modification of the 2MSiPA ajacent to the anticodon in E. coli tRNA$_{tyr}$ (Burrows et al. 1968; Harada et al. 1968) is essential to insure efficient binding of the tRNA to ribosomes. We wish to report here our initial studies concerning the biosynthesis of 2MSiPA and of 2'-omethylguanosine.

Materials and Methods

Methyl ^{14}C SAM (52 mC/μmole) was purchased from the Radiochemical Center,

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SAM, S-Adenosylmethionine; 2MSiPA, 2-methylthio, ^{6}N (γ,γ, dimethylallyl) adenosine; 2MSiPAp, 2-methylthio, ^{6}N (γ,γ, dimethylallyl) adenosine 2',3' phosphate; 2SiPA, 2-thio, ^{6}N (γ,γ, dimethylallyl) adenosine; iPA, ^{6}N (γ,γ, dimethylallyl) adenosine; GmG, 5' (2-omethyl) guanosylguanosine 2',3' phosphate; XY, dinucleoside diphosphates; TEC, 30% triethylamine carbonate pH10; $R_{3'AMP}$, mobility relative to 3'AMP, etc.

Amersham. iPA 5' phosphate was a gift from Dr. Nelson Leonard. Authentic GmGp and 2MSiPAp were prepared from pure ^{32}P-labelled E. coli $tRNA_{tyr}$ by alkaline digestion (Goodman et al. 1968).

Methyl-deficient $tRNA_{tyr}$ was prepared from E. coli strain 58-161 grown in minimal-salts medium supplemented with methionine (3μg/ml) and harvested 3 hrs. after growth had ceased (7 x 10^8 cells/ml). Transfer RNA_{tyr} was isolated and purified as previously described (Gefter and Russell, 1969) (see Fig.1). Cell-free extracts of E. coli CA274 were prepared by grinding of frozen cells with twice the weight of alumina. The paste was extracted with 3 volumes of 10% glycerol containing (final concentration) tris-HCl pH 8.0 (0.01M), $MgCl_2$ (0.01M), EDTA (0.001M), β-mercaptoethanol (0.001M) and pancreatic DNAase (5μg/ml). The extract was centrifuged at 30,000 x G for 15 min. and the supernatant fluid removed and centrifuged again at 30,000 x G for 5 min.

The incubation mixtures (2.0 ml) used for the synthesis of GmG contained tris-HCl pH8, 100μmoles; $MgCl_2$, 20μmoles; ATP, 4μmoles; PEP, 3.5μmoles; Pyruvate kinase, 5μg; β-mercaptoethanol, 40μmoles; ^{14}C-methyl, SAM, 200 mμmoles; methyl-deficient $tRNA_{tyr}$, 2.0 $O.D_{260}$ and cell extract, 3.6 mg. The mixture was incubated at 37°C for 30 min. The reaction was terminated by the addition of 0.2 ml of 1M K acetate pH5 and 1.0 ml of phenol.

Incubation mixtures for the synthesis of 2MSiPA were the same as above except that β-mercaptoethanol was omitted.

Methylated tRNA was isolated by ethanol precipitation and then hydrolyzed in 0.3N KOH for 18 hrs. at 37°C.

Chromatographic solvent systems used were Propan-2-ol, H_2O, NH_3, 7:2:1 (Fittler et al. 1968) and Propan-2-ol, HCl, H_2O (Wyatt, 1951). ^{14}C-labelled products and ^{32}P markers were located by autoradiography or by cutting chromatograms into strips and counting them in a liquid scintillation counter.

Results

Isolation of methyl-deficient $tRNA_{tyr}$ As seen in figure 1, methyl starvation of E. coli 58-161 leads to the accumulation of a species of $tRNA_{tyr}$

differing in mobility from fully methylated tyrosyl tRNA. For preparation of tRNA substrate, the methyl-deficient tyrosyl tRNA was further purified by chromatography on a column of benzoylated DEAE cellulose as previously described (Gefter and Russell, 1969). The purified preparation was used as a substrate for the synthesis of 2'-omethylguanosine and 2MSiPA. Using a cell-free extract and SAM, this preparation was capable of accepting 0.8 moles of methyl group per mole of tRNA.

