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# In vitro methylation of yeast $tRNA^{Asp}$ by rat brain cortical tRNA-(adenine-1) methyltransferase

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*ABSTRACT*

*Rat brain cortices from young animals contain large amounts of tRNA(adenine-1)methyltransferase(s). The enzyme(s) can methylate E.coli tRNA and to a lower degree yeast tRNA. Among yeast tRNA species which can be methylated we have selected $tRNA^{Asp}$ as a substrate for the brain enzyme. The digestions of in vitro methylated [Me-$^3$H]-$tRNA^{Asp}$ with pancreatic and/or $T_1$ ribonucleases followed by chromatographies on DEAE-cellulose, 7 M urea, suggested that the methylation of $tRNA^{Asp}$ occurred at a single position within the D-loop. Further digestion of the radioactive oligonucleotide recovered after DEAE-cellulose chromatography by phosphomonoesterase and snake venom phosphodiesterase enzymes followed by bidimensional thin layer chromatography enabled us to determine the location of the adenine residue which becomes methylated by the brain enzyme. This one resulted to be the adenine 14 in the D-loop of yeast $tRNA^{Asp}$.*

## INTRODUCTION

Studies on tRNA(adenine-1)methyltransferases (tRNA-AMT) to determine the site specificity requirements of this family of enzymes have been done in the past (1-4). For instance in *B.subtilis* there is a tRNA-AMT which methylates adenine moieties which are located in the D-loop ($A_{22}$) of certain tRNAs (1,2). Studies carried out in higher plants showed that at least three tRNA-AMT enzymes are present there (3). The cytoplasmic one, able to methylate an adenine residue located in the TψC loop ($A_{58}$) of yeast $tRNA^{Asp}$, whereas the mitochondrial and chloroplastic enzymes both methylate, in addition to the same residue $A_{58}$, the adenine residue in position 7 (acceptor stem).

In mammals, tRNA-AMT enzymes also have attracted the attention of many groups. In that way, it has been reported that rat liver enzyme methylates *E.coli* tRNAs in position 58 (4,5,6), as occurred with enzyme preparations derived from higher plants. A similar situation was seen to happen when colon or kidney ex-

 

tracts from rats were used to methylate several *E.coli* tRNAs (7).

In a previous study we demonstrated the presence of high levels of tRNA-AMT in rat brain tissue from young animals (8). The enzyme which seems to be distributed mostly in the neuronal compartment, methylates *E.coli* tRNAs and yeast tRNAs provided that the methyl donor S-adenosyl-L-methionine and spermidine are present in the incubation mixture. Therefore, we considered relevant to study the site specificity requirements of the enzyme to compare its properties to other mammalian enzymes.

## MATERIAL AND METHODS

Bulk tRNA from yeast and S-adenosyl-L-methionine were from Boehringer, Mannheim, GFR. S-adenosyl-L-[Me-$^{14}$C]-methionine (specific activity 46 mCi/mmol) and S-adenosyl-L-[Me-$^{3}$H]-methionine (specific activity 15 mCi/mmol) were from CEA, Gif-sur-Yvette, France. Phosphocellulose (P-11) and DEAE-cellulose paper (DE-81) for high voltage electrophoresis were from Whatman, Maidstone, G.B. DEAE-cellulose was from Schleicher and Schuell, Keene, N.H., USA. Cellulose plates for thin layer chromatography were from Schleicher and Schüll, Dassel, GFR. Spheroidal hydroxylapatite was from Merck, Darmstadt, GFR. Adenosine, 1-methyladenosine, 6-methyladenosine, adenosine 5'-phosphate and spermidine phosphate were from Sigma Chemical Co., St. Louis, Mo, USA.

Pancreatic RNAase, phosphomonoesterase (BAPF), snake venom phosphodiesterase and $T_1$ RNAases were from Worthington Biochem. Corp., New Jersey, USA. Yeast $tRNA^{Asp}$ was obtained as described elsewhere (9). Yeast $tRNA^{Trp}$ was a kind gift from Dr. G. Keith.

