METHYLATION OF YEAST ASPARTIC ACID TRANSFER RNA BY RAT LIVER EXTRACTS

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1. Introduction

It is now well-established that the methylated nucleosides in tRNA are formed by the action of enzymes transferring methyl groups to preformed nucleic acid [1, 2]. Furthermore, this reaction is species-specific and enzymes from mammalian tissues are known to be able to incorporate additional methyl groups into tRNA from yeast and bacteria [1–6]. Extracts prepared from rat liver produce at least 7 methylated bases when acting on tRNA from *E. coli* in the presence of polyamines or NH_4^+ [3, 4]. The use of purified tRNA preparations as substrates for methylation may produce a much simpler pattern of methylated products and permit detailed analysis of methylation reactions [5–7]. This paper describes the methylation of $tRNA^{Asp}$ from brewers yeast by rat liver methylases. It was found that the product of this reaction was almost exclusively N^2-methylguanine and the site of methylation was the guanosine residue at position 26 from the 5′-OH end of this tRNA.

2. Methods

S-adenosyl-L-Me-^{14}C-methionine (50–60 mCi/mmole) was purchased from The Radiochemical Centre, Amersham, Bucks., England. The methylated bases and nucleosides used as markers for chromatography were obtained from Sigma Chemical Company, London and the Cyclo Chemical Company, California, USA. DEAE-cellulose used was Cellex-D from Bio-Rad Laboratories, Richmond, California, USA. DEAE-Sephadex A-25 was purchased from Pharmacia, Uppsala, Sweden. Purified $tRNA^{Asp}$ from brewers yeast was a generous gift from Dr. I. Gillam and Prof. G.M. Tener, Univ. of British Columbia, Canada. The preparation was at least 95% pure $tRNA^{Asp}$ [8].

The preparation of rat liver tRNA methylase, the assay of methylase activity and the determination of the methylated bases produced in this reaction were as previously described [3, 7]. The preparation used as a source of tRNA methylases in the present experiments was the crude ammonium sulphate precipitate. The labelled, methylated $tRNA^{Asp}$ was digested with pancreatic ribonuclease or ribonuclease T_1 (Sigma Chemical Company, London) and the oligonucleotides produced were fractionated on columns of DEAE-cellulose or DEAE-Sephadex respectively, in the presence of 7 M urea, 0.02 M Tris-HCl, pH 7.5 and a gradient of NaCl according to published methods [9–11]. Samples of 1 ml of the fractions from the column eluate were assayed for radioactivity in the presence of 15 ml of a mixture of 2 parts (by volume) of 0.6% (w/v) 2,5-diphenyloxazole in toluene to 1 part of Triton-X100. The efficiency of counting for ^{14}C was about 60%.

3. Results and discussion

The methylation of heterogenous preparations of tRNA from *E. coli* by rat liver extracts proceeds at a limited rate in the presence of Mg^{2+} but is greatly increased by the addition of polyamines or a high concentration of NH_4^+ [3, 12]. Similar results to those previously published are shown in table 1 which also shows that the liver extracts catalysed the methylation

Table 1
Methylation of $tRNA^{Asp}$ and unfractionated tRNA in the presence of various cations.

Cation added	Methylation of tRNA (pmoles/15 min/mg protein)	
	Unfractionated tRNA	$tRNA^{Asp}$
2 mM Mg^{2+}	32	31
0.3 M NH_4^+	155	28
15 mM Putrescine	150	25
1.5 mM Spermidine	136	22

The assay medium contained 10 μmoles Tris-HCl, pH 8.6, 5 nmoles *S*-adenosyl-L-Me-^{14}C-methionine (50 mCi/mmole), about 0.5 mg liver protein, the cation conc. shown and either 200 pmoles yeast $tRNA^{Asp}$ or 50 μg tRNA from *E. coli* K 12 (sub-methylated) in a total volume of 0.1 ml.

Table 2
Methylated bases present in $tRNA^{Asp}$ after methylation by rat liver enzymes *in vitro*.