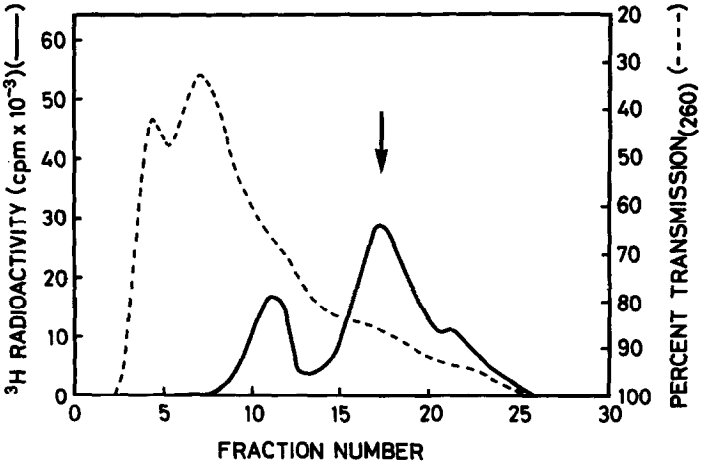

Figure 1 Reverse phase column chromatography of tRNA isolated from E. coli strain 58-161 after 3 hrs. of methyl starvation. The arrow indicates the mobility of tyrosine acceptor activity isolated from unstarved cells.

Identification of 2'-omethylguanosine Following incubation of methyl-deficient tyrosyl tRNA with a cell-free extract and ^{14}C-SAM, the tRNA was isolated and hydrolyzed in alkali. The digest was applied to DEAE paper and subjected to electrophoresis at pH 3.5 at 3,000 volts for 3 hrs. Several radioactive products were present. (The major component being ribothymidylate). ^{14}C material moving as a discrete band coincident with authentic GmGp was eluted with TEC and dried. It was then subjected to paper chromatography in propan-2-ol, HCl, water andhad a mobility identical to that of authentic GmGp. The ^{14}C product was again eluted and divided into two equal samples. One was digested with snake venom phosphodiesterase and the other

remained untreated. Both samples were applied to Whatman 3MM paper and subjected to electrophoresis at pH 3.5. The radioactivity from the digested sample migrated towards the cathode and had a mobility similar to 2-deoxyguanosine. The untreated material migrated towards the anode and had a mobility identical to that of GmGp (figure 2 and table 1). Incubations using heated extract (100°C for 5 min.) served as control samples. Under these conditions, no detectable GmGp was synthesized.

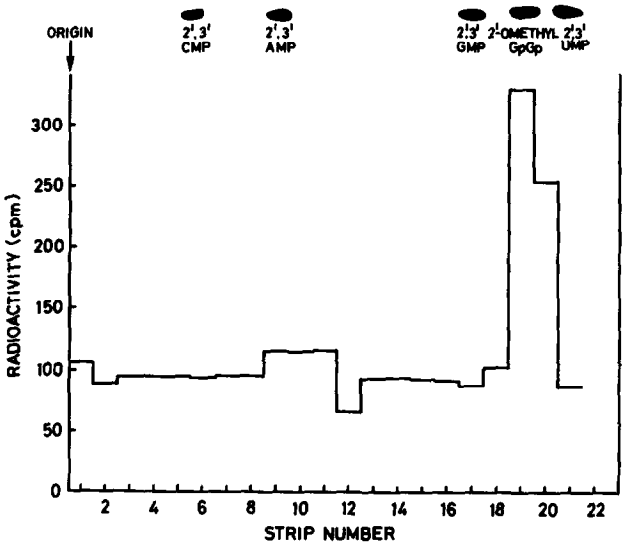

Figure 2 Electropherogram (pH 3.5, 3MM paper) of ^{14}C, *in vitro* synthesized GmGp 2',3' phosphate. The mobility of the ^{14}C-labelled product was determined by cutting the electropherogram into 1 cm strips and determining their ^{14}C content.

The precursors of the sulfur and methyl groups of 2MSiPA Tyrosyl tRNA was isolated from *E. coli* CA274 grown in the presence of $^{32}PO_4$, ^{35}S-cysteine, and unlabelled methionine and SO_4. The tRNA was digested with pancreatic ribonuclease and the resultant oligonucleotides separated by two-dimensional paper electrophoresis (Sanger, Brownlee and Barrell, 1965). Two oligonucleotides, G_4, 4 thio U and A,2MSiPA,A Ψ, were labelled with ^{35}S. The latter was hydrolyzed in alkali and the ^{35}S shown to be present in 2MSiPAp. The specific activities ($^{35}S/^{32}P$) of 4 thio Up and 2MSiPAp were nearly equal.