Numbering of nucleotides : The numbering of nucleotides we have adopted for tRNAs is that of cytoplasmic yeast $tRNA^{Phe}$ which contains 76 nucleotides (8 nucleotides in the D-loop and 5 nucleotides in the variable loop). The terminal end always carries number 76 even in tRNAs having less nucleotides than yeast $tRNA^{Phe}$ like $tRNA^{Asp}$ which has only 75 nucleotides (10).

Preparation of rat brain tRNA-AMT : Sprague-Dawley rats (7-days old) were decapited and the brain cortices removed and processed as described elsewhere (8). After hydroxylapatite chromatography, tRNA-AMT was fractionated by phosphocellulose

(30 x 2 cm) chromatography. The column was equilibrated before sample loading with a buffer solution containing 0.1 M potassium phosphate (pH 6.5), 0.5 mM EDTA, and 10 mM β-mercaptoethanol. The elution of the enzyme fractions was done as described elsewhere (11). The active fractions were precipitated with ammonium sulfate to 80% saturation and the protein insoluble material recovered by centrifugation at 15,000 x g for 10 min. The supernatant was discarded and the pellet resuspended in a buffer solution 10 mM Tris-HCl (pH 8.0) containing 1 mM dithiothreitol and 0.1 mM EDTA. The remaining ammonium sulfate was eliminated from the protein solution by extensive dialysis against the same buffer solution. The final solution was stored in small aliquots at -70°C.

Methylation of yeast $tRNA^{Asp}$ : The incubation mixture contained in a final volume of 0.5 ml ; 50-75 µg $tRNA^{Asp}$, 0.2 µCi S-adenosyl-L-[Me-$^{14}$C] -methionine or 2 µCi S-adenosyl-L-[Me-$^{3}$H] -methionine (1.5 Ci/mmol), 2.5 mM spermidine phosphate, 1 mM dithiothreitol and 0.15 mg of enzyme protein. The mixture was incubated at 37°C for 3 h and the [Me-$^{3}$H] - tRNA or [Me-$^{14}$C] -tRNA formed was freed of protein by phenol extractions, precipitated by ethanol and stored at -20°C (12).

Total enzymatic hydrolysis of [Me-$^{3}$H] -$tRNA^{Asp}$ into nucleosides and their subsequent separation by bidimensional thin layer chromatography on cellulose coated aluminium foil was carried out according to Rogg *et al.* (13).

Analysis of labeled nucleotide sequences : Hydrolysis of [Me-$^{3}$H]-$tRNA^{Asp}$ by pancreatic or $T_1$ RNAases were performed according to Gangloff *et al.* (14). $tRNA^{Trp}$ from yeast to be used as source of A-$m^1$A-Up marker was hydrolyzed by pancreatic RNAase according to Gangloff *et al.* (14). Fractionation of the resulting oligonucleotide mixture was achieved by DEAE-cellulose chromatography in the presence of 7.0 M urea at pH 7.2 as previously described (15). The radioactivity was determined after mixing aliquots (100 µl) from each fraction with 3 ml of Unisolve I Scintillator (Koch-Light Labs, Colnbrook, Bucks, GB).

Further digestion of the oligonucleotides was performed by the action of phosphomonoesterase followed by digestion with snake venom phosphodiesterase (14). In this case removal of the

phosphomonoesterase must be done before digestion with phosphodiesterase (16). The final nucleotide and nucleoside mixture was fractionated by bidimensional thin layer chromatography as described elsewhere (14) in the presence of non-radioactive nucleotides and nucleosides used as standards. Protein measurements were done according to Lowry *et al.* (17) using bovine serum albumin as standard.

## RESULTS

Rat brain tRNA-AMT was fractionated by hydroxylapatite and phosphocellulose columns (see Methods section). Phosphocellulose chromatography resolved two peaks of methyltransferase activity, (Fig.1) only the second one, was used as the source of tRNA-AMT. The enzyme methylates bulk yeast tRNA but to about 1/3 the extent obtained with bulk *E.coli* tRNA.