Methylated Base	Radioactivity present	
	(dpm)	(% total incorporation)
1-Methyladenine	171	2
N^2-Methylguanine	7905	96
5-Methylcytosine	148	2

$tRNA^{Asp}$ was methylated as described in table 1 in a scaled-up reaction mixture of total volume 0.5 ml. The cation present was 0.25 M NH_4^+. After incubation at 37° for 15 min the tRNA was re-isolated and the methylated bases which had been labelled analysed as previously described [3, 7]. The total radioactivity present in the isolated tRNA was 8,286 dpm and 99% of this was accounted for in the products shown.

of purified yeast $tRNA^{Asp}$ but in this case the rate of reaction was approximately the same whether Mg^{2+}, NH_4^+ or polyamines were added. Analysis of the methylated bases labelled after incubation of $tRNA^{Asp}$ with *S*-adenosyl-L-Me-^{14}C-methionine and rat liver extracts revealed that 96% of the incorporated radioactivity was present in N^2-methylguanine (table 2). The remainder was equally distributed between 5-methylcytosine and 1-methyladenine and could well be due to methylation of impurities in the $tRNA^{Asp}$ preparation. Thus although enzymes capable of producing at least 7 methylated bases (1-methyladenine, 5-methylcytosine, N^2-methylguanine, N^2,N^2-dimethylguanine, 1-methylguanine, 7-methylguanine and 5-methyluracil) are present in the rat liver extracts used as source of methylases [3, 4] only the enzyme(s) forming N^2-methylguanine recognises a suitable site for methylation in yeast $tRNA^{Asp}$. The finding that this reaction proceeds equally well in the presence of Mg^{2+} as in the presence of the other cations which

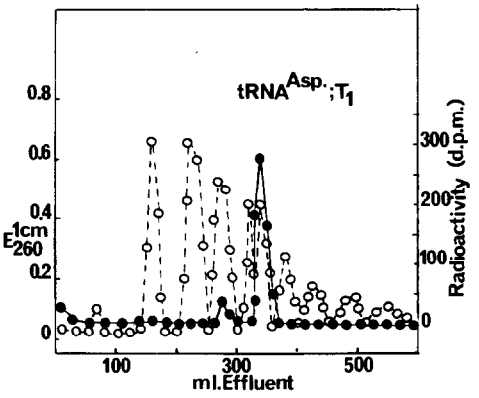

Fig. 1. DEAE-Sephadex chromatography of ribonuclease T_1 digest of ^{14}C-methylated $tRNA^{Asp}$. $tRNA^{Asp}$ was methylated as described in table 1, added to 0.5 mg of *E. coli* tRNA and digested with 50 units of ribonuclease T_1 at 37° for 6 hr. The digest was applied to a column (80 cm × 1 cm) of DEAE-Sephadex A-25, the column washed with 50 ml of 20 mM Tris-HCl, pH 7.5, 7 M urea and the oligonucleotides then separated by elution with a gradient of 0.1–0.45 M NaCl in this buffer.

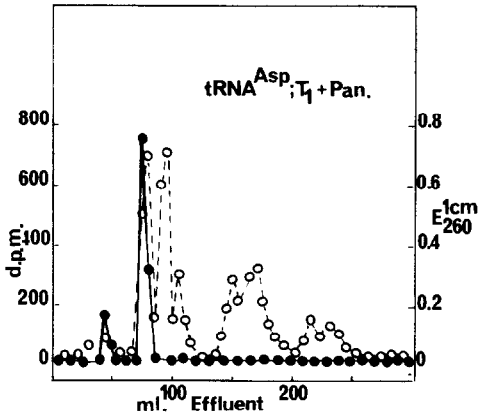

Fig. 2. DEAE-cellulose chromatography of ^{14}C-methylated $tRNA^{Asp}$ digested with pancreatic and T_1 ribonuclease. Methylated $tRNA^{Asp}$ and carrier tRNA (fig. 1) was digested with 50 units ribonuclease T_1 and 23 units pancreatic ribonuclease at 37° for 6 hr. The digest was then chromatographed on a column (60 cm × 1 cm) of DEAE-cellulose with a linear gradient of 0.1–0.45 M NaCl in 7 M urea, 20 mM Tris-HCl, pH 7.5 as described above.

