TETRAHYDROFOLATE-DEPENDENT 5-METHYLURACIL-tRNA TRANSFERASE ACTIVITY IN *B. SUBTILIS*

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1. Introduction

The biosynthetic pathway of ribothymidine in *B. subtilis, M. lysodeikticus* and *S-faecalis* involves as coenzyme a tetrahydrofolate (FH_4) derivative for tRNA [1–3] but *S*-adenosyl-l-methionine (SAM) for tRNA [4]. The problem whether the FH_4-dependent transmethylation reaction is a post-transcriptional event was as yet unsolved. All FH_4-dependent pyrimidine methyltransferases so far known, as the 5,10-methylene FH_4-dependent deoxyuridylate methyltransferase, the deoxycytydylate hydroxymethyltransferase or the thymidylate synthetase use monomers as substrates [5]. The methylated pyrimidine nucleotide is then incorporated into DNA.

In the present work experimental evidence is presented showing that extracts of *B. subtilis* contain a specific FH_4-dependent 5-methyluracil-tRNA transferase. The enzyme mediates the transfer of one-carbon groups from formaldehyde via a tetrahydrofolate derivative to m^5U-deficient tRNA from *E. coli* IB5 Trm$^-$. The product of the transmethylation reactions was identified as 5-methyluracil.

2. Materials and methods

Chemicals were obtained from the following sources: [^{14}C] formaldehyde (spec. act. = μCi/μmole), *S*-adenosyl-[methyl-^{14}C] l-methionine (spec. act. = 53 μCi/μmole): Radiochemical Centre Amersham. Tetrahydrofolic acid: Sigma Chemical Company. All other chemicals were from sources as described previously [1,2,6].

E. coli IB5 Trm$^-$ derived from *E. coli* K12, strain CP59, was a kind gift from Dr Björk, Umea, Sweden.

2.1. *Crude extracts*

B. subtilis W 23 and *E. coli* MRE 600 were grown in maximal medium (10 g trypton; 5 g yeast extract; 10 g NaCl; 1 g glucose per litre; pH = 7.0) and harvested during exponential growth. The cells were rapidly chilled and disrupted immediately at 0–4°C with the two-fold amount of Alcoa in a precooled mortar. The resulting paste was extracted with the two-fold amount of buffer: 0.01 M Tris–HCl pH 7.8; 0.01 M Mg-acetate, 0.06 M mercaptoethanol, 0.06 M KCl. After centrifugation at 12 000 *g* for 20 min the supernatant was used directly as enzyme source.

2.2. *m^5U-deficient tRNA from E. coli IB5 Trm$^-$*

E. coli IB5 Trm$^-$ was grown in a minimal medium containing/liter 0.2 g $MgSO_4 \times 7\ H_2O$; 2 g citric acid; 10 g KH_2PO_4; 3.5 g $NaNH_4HPO_4 \times 4\ H_2O$; 2 g glucose; 1 mg thiamin; 100 mg threonine; 100 mg leucine; 100 mg histidine and 100 mg arginine; pH = 7.0 [6]. The cells were grown up to the stationary phase (8–10 hr after inoculation). tRNA was isolated by the phenol method and purified on DEAE cellulose as described previously [7].

2.3. *tRNA-methyltransferase assays*

(a) Assay with [^{14}C] formaldehyde and FH_4: 9 μmol FH_4 was dissolved together with 50 μmol = 100 μCi [^{14}C] formaldehyde in a final volume of 600 μl 0.01 M phosphate buffer pH 7.0 and incubated at 25°C for 30 min to allow non-enzymatic formation of formyltetrahydrofolic acid [8]. The reaction mixture was used immediately or kept frozen at –20°C for further assays.

The assay contained in a final volume of 2 ml/0.01 M phosphate buffer pH 7.5: 0.3 μmol of tetrahydrofolic

acid; 1.6 μmol = 3.3 μCi of [^{14}C] formaldehyde (20 μl of the mixture described above); 20 A_{260} units of m^5U-deficient tRNA; 20 μmol of $MgSO_4$; extracts of *B. subtilis* respectively E. coli 400 μl, corresponding to about 8 mg protein. In some experiments NADH + H^+ of NADPH + H^+ was added at a final concentration of 5 μmol. The assay mixture was incubated for 60 min at 37°C. The tRNA was recovered and purified on DEAE cellulose, eluted and precipitated as described previously [7].

(b) Assay with (methyl-^{14}C) SAM

The SAM-dependent tRNA-transmethylase assay, the purification, precipitation, hydrolysis of tRNA and the analysis of the methylated bases was carried out as described in detail in a previous communication [7].

