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7-Methylguanine specific tRNA-methyltransferase from Escherichia coli

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ABSTRACT

A 7-methylguanine (m^7G) specific tRNA methyltransferase from *E. coli* MRE 600 was purified about 1000 fold by affinity chromatography on Sepharose bound with normal *E. coli* tRNA. The purified enzyme catalyzes exclusively the formation of m^7G in submethylated bulk tRNA of *E. coli* K12 met^- rel^-. The purified enzyme transfers the methyl group from S-adenosylmethionine to initiator tRNA of *B. subtilis* and 0.8 moles m^7G residues are formed per mole tRNA. It is suggested that the enzyme specifically recognizes the extra arm unpaired guanylate residue.

INTRODUCTION

All tRNA species from bacteria and higher organisms contain a certain number of methylated nucleosides. The transfer of methyl groups occurs at the polynucleotide level (1) and is catalyzed by specific tRNA methyltransferases. Usually S-adenosylmethionine serves as coenzyme (2). In special transmethylation reactions e. g. the formation of 5-methyluracil in tRNA of several grampositive microorganisms the methyl group is donated by a tetrahydrofolate derivative (3-5).

Methyltransferases were first enriched with submethylated bulk tRNA from *E. coli* K12 met^- rel^- (6) or with heterologous tRNAs as substrates (7,8). Many attempts to purify and characterize specific tRNA methyltransferases failed because the cognate tRNAs are unknown or often not available in a methyl-deficient state. Moreover the enzymes are highly unstable.

Affinity chromatography with $tRNA^{Glu}$-Sepharose was used by Taya and Nishimura to purify a SAM-dependent 2-thio-5-methyl-aminomethyl-uridine specific tRNA methyltransferase from *E. coli*. The enzyme was found to methylate methyldeficient

$tRNA^{Glu}$ from *E. coli* in a homologous system in vitro (9).

In this paper we describe the purification of a methyltransferase from *E. coli* MRE 600 by affinity chromatography on a column of Sepharose 4B bound with normal bulk tRNA from *E. coli* MRE 600. This procedure allows the separation of tRNA methyltransferases from the bulk of other proteins. The purified enzyme exhibits base specificity for the methylation of guanine to 7-methylguanine in submethylated bulk tRNA of *E. coli*.

Initiator-tRNA of *B. subtilis* differs from initiator-tRNA of *E. coli* in that an unmodified guanine residue instead of 7-methylguanine is present in the extra arm and a cytidine residue instead of a ribose methylated cytidine residue (Cm) in the anticodon loop. Otherwise $tRNA_f^{Met}$ of *B. subtilis* shows - with minor exceptions - sequence homology to $tRNA_f^{Met}$ of *E. coli* (10,11). The initiator tRNA of *B. subtilis* was isolated and found to serve as specific substrate for the m^7G specific tRNA methyltransferase from *E. coli*. This tRNA was therefore used in the purification procedure of this enzyme.

MATERIALS AND METHODS

Chemicals

Chemicals were from the following sources: (Methyl-^{14}C)-S-adenosylmethionine (specific activity 55 mCi/mMol) from Radiochemical Center Amersham; protamine sulfate, acrylamide, N, N-methylene-bisacrylamide, riboflavin and Comassie brilliant blue from Serva, Heidelberg; DE 32 DEAE-cellulose and paper filters 3MM (Ø 23 mm) from Whatman, Hormuth + Vetter, Heidelberg; bulk tRNA (*E. coli* MRE 600), katalase and ferritin from Boehringer, Mannheim; CNBr-activated Sepharose 4B, Sephadex G-25, Sephadex G-200 and Dextran Blue from Pharmacia, Stockholm; serumalbumine and transferrin from Behringwerke, Marburg; ribonuclease T_2 from Calbiochem, San Diego. All other chemicals were obtained from commercial sources.

Preparation of submethylated bulk tRNA

E. coli K12-58-161 F^- met^- rel^- was grown in a 20 liter incubator in minimal medium with 3 mg methionine per liter

(6) to A_{578} of 0.7 (stationary phase, about 24 hrs after inoculation). Usually nucleic acids were extracted from 10-20 g wet weight bacteria by treatment with phenol and subsequently precipitated with ethanol. About 2000 A_{260} of the nucleic acid extract was applied to a 720 x 15 mm column of DEAE-cellulose which was equilibrated with 0.02 M Tris-HCl buffer, pH 7.5, containing 0.01 M $MgCl_2$ and 0.375 M NaCl. The flow rate was 24 ml/hr at 20°C. Elution was performed with a linear gradient from 0.375 to 0.525 NaCl in 0.02 M Tris-HCl, pH 7.5 containing 0.01 M $MgCl_2$. The tRNA fractions were pooled, concentrated by evaporation and dialyzed two to three times against water at 0°C. The tRNA was finally precipitated with a threefold volume of ethanol/potassium acetate.