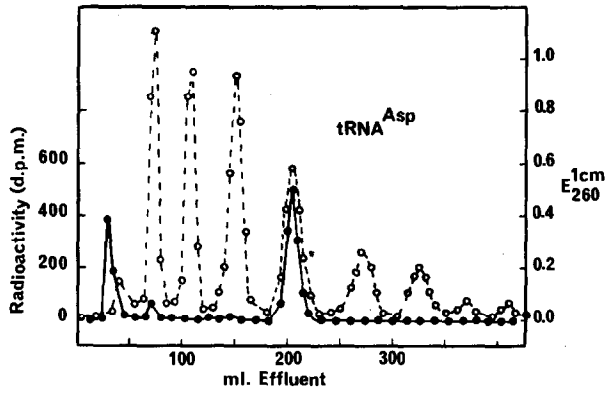

Fig. 3. DEAE-cellulose chromatography of pancreatic ribonuclease digest of ^{14}C-methylated tRNA^{Asp}. Methylated tRNA^{Asp} (and carrier tRNA) was digested with 23 units of pancreatic ribonuclease for 6 hr at 37° and the digest chromatographed as described for fig. 2.

stimulate tRNA methylases is in agreement with the data of Leboy [4] that Mg^{2+} supported the formation of methylated guanines at rates comparable to those found in the presence of polyamines or NH_4^+ whereas the formation of other methylated bases was much greater when the latter compounds replaced Mg^{2+} in the assay medium. It should be noted that the results shown in table 2 which indicate that N^2-methylguanine was virtually the only product formed by liver extracts acting on tRNA^{Asp} were obtained following methylation of the tRNA in the presence of NH_4^+ which would allow the formation of other methylated bases if suitable sites for methylation were present.

The site within the nucleotide sequence of tRNA^{Asp} at which N^2-methylguanine was produced by rat liver extracts was investigated by digesting the ^{14}C-methylated-tRNA^{Asp} with pancreatic ribonuclease and ribonuclease T_1 and then isolating the labelled oligonucleotides produced by chromatography on DEAE-cellulose or DEAE-Sephadex [9–11]. As shown in fig. 1 the labelled N^2-methylguanine was found to be present in a tetranucleotide following treatment with ribonuclease T_1 suggesting that the sequence methylated was GpXpXpXp2MeGp (where X represents a nucleoside not attacked by ribonuclease T_1). When the labelled tRNA^{Asp} or this tetranucleotide were treated with pancreatic ribonuclease and ribonuclease T_1 the labelled N^2-methylguanosine was liberated as a mononucleotide suggesting that the base preceeding the methylated guanine was a pyrimidine (fig. 2). Treatment of the methylated tRNA^{Asp} with pancreatic ribonuclease alone liberated another tetranucleotide containing the radioactivity (fig. 3) suggesting that the N^2-methylguanosine was present as one of the purines of the sequence PypPupPupPupPyp (where Py is a pyrimidine and Pu is a purine). Combining the information given above suggests that the N^2-methylguanosine was present in an oligonucleotide of composition GpXpXpPyp2MeGpPupPupPyp. A sequence which could be methylated to produce such an oligonucleotide is indeed present in the total nucleotide sequence of tRNA^{Asp} from brewers yeast [13]. This sequence occurs from residue 22 to residue 29 from the 5′-OH end of tRNA^{Asp} and is GpApApUpGpGpGpCp. The data therefore strongly suggest that the site of formation of N^2-methylguanosine by rat liver enzymes acting on yeast tRNA^{Asp} was the guanosine residue at position 26 of the molecule. This residue occupies the position between the stem of the dihydrouridine loop and the anticodon loop. This position within the tRNA molecule is known to be occupied by the methylated nucleoside, N^2,N^2-dimethylguanosine in tRNA^{Tyr}, tRNA^{Ile}, tRNA^{Phe}, tRNA^{Ala} and tRNA^{Ser} from yeast, tRNA^{Phe} from wheat and tRNA^{Ser} from rat liver [14]. N^2-methylguanosine is also produced at a similar position in *E. coli* tRNA^{fMet} by enzymes from rat liver [6] and from rat kidney and mouse colon (Pegg, unpublished observations) but in this case the sequence methylated is UpCpGpUpCp. It is not clear why the methylation *in vitro* by mammalian enzymes proceeds only to the extent of the mono-

methyl derivative but it may be that there is a greater specificity for a particular nucleotide sequence for the second methyl group to be added (either by the same enzyme or a different methylase [15]) since N^2,N^2-dimethylguanosine in known tRNA sequences is always preceded by the dinucleotide GpCp and is followed by C, Ψ or A [14].