The reaction requires $Mg^{++}$ ions or polyamines (8) to occur and various tRNAs differ in their methyl acceptor properties when using the brain enzyme (results not shown). Among tRNAs that can be methylated we selected yeast $tRNA^{Asp}$ because we know

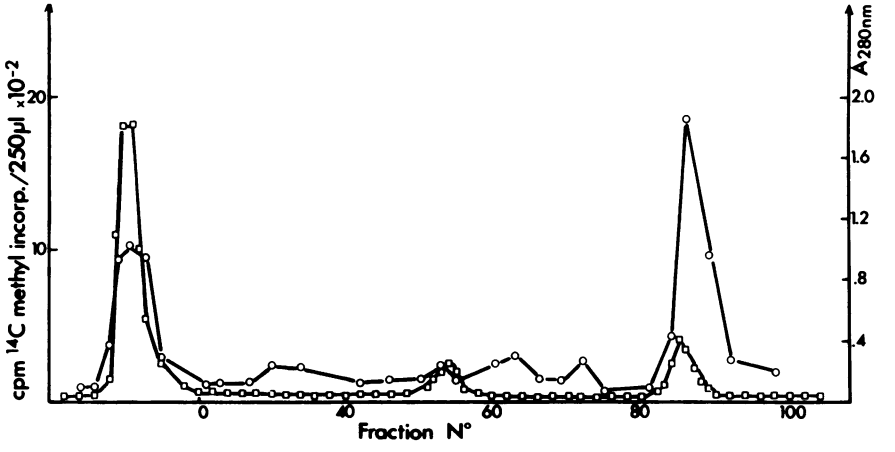


Fig. 1. Phosphocellulose chromatography of rat brain cortical tRNA-AMT. The enzyme fractions obtained after chromatography on hydroxylapatite columns were then fractionated by phosphocellulose (see Methods Section). The tRNA-AMT activity in the fractions (4 ml/fraction) was assayed in the presence of bulk yeast tRNA (see Methods Section). □—□—□ $A_{280}$ ; o—o—o [Me-$^{14}$C] -$tRNA^{Asp}$ formed.

its primary structure (14) and also because of its relatively high content of non-modified adenine making this tRNA a suitable substrate for tRNA-AMT.

tRNA was methylated *in vitro* in the presence of S-adenosyl-L- [Me-$^{3}$H] -methionine and then completely digested with $T_1$ RNAase.

The hydrolysate was resolved by chromatography on DEAE-cellulose, 7 M urea. The elution profile obtained is shown in Fig.2, it agrees well with that obtained with a $tRNA^{Asp}$ hydrolysate, chromatographed under similar conditions (3,14). In addition, it allowed us to assign the composition of the various U.V. absorbing peaks. Fig.2 shows that approximately 80% of the radioactivity recovered eluted in the region corresponding to tetranucleotides. Oligonucleotides carring a positively charged $m^1A$ modification generally elute more than one chain lenght earlier than the non-methylated homologous nucleotides, therefore the methylated oligonucleotide is at least 5 nucleotides long. Fig.2 shows that after $T_1$ digestion of $tRNA^{Asp}$ only 3 oligonucleotides behave as if they contain 5 or more nucleotides ; U-U-ψ-A-A-D-Gp, pU-C-C-Gp and T-ψ-C-A-A-U-U-C-C-C-C-Gp, the