Cells grown on ^{35}S methionine showed no detectable ^{35}S in either 4 thio U or 2MSiPAp. The precursor of the methyl group of 2MSiPA has been shown to be methionine (Goodman et al. 1968).

In vitro synthesis of 2MSiPA Incubation of methyl-deficient $tRNA_{tyr}$, ^{14}C SAM and a cell-free extract of E. coli leads to the synthesis of ^{14}C-labelled 2MSiPAp. Following alkaline digestion of the methylated tRNA, ^{14}C 2MSiPAp was isolated by electrophoresis at pH 3.5 on DEAE paper. A discrete band of radioactivity having a mobility identical to the marker and well separated from other products was eluted with TEC and dried. The material was dissolved in water and applied to Whatman 3MM paper and subjected to electrophoresis at pH 3.5. Five radioactive components were resolved. One of these had a mobility identical to 2MSiPAp and a second major component had a mobility characteristic of an oxidized form of 2MSiPAp (2MSiPAp can be converted to this form by treatment with 0.02% $KMnO_4$ at 25°C for 5 min.). The nature of the other components is unknown. The material having a mobility

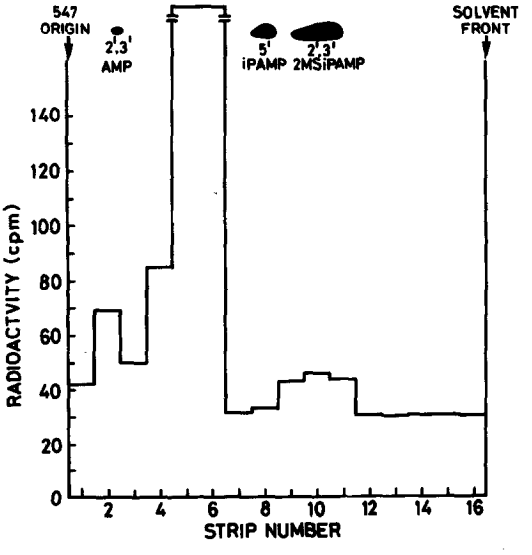

Figure 3 Chromatography (propan-2-ol, H_2O NH_3 2:2:1) of synthetic 2MSiPAp. The chromatogram was dried and cut into 1.5 cm strips and the ^{14}C content determined. Each strip was counted to within 2.5% standard deviation.

of 2MSiPAp was eluted with TEC and subjected to chromatography in propan-2-ol, water, conc. NH_3, which resolved two components. The minor component had a mobility identical to 2MSiPAp. The major component migrated slower (figure 3). This is believed to be a derivative of 2MSiPAp since authentic 2MSiPAp taken sequentially through the electrophoretic and chromatographic systems also shows a similar slow moving component as a major product.

The mobilities of synthetic 2MSiPAp in the various systems employed is summarized in table 1. Control incubations employing heated extract (100°C for 5 min.) did not lead to any detectable 2MSiPAp.

	SYSTEM					
METHYL-LABELLED PRODUCT	ELECTROPHORESIS pH 3.5 (DEAE PAPER)		ELECTROPHORESIS pH 3.5 (3MM PAPER)		PROPAN-2-OL WATER, AMMONIA	PROPAN-2-OL HCl, WATER
	$R_{3'AMP}$	$R_{3'GMP}$	R_{AMP}	R_{GMP}	R_F	R_F
GmGp	0.50	0.46	2.1	1.2	-	0.24
2MSiPAp	0.76	0.71	1.3	0.77	0.56	-

Table 1 Mobility of in vitro products in the systems indicated. In each case the mobility of the methyl-labelled product was identical to that of the authentic markers.

Discussion

2'-omethylguanosine has been characterized as GmGp, the dinucleotide expected from $tRNA_{tyr}$. Reports on the synthesis of 2'-omethyl nucleosides have not included the synthesis of GmG in tRNA (Svensson et al. 1968; Nichols and Lane, 1968).

We have observed that drying of 2MSiPAp from TEC leads to breakdown of this nucleotide. This perhaps can account for the multiple components observed in each system as well as the low yield of ^{14}C 2MSiPAp obtained. We are now engaged in the purification of the two tRNA methylases described.

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