Preparation of $tRNA_f^{Met}$ from *B. subtilis*

$tRNA_f^{Met}$ was purified and characterized as described in a previous communication (12).

Determination of protein

In crude extracts the protein content was measured according to Lowry (13). In purified protein fractions, devoid of nucleic acids, the content was estimated by Δ A at 215 and 225 nm (14). Bovine serumalbumine was used as standard in both procedures.

Standard assay for methyltransferase activity

Methyltransferase activity was measured in the following assay: 50 µl TMMg-buffer (0.1 M triethanolamine, pH 8.0; 0.02 M 2-mercaptoethanol; 0.01 M $MgCl_2$); 50 µl substrate solution, 0.1 or 2.0 A_{260} of tRNA from *E. coli* met$^-$ or $tRNA_f^{Met}$ from *B. subtilis* (dissolved in 50 µl aqua dest) as indicated in the legends of Fig. 3 and 4; 10 µl S-adenosyl-(methyl-^{14}C) l-methionine (16 nMol = 0.05 µCi); 50 µl enzyme fraction. The assay was incubated for 120 min at 37°C. The reaction was stopped by pipetting 100 µl portions to filter discs. The discs were further treated according to Kuchino et al. (7) and counted in toluene scintillator (5 g PPO + 0.3 g dimethyl-POPOP per liter) in a scintillation spectro-

meter (Packard 3320 or 3375). For kinetic measurements the specific activity of SAM was about four times higher (19 nMol = 0.2 µCi) and samples of 50 µl were removed from the reaction mixture at appropriate intervals of time and pipetted to the filter discs.

Analysis of ^{14}C-methylated tRNA

The methylated constituents of tRNA were analyzed after perchloric acid hydrolysis according to Kahle (15) or after ribonuclease T_2 digestion according to Nishimura (16).

Preparation of Sepharose-tRNA

The matrix-tRNA used for affinity chromatography was prepared according to Remy et al. (17) via coupling of hydrazin-Sepharose 4B with perjodate oxydized bulk tRNA from *E. coli* MRE 600. The substituted matrix contained 5.7 mg tRNA/ml gel.

Disc-electrophoresis

For disc-electrophoresis the standard gel system 1a of Maurer (18) with 5.6 % polyacrylamide was used. 50 - 80 µg protein was applied per gel. The proteins were stained with Comassie Brilliant Blue. Gel slicing was performed by a razor blade set (19).

Molecular sieve chromatography

Molecular weights and the elution behaviour of standard and unknown proteins which exhibit tRNA methyltransferase activity were compared by gel filtration on a Sephadex G-200 column (d = 5 mm, l = 400 mm, flow rate = 0.84 ml/hr, t = 1°C). The column was equilibrated with 0.05 M Sörensen-phosphate buffer pH 8.0 containing 0.5 mM 2-mercaptoethanol according to Andrews (20).

Large scale preparation of crude extracts and protein fractions

500 g wet weight of *E. coli* MRE 600 were kindly supplied by Boehringer. The cells were harvested at the end of the exponential growth phase and suspended in 0.01 M Tris-HCl, pH 7.5 which contained 0.01 M $MgCl_2$. The cells were immedia-

tely disrupted by high pressure dispersion at 500 atm (dispergator: Manton-Gaulin Manuf. Co. Inc., Everett, Mass., USA). These operations and all others were carried out at 0°C. After centrifugation at 13000 x g for 120 min 4000 ml of an opalescent crude extract was obtained with a protein content of 14 mg/ml. Nucleic acids were precipitated by adding 2700 ml of a 0.5 % aqueous solution of protamine sulfate to the crude extract (6) and centrifuged at 3000 x g for 45 min. The yellow coloured supernatant, 6700 ml corresponding 27 g protein, was saturated with ammonium sulfate and stored at -2°C (protein fraction A). The pellet was extracted with 2300 ml 0.3 M potassium succinate pH 6.0 and centrifuged at 3000 x g for 45 min to separate associated proteins from nucleic acids. The colourless supernatant contained 7.5 g protein and was saturated with ammonium sulfate and stored at -2°C (protein fraction B).