3. Results and discussion

Ribothymidine deficient tRNA was isolated from the *E. coli* mutant IB5 Trm$^-$ described by Björk and Isaksson [6]. This tRNA accepted in vitro with SAM as coenzyme and methyltransferases of *E. coli* 0.7–0.8 mol methyl groups per mol tRNA. From the total recovered radioactivity 94% represented ribothymidine (m^5U). Trace amounts of 7- or 1-methylguanine were found (fig.1 and table 1 first column). No radioactivity was observed in m^5U when the tRNA was tested with *E. coli* enzymes in the FH_4-dependent transmethylase assay (table 1 second column).

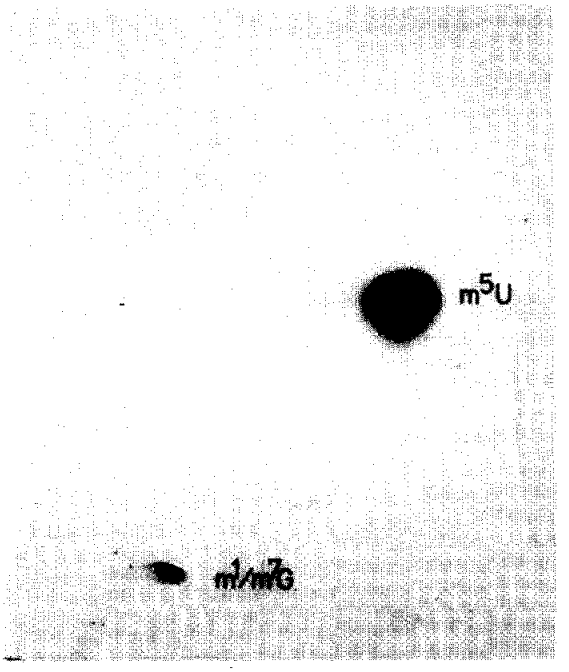

Fig.1. Autoradiography of the pattern of [methyl-^{14}C]-labeled bases of m^5U-deficient tRNA upon in vitro methylation with *E. coli* extracts and [methyl-^{14}C] SAM as coenzyme.

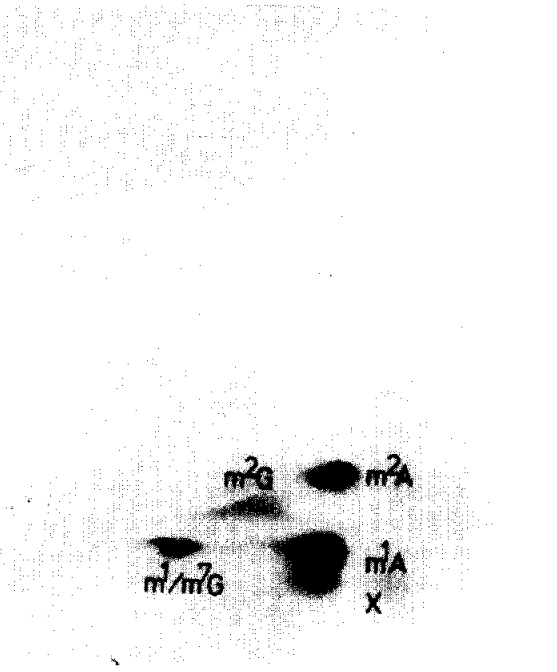

Fig.2. Autoradiography of the pattern of [methyl-^{14}C]-labeled bases of m^5U-deficient tRNA upon in vitro methylation with *B. subtilis* extracts and [methyl-^{14}C] SAM as coenzyme

The m^5U deficient tRNA from *E. coli* was tested as acceptor of methyl groups with *B. subtilis* extracts as source of enzymes either with [methyl-^{14}C] SAM or with [^{14}C] formaldehyde plus FH_4 as coenzyme. The SAM-dependent transmethylation reaction resulted in the formation of m^1/m^7G, m^1A and m^2A. In addition two as yet unidentified labeled bases were found in trace amounts. A SAM-dependent m^5U-tRNA transferase activity was not detectable in extracts of *B. subtilis* (fig.2 and table 1 column 3). However with the FH_4-dependent in vitro system methyl groups derived from formaldehyde, are transferred via FH_4 to uracil residues of m^5U deficient tRNA. No labeled m^5U was found when either m^5U lacking tRNA was

Table 1
Enzymatic methylation of m^5U deficient tRNA from *E. coli* IB5 Trm^- with [methyl-^{14}C] SAM or [^{14}C] formaldehyde and FH_4: Extracts of *E. coli* MRE 600 or *B. subtilis* W 23 were used as enzyme source.