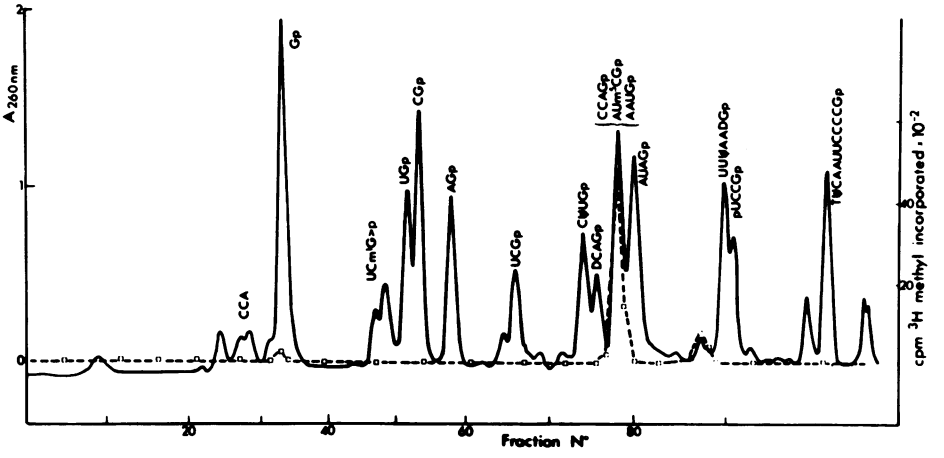


Fig. 2. DEAE-cellulose, 7 M urea chromatography of a $T_1$ RNAase digest from yeast $tRNA^{Asp}$. The tRNA was methylated *in vitro* by the brain enzyme as described in the Methods Section, 200 µg of $tRNA^{Asp}$ were digested with 60 units of $T_1$ RNAase as described elsewhere (14). The digests were fractionated on a DEAE-cellulose column 0.3 x 150 cm using a NaCl linear gradient (0-0.33 M) in 0.02 M Tris-HCl (pH 7.2). The gradient total volume was 200 ml (2 ml/fraction). ——— $A_{260}$ ; ----- radioactivity.

first and the third one only carrying adenine residues. The positioning of the radioactive peak in Fig.2 suggests that methylation of $tRNA^{Asp}$ took place on the heptanucleotide sequence occurring in the D-loop, since a methylated dodecanucleotide would elute around the octa- or nonanucleotide region, as previously shown (3).

Alternatively, $[Me\text{-}^{3}H]\text{-}tRNA^{Asp}$ was digested with pancreatic RNAase and the hydrolysate also fractionated by DEAE-cellulose, 7 M urea. The result of this experiment is summarized in Fig.3 which again shows that a large portion of the radioactivity eluted as a single peak, this time though in front of the dinucleotide peak. Consequently, the methylated material must be the trinucleotide A-A-Dp belonging to the U-U-ψ-A-A-D-Gp sequence. We therefore assumed that the position 14 or 15 in yeast $tRNA^{Asp}$ is the one involved in the methylation reaction. Thus, subsequent experiments were carried out to confirm the identity of the methylated adenine in the D-loop.

The radioactive peak obtained after DEAE-cellulose, 7 M urea (Fig.2) was pooled and desalted by passage on a DEAE-cellulose column, eluted with 1 M triethylammonium carbonate as previously described (18). The resulting solution was digested with pancreatic RNAase and the hydrolysate was fractionated by high voltage electrophoresis on DEAE-cellulose paper (7% formic

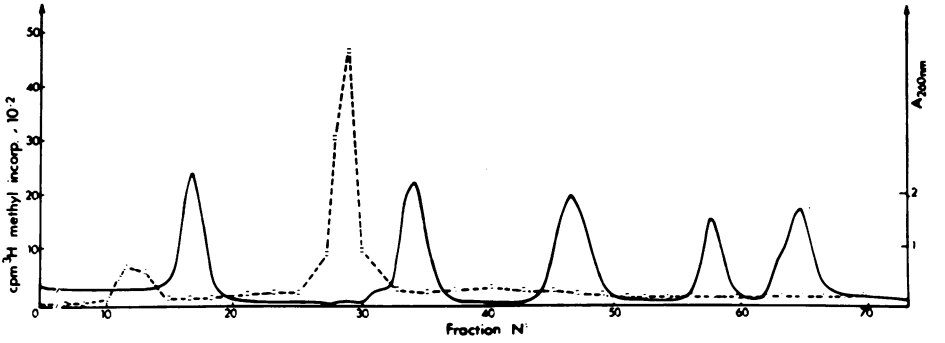


Fig. 3. DEAE-cellulose, 7 M urea chromatography of a pancreatic RNAase digest from yeast $tRNA^{Asp}$. The tRNA was previously methylated *in vitro* as described in the Methods Section, 200 µg $tRNA^{Asp}$ were digested with 20 µg of pancreatic RNAase as described elsewhere (14). For experimental details see the legends to Fig.2. ——— $A_{260}$ ; – – – – radioactivity.