RESULTS

Purification procedure

Protein fraction B, obtained as described under methods, was used as source of m^7G specific tRNA methyltransferase. The protein fraction was desalted on a Sephadex G-25 column equilibrated with 0.05 M Tris-HCl, pH 8.0. 850 mg of the desalted protein was applied to a column of Sepharose bound with normal tRNA from *E. coli*.

The bulk of proteins, except tRNA methyltransferases and some other tRNA interacting proteins, are not bound to the matrix and are washed out with the equilibration buffer (Fig.1). Most of the bound proteins were then eluted in two steps with 0.04 M and 0.1 M phosphate buffer at pH 8.0. Fractions of 3.3 ml were collected and aliquots of each fraction were assayed for the presence of tRNA methyltransferase activity with bulk submethylated tRNA from *E. coli* K12 met^- and in selected fractions with $tRNA_f^{Met}$ from *B. subtilis* as substrate. With the tRNA from *E. coli* methyltransferase activity was found between fractions 55-85. The overall yield of tRNA methyltransferase activity eluted from the column was about 50 %.

As shown in Fig. 1 tRNA methyltransferase activity with specificity for $tRNA_f^{Met}$ of *B. subtilis* was eluted with 0.1 M phosphate buffer between fractions 78-85.

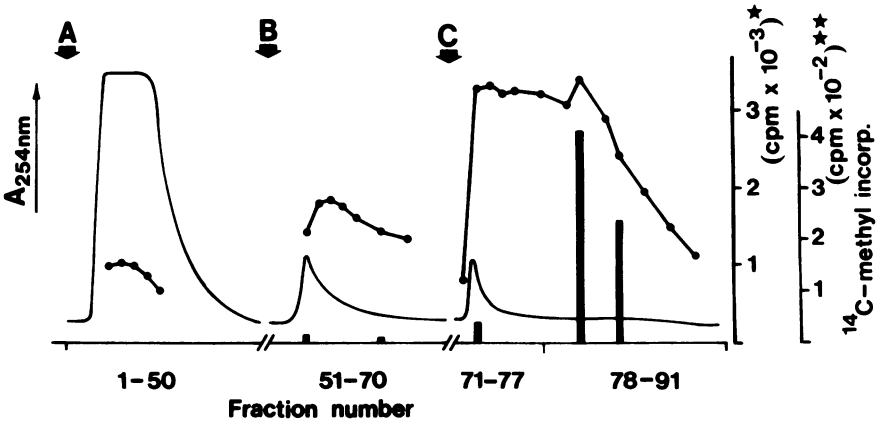

Fig. 1: Chromatography of desalted protein fraction B on tRNA-Sepharose.

850 mg protein was applied to a column (9 x 40 mm) that contained 15.4 mg bound tRNA. The column was equilibrated with buffer A (0.05 M Tris-HCl pH 8). The proteins were eluted stepwise with buffer B (0.04 M Sörensen-phosphate pH 8) and buffer C (0.1 M Sörensen-phosphate pH 8). Fractions of 3.3 ml each were collected, A_{254} nm was measured (———) and 50 µl aliquots were assayed for the presence of tRNA methyltransferases (•—•—•) 1 A_{260} submethylated bulk tRNA from *E. coli* K12 met^- rel^- ★was methylated with the enzyme fraction for 2 hrs at 37°C★. The filled columns show the extent of methylation after 10 min with initiator-tRNA (*B. subtilis*) as substrate and 5 µg protein from fractions 53, 60, 72 and 80, 84.★★

Base analysis of $^{14}CH_3$ labeled tRNA

Protein fraction B and several methyltransferase active fractions obtained during chromatography (Fig. 1) were assayed with respect to base specificity using submethylated tRNA of *E. coli* and initiator-tRNA of *B. subtilis* as substrates. The resulting $^{14}CH_3$-labeled tRNAs were purified (21) and hydrolyzed either with perchloric acid or digested by ribonuclease T_2. The resulting bases respectively nucleotides were analyzed by thin layer chromatography as cited in materials and methods.