It is clear from the results of this study and from previous investigations of the specificity of tRNA methylases [6, 14, 16] that these enzymes attack particular sites within the tRNA molecule which are defined by the structure of the molecule as well as by the sequence of nucleotides around the methylatable site. The sequence Up2MeGpGp contains the methylated base found when $tRNA^{Asp}$ is methylated by liver enzymes but the sequence UpGpGp occurring at residues 18–20 in the dihydrouridine loop of $tRNA^{fMet}$ from *E. coli* and at residues 41–43 in the anticodon arm of yeast $tRNA^{Phe}$ are not methylated by rat liver enzymes ([6]; Pegg, unpublished observations). However, although certain methylated bases occur at unique sites within the tRNA molecules which have been sequenced [14], these sites cannot invariably be methylated by mammalian enzymes when occupied by the relevant unmodified base. Thus although the first nucleoside of the dihydrouridine arm is N^2-methylguanosine in a number of tRNAs the guanosine residue at this site in $tRNA^{Asp}$ and $tRNA^{fMet}$ is not methylated by liver extracts. Similarly a guanosine between the stems of the anticodon arm and the GTΨC arm of many tRNAs which do not have the extra arm in this region is often modified to 7-methylguanosine [14] but $tRNA^{Asp}$ is not methylated by liver enzymes at this position.

The formation of 1-methyladenosine when the rat liver extract employed as a source of methylases in these studies was incubated with $tRNA^{Glu}$, $tRNA^{fMet}$ and $tRNA_3^{Ser}$ from *E. coli* proceeded at a rate at least 60 times greater than when yeast $tRNA^{Asp}$ was added and the formation of 1-methyladenosine when the latter tRNA was a substrate for methylation was so low that it could be accounted for by the presence of other tRNAs as impurities in the $tRNA^{Asp}$ preparation. However, the sequence PypAplMeApUp which could be produced by methylation of $tRNA^{Asp}$ at the second nucleoside after the TpΨpCp sequence (the site at which 1-methyladenosine is uniquely found in tRNAs [14]) is known to occur in rat liver tRNA and to be formed by the action of rat liver methylases on unfractionated yeast tRNA [14, 16]. The reason why this reaction does not in fact occur may have some relevance to the important question posed by Staehelin [14] as to why some tRNAs are modified by methylation *in vivo* but others are not, even though enzymes capable of carrying out the analogous reaction on other tRNAs are present within the cell. The possibility of the existence of tRNA-specific inhibitors of tRNA methylases should not be ignored in this respect [17] and further studies of the methylation of $tRNA^{Asp}$ by enzyme extracts more extensively purified from normal and neoplastic tissues would be of interest.

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References

[1] E. Borek and P. Srinivasan, Ann. Rev. Biochem. 35 (1966) 275.
[2] J.L. Starr and B.H. Sells, Physiol. Rev. 49 (1969) 623.
[3] A.E. Pegg, Biochim. Biophys. Acta 232 (1971) 630.
[4] P.S. Leboy, FEBS Letters 16 (1971) 117.
[5] B.C. Baguley, W. Wehrli and M. Staehelin, Biochemistry 9 (1970) 1645.
[6] K. Kuchino and S. Nishimura, Biochem. Biophys. Res. Commun. 40 (1970) 306.
[7] A.E. Pegg, Biochim. Biophys. Acta, in press.
[8] I. Gillam, D. Blew, R.C. Warrington, M. von Tigerstrom and G.M. Tener, Biochemistry 7 (1968) 3459.
[9] R.V. Tomlinson and G.M. Tener, J. Am. Chem. Soc. 84 (1962) 2644.
[10] J.T. Madison, G.A. Everett and H.K. Kung, J. Biol. Chem. 242 (1967) 1318.
[11] T. Seno, M. Kobayashi and S. Nishimura, Biochim. Biophys. Acta 169 (1968) 80.
[12] P.S. Leboy, Biochemistry 7 (1970) 1577.
[13] G. Keith, J. Gangloff, J.-P. Ebel and G. Dirheimer, Compt. Rend. 271 (1970) 613.
[14] M. Staehelin, Experientia 15 (1971) 1.
[15] J.H. Phillips and K. Kjellin-Straby, J. Mol. Biol. 26 (1967).
[16] B.C. Baguley and M. Staehelin, Biochemistry 8 (1969) 257.
[17] S.J. Kerr, Biochemistry 9 (1970) 690.