acid) as described by Gangloff *et al.* (14). In a parallel experiment we isolated by high voltage electrophoresis the trinucleotide A-m$^1$A-Up resulting from pancreatic RNAase digestion of yeast tRNA$^{Trp}$ (19). The non-radioactive trinucleotide thus obtained and the radioactive methylated trinucleotide recovered after pancreatic RNAase digestion were fractionated together by high voltage electrophoresis. A previous study showed that under these experimental conditions A-A-Dp migrate toward the anode slightly ahead of A-A-Up (14). We found after high voltage electrophoresis that (Fig.4) the methylated radioactive trinucleotide migrated in front of the trinucleotide A-m$^1$A-Up used as U.V. marker. We therefore conclude that the radioactive modified trinucleotide obtained after tRNA$^{Asp}$ methylation was m$^1$A-A-Dp or A-m$^1$A-Dp. The radioactive peak after being eluted from the DEAE-cellulose paper was digested with phosphomonoesterase and phosphodiesterase (see Methods). The final hydrolysate mixture containing nucleotides and a nucleoside from the 5'-end were resolved by bidimensional thin layer chromatography. Analysis of the radioactivity recovered after thin layer chromatography (Table I) indicates that 77% of it comigrated with the nucleoside m$^1$A (non radioactive m$^1$A was added as a marker). Also, a minor position of the radioactivity (22%) was found to migrate together with N$^6$-methyladenosine which is known to be a conversion product of m$^1$A at alkaline pH (20). These results led

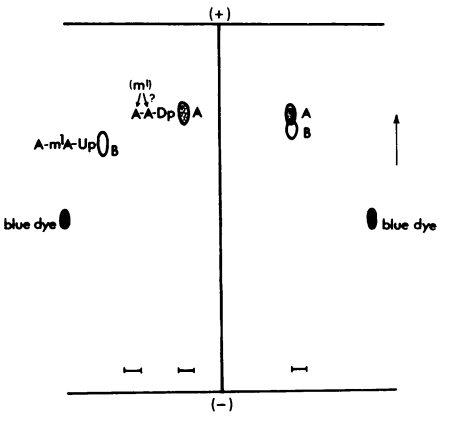


Fig. 4. High voltage electrophoresis of Me-$^3$H -oligonucleotide recovered from DEAE-cellulose chromatography and digested by pancreatic RNAase. The fractionation was carried out on DEAE-cellulose paper (DE-81) with 7% formic acid at 900 volts during 13 h (14). The U.V. absorbing material and the radioactivity were recovered from the paper with 1 M triethylammonium carbonate buffer pH 8.0. The left side represents the separation of (m$^1$)A-A-Dp (A) and A-m$^1$A-Up (B) without mixing samples at the beginning. The right side shows the same separation after mixing A and B.

Table I. BIDIMENSIONAL THIN LAYER CHROMATOGRAPHY OF A [Me-$^3$H] -TRINUCLEOTIDE DIGESTED WITH PHOSPHOMONOESTERASE AND SNAKE VENOM PHOSPHODIESTERASE

| Compound | CPM * | % |
|---|---|---|
| Adenosine | - | - |
| 1-Methyladenosine | 24,460 | 77,7 |
| 6-Methyladenosine | 7,016 | 22,3 |
| 1-Methyladenosine 5' phosphate | - | - |

* Total CPM recovered after bidimensional thin layer chromatography. Radioactivity from the plates was scrapped off, water extracted and recovered by low speed centrifugation (1,000xg, 5 min). Radioactivity in the solution was measured after mixing aliquots (100 µl) with Unisolve I, scintillator. Adenosine, 1-methyl adenosine and 6-methyl adenosine were added as standards to the radioactive mixture. Solvent mixture : First dimension (25% $NH_3$)-propanol-water ; (30:60:10, v/v) ; Second dimension HCl conc.-isopropanol-water; (17.6:68:14.4) ; v/v).

us to conclude that in tRNA$^{Asp}$ the adenine residue located in position 14 corresponding to the trinucleotide m$^1$A-A-Dp (Fig.5) becomes methylated by the brain enzyme.