The methyltransferases present in protein fraction B methylate in submethylated tRNA from *E. coli* U to m^5U, G to m^7G, C to C_m and A to methylated adenines; in addition two minor unidentified products are formed. With 0.04 M buffer m^7G and mA specific tRNA methyltransferase activities are eluted from the tRNA Sepharose column together with two methyl-

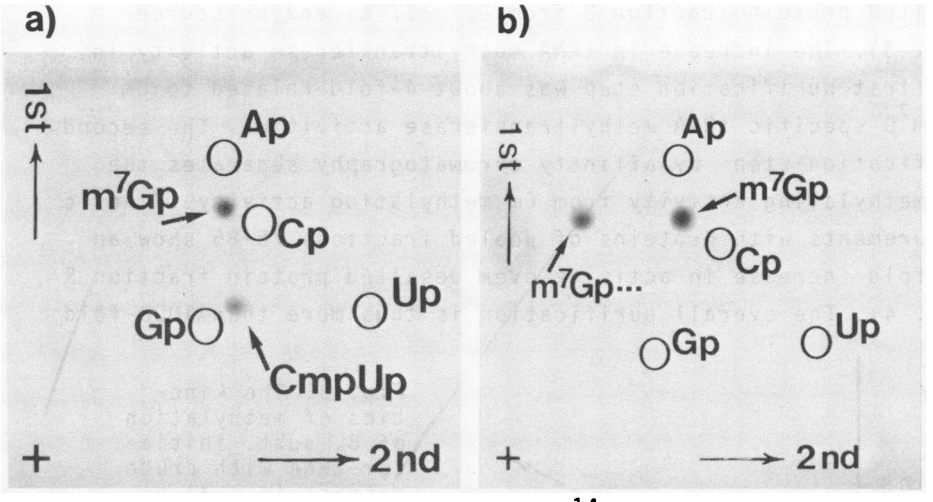

Fig. 2: Autoradiography of (methyl-^{14}C) labeled nucleotides from initiator-tRNA of *B. subtilis* obtained by T_2-digestion after in vitro methylation with protein fraction B (2 a) or pooled fractions 78-85 from tRNA Sepharose chromatography (2 b) and S-adenosyl(methyl-^{14}C)l-methionine.
The radioactive spot migrating slower in the second dimension corresponds to m^{7}G... . This dinucleotide results from an incomplete digestion of tRNA by ribonuclease T_2 (22).

transferases of unidentified specificity (results are not shown). In fractions 78-85 only m^{7}G methyltransferase activity was found with submethylated bulk tRNA from *E. coli*. The m^{5}U specific tRNA methyltransferase activity, which represents about 50 % of total methyltransferase activity in protein fraction B, remains bound to the column and cannot be eluted in the course of affinity chromatography under the conditions described. This explains, why the overall yield of tRNA methyltransferase activity, eluted from the column, is not higher than 50 %.

Enzymes present in protein fraction B methylate in initiator-tRNA of *B. subtilis* G to 7-methylguanine and cytidine to O'-methylcytidine (Fig. 2 a). The purified enzyme fractions (78-85) exhibit exclusively m^{7}G activity (Fig. 2 b).

Measurement of m^{7}G methylating activity

To determine the degree of purification during the isolation procedure methyltransferase activity was measured with $tRNA_f^{Met}$ of *B. subtilis* as substrate and the crude extract or

desalted protein fraction B from *E. coli* as enzyme source (Fig. 3). The increase in tRNA methyltransferase activity in the first purification step was about 4-fold related to Cm and m^7G specific tRNA methyltransferase activities. The second purification step by affinity chromatography separates the m^7G methylating activity from Cm methylating activity. Kinetic measurements with proteins of pooled fractions 78-85 show an 200-fold increase in activity over desalted protein fraction B (Fig. 4). The overall purification is thus more than 1000-fold

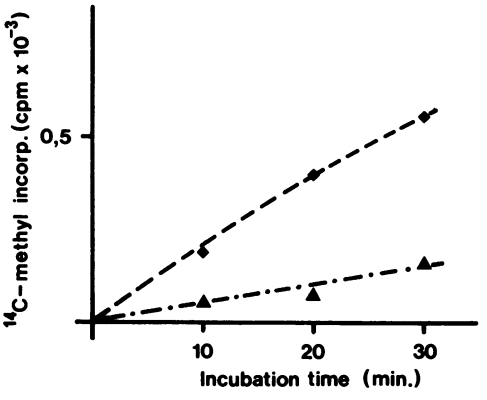

Fig. 3: The kinetics of methylation of B. subt. initiator-tRNA with crude extract (▲——▲) or protein fraction B (◆——◆) from E. coli MRE 600. The assay contained 0.1 A_{260} tRNA and 250/ug protein. All other assay conditions are described under methods