Product	Percentage of total radioactivity recovered			
	E. coli		*B. subtilis*	
	SAM	FH_4	SAM	FH_4
m^5U	92.4	0	0	100
m^7/m^1G	6.8	–	17.3	–
m^1A	0	–	63.1	–
m^2A	0	–	15.6	–
m^2G (traces) Methyl. bases unidentified	0.8	–	4.0	–

replaced by fully methylated tRNA from *E. coli* or when an extract of *E. coli* MRE 600 was used as enzyme source.

The conditions used in this in vitro transmethylation reaction were chosen as described for the assay of 5-deoxycytidylate methyltransferase [9]. In this test system [^{14}C] formaldehyde is in excess over FH_4 to allow extensive formylation of FH_4 to 10-formyl FH_4. The formylation occurs at pH 7.0 in phosphate buffer without enzymes [8]. Under these conditions formaldehyde remains in the reaction mixture. Formaldehyde interacts with the amino groups of the bases of nucleic acids [10]. Therefore the tRNA isolated from the transmethylation assay contains considerable amounts of radioactivity. During purification and hydrolysis of the tRNA the formylated amino groups become hydrolyzed quantitatively, and the formaldehyde evaporates. As can be seen from the autoradiography in fig. 3 no label remains in the position of the main bases.

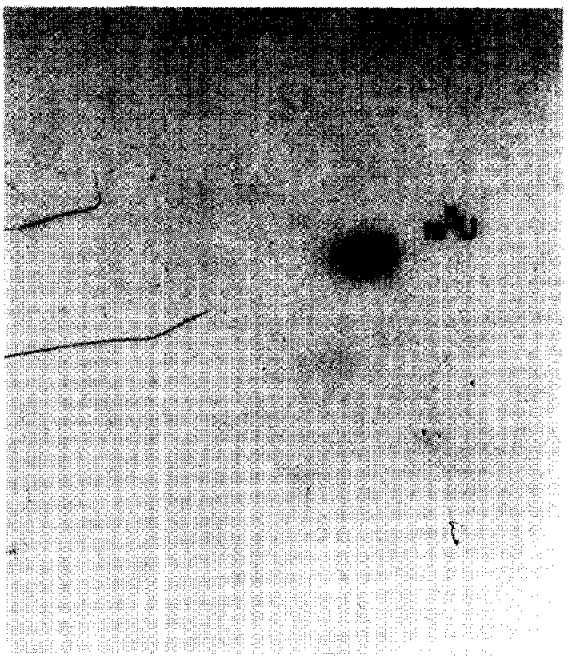

Fig. 3. Autoradiography of [methyl-^{14}C]-labeled m^5U obtained from hydrolysates of m^5U-deficient tRNA upon in vitro methylation with *B. subtilis* extracts and [^{14}C] formaldehyde + FH_4 as coenzyme.

The tetrahydrofolate derivative involved in the transfer of methyl groups to tRNA is not yet known. Formyl FH_4 or 5,10-methenyl FH_4 are converted via 5 10-methylene FH_4 to 5-methyl FH_4. As methyl donor 5,10-methylene FH_4 or 5-methyl FH_4. As methyl donor 5,10-methylene FH_4 or 5-methyl FH_4 could serve. The formation of the final methyl donor from 10-formyl FH_4 probably involves several enzymatic reactions. This circumstance might explain the relative low extent to which m^5U is labeled in the tetrahydrofolate-dependent transmethylation reaction which is in the order of 1–4% estimated from the specific activity of m^5U in tRNA. In preliminary experiments the addition of NADH + H^+ or NADPH + H^+ or ATP was without effect, probably because the extact contained sufficient amounts of these coenzymes. A

detailed analysis on the conditions of the FH_4-dependent m^5U-tRNA methyltransferase assay is now under way.

Previous results in our laboratory revealed: (a) formate or serine serve as methyl donors in the biosynthetic pathway of m^5U for tRNAs in *B. subtilis* [1]; (b) trimethoprim which prevents the reduction of dihydrofolic acid to tetrahydrofolic acid causes the accumulation of m^5U deficient tRNA in *B. subtilis* provided that the growth medium contains purines, thymidine, methionine and glycine. From this result we concluded that a tetrahydrofolate derivative is involved in the biosynthesis of m^5U. Whether the SAM-independent formation of m^5U is a post-transcriptional event was as yet unclear. The data presented here conclusively show the presence of a tetrahydrofolate-dependent m^5U-tRNA tranferase in extracts *B. subtilis*. This is at the present state of knowledge the first case in which an enzyme uses tetrahydrofolate as coenzyme and transfers methyl groups to a pyrimidine nucleotide at the macromolecular level.

Acknowledgements

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