## DISCUSSION

In recent years many laboratories studied the nucleotide positions within the tRNA structure recognized by methyltransferases. In that way, it was shown that tRNA-AMT present in *B.subtilis* methylates the unpaired adenine residue located in

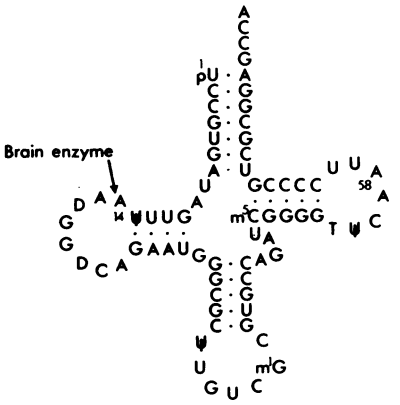


Fig. 5. Nucleotide sequence of yeast tRNA$^{Asp}$ (14).

the 3'terminal position of the D-loop occurring in several tRNAs (1,2). On the other hand, the rat liver tRNA-AMT which has been purified in many laboratories seems to be specific for the adenine moieties found in position 58 from different tRNAs (3-5, 6). Similar results were obtained by Pegg (7) who analyzed the site specificity of crude extracts from mouse colon or rat kidney as a source of tRNA-AMT to methylate several *E.coli* tRNAs. Unexpectedly, as shown by our results, rat brain cortical tRNA-AMT failed to methylate the adenine 58 located in the TψC-loop of yeast $tRNA^{Asp}$ but in turn methylated the adenine residue in position 14.

Although the presence of $m^1A_{14}$ in the D-loop is being demonstrated before in beef liver $tRNA^{Phe}$ (21,22) this is the first time in which an enzyme responsible for such modification is being characterized. However, if we inspect the results obtained by Dubois *et al.* with tRNA-AMT preparation derived from chloroplasts of *Phaseolus vulgaris* (3), we observe that after $T_1$ RNAase digestion of *in vitro* methylated $tRNA^{Asp}$ a small but measurable amount of radioactivity elutes in the position corresponding to the tetranucleotide peak, but the authors did not characterize the identity of this methylated oligonucleotide. This radioactivity represents about 27% of the major radioactive methylated dodecanucleotide peak characterized in that study. From our results we conclude that the minor radioactive peak detected in that opportunity corresponded to the methylated heptanucleotide determined in the present study. Moreover, in a recent work (23), yeast $tRNA^{Phe}$ has been successfully methylated *in vitro* with a methyltransferase preparation from chloroplasts similar to that used previously to methylate yeast $tRNA^{Asp}$. The analysis of the modified nucleoside obtained under these experimental conditions indicates that methylation occurred in the adenine moiety in position 14 from the 5'-end, thus confirming the existence of the enzyme activity responsible for that modification in *Phaseolus vulgaris* ; It would be of interest to know whether the chloroplastic enzyme occurring in *Phaseolus vulgaris* and the brain enzyme characterized in the present study can both methylate other tRNA structures to confirm the homology between the two enzymes.

To conclude, we must point out that at present time we cannot rule out the possibility that rat brain cells contain also a tRNA(adenine-1)methyltransferase activity specific for the TψC loop. However, in our experiments after phosphocellulose chromatography, all the tRNA(adenine-1)methyltransferase detected in both activity peaks (Fig. 1) methylated only the position 14 from the D-loop. Future studies will be oriented to demonstrate whether rat brain crude extracts contain or not the TψC specific enzyme.

ACKNOWLEDGMENTS

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Abreviation

$m^1A$ : 1-methyladenosine

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