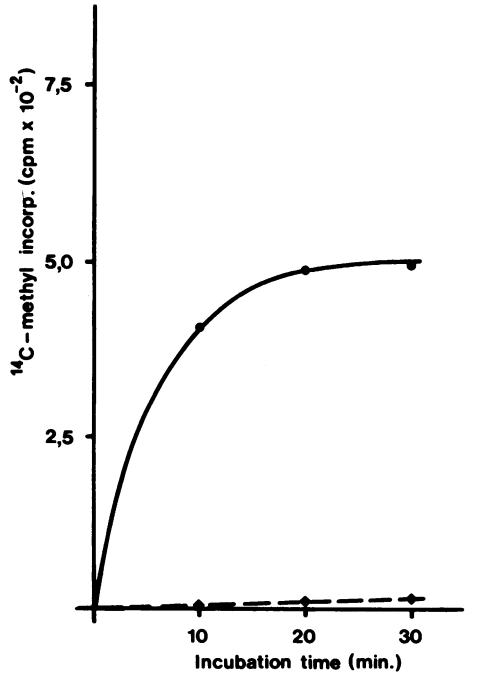

Fig. 4: The kinetics of methylation of B. subt. initiator-tRNA with protein fraction B (◆——◆) and pooled fractions 78-85 (●——●) from tRNA-Sepharose chromatography. The assay contained 0.1 A_{260} tRNA and 5/ug protein. All other assay conditions are described under methods.

because the activity of the cytidine methyltransferase, present in the crude extract and protein fraction B but separated in the last step cannot exactly be determined (Table 1). The relative high yield of m^7G specific methyltransferase activity (47 %) at the end of the purification is explained by the stabilizing effect of the matrix bound tRNA on the enzyme.

Table 1: Purification of a m^7G-specific methyltransferase from *E. coli* MRE 600

	total protein mg	total activity U	specific activity mU/mg	purification fold	yield protein %	yield activity %
crude extract	6332	299.5	4.73		100	100
protein fraction B	848	165.6	19.53	4.13	13.4	55
pooled fractions 78-91	3.56	140.6	3950	835	0.06	47

A unit of methyltransferase (U) is defined as the amount of enzyme, which catalyzes the incorporation of 1 nMol ^{14}C-labeled methyl group into initiator-tRNA per min under the conditions described.

Storage of m^7G specific tRNA methyltransferases

The activity of pooled fractions 78-85 from affinity chromatography was stable for at least several months when small samples were frozen in liquid nitrogen and stored at -70°C.

Disc-electrophoresis

Analytical disc-electrophoresis of 80 µg protein from fractions 78-85 in 5.6 % polyacrylamide gels at 1°C show three distinct bands besides the marker protein (Fig. 5). A duplicate unstained gel was sliced into 2 mm pieces. These slices were used without further treatment and tested for the presence of methylating activity in the standard assay with $tRNA_f^{Met}$ from *B. subtilis*. Two active bands were detected with this technique.

Molecular weight estimation of m^7G methylating activity

Diffusion chromatography on a Sephadex G-200 gel column was used to estimate the molecular weight of m^7G specific methyl-

transferase activity (Fig. 6). The column was adjusted with four standard proteins ofknown molecular weight (katalase, ferritin, transferrin, hydroxynitrile lyase). The void volume of the gel bed was calculated with Dextran Blue and found to be constant during the whole procedure. 350 µg protein of pooled

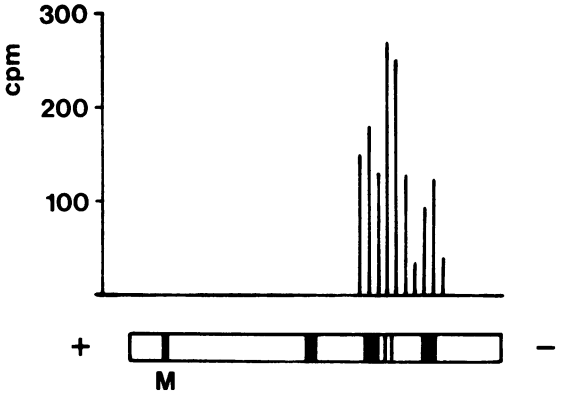

Fig. 5: Disc-electrophoresis of 80 µg protein from fractions 78-85 obtained during tRNA-Sepharose chromatography. Each column in the upper graph represents cpm obtained when 2 mm slices were tested for the presence of methylating activity in the standard assay with $tRNA_f^{Met}$ as substrate. A duplicate gel after staining with Comassie Brilliant Blue shows the location of the proteins (lower part of this figure).

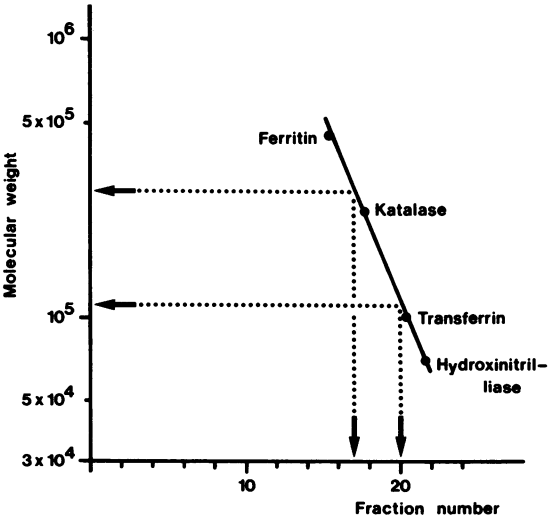

Fig. 6: Plot of the migration distance (elution volume) against log molecular weight for gelfiltration of standard proteins and m^7G methyltransferase activities on a Sephadex G-200 column. The void volume was estimated with Dextran Blue.

fractions 78-85 was applied to the column. Methylating activity was found in two fractions, that correspond the molecular weights of 1 x 10^5 respectively 3 x 10^5 Daltons.

DISCUSSION

In bacteria the methylation of tRNA mainly occurs at the precursor level (23). However submethylated tRNA that accumulates in *E. coli* K12 met$^-$ rel$^-$ during methionine starvation can be fully methylated in vitro with crude extracts from *E. coli* as enzyme source and S-adenosylmethionine as donor of methyl groups (1). These results suggest that methyltransferases recognize structures in a tRNA molecule, that are already present at the precursor level and are preserved during processing. The purified enzyme described here methylates in submethylated tRNA of *E. coli* G residues to 7-methylguanine. Sequence analyses of several tRNA species of *E. coli* show that m^7G is located in the extra arm (24). Thus the enzyme described here might recognize the extra arm unpaired methyldeficient guanylate residue.

So far studied tRNA methyltransferases are capable of recognizing a certain sequence in an intact heterologous tRNA molecule and introduce a methyl group into a specific base at a well defined position (7,25,26). $tRNA_f^{Met}$ of *B. subtilis* was found to be homologous to the initiator-tRNA of *E. coli* with respect to the sequences from position (counted from the 5' end) 19-50 with the exception that in tRNA of *B. subtilis* a cytidine residue is present instead of the ribose methylated cytidine at position 33 (anticodon loop) and a guanine residue instead of 7-methylguanine at position 47 (extra arm). The initiator-tRNA of *B. subtilis* was therefore isolated and tested whether it could serve as substrate for the m^7G specific tRNA methyltransferase from *E. coli*. The results show that this tRNA can be recognized by the *E. coli* enzyme and we therefore assume, that the enzyme methylates G to m^7G in the extra arm of initiator-tRNA of *B. subtilis*.

An important aspect to be mentioned here is the observation that $tRNA_f^{Met}$ is not methylated in G whereas a m^7G specific tRNA methyltransferase activity is present in *B. subtilis* (27). The

enzyme is capable of methylating in submethylated bulk tRNA G residues to m^7G. Nucleoside, respectively nucleotide analysis of $tRNA^{Phe}$ of *B. subtilis* shows the presence of one m^7G residue per one tRNA molecule. The m^7G residue is located in the extra arm (28). Thus in *B. subtilis* an enzyme specific for the methylation of G to m^7G in the extra arm is present, but does not methylate the extra arm unpaired guanylate residue in initiator tRNA. Our results show that m^7G specific activity of *E. coli* extracts can be eluted from the tRNA-Sepharose columns in two peaks, both active with submethylated bulk tRNA from *E. coli*. However initiator tRNA of *B. subtilis* is methylated only by the enzyme eluted by higher salt concentration. This suggests that in *E. coli* at least two m^7G specific tRNA methyltransferases occur with different specificities for the extra arm unpaired guanylate residue in different tRNA species.

The initiator tRNA of *B. subtilis* was used throughout the purification procedure. This allowed us to calculate the degree of purification in each step. During disc-electrophoresis and gel-filtration the m^7G specific tRNA methyltransferase splits - in repeated experiments from different preparations - into two methylating activities. Whether the active fractions differ in molecular weight and electrophoretic mobility because of proteolysis during the separation procedure or whether the enzyme is built up by several active subunits is under current investigation.

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