Volume 1 number 2 February 1974 Nucleic Acids Research

Acceptor activity of hypermethylated E. coli $tRNA_f^{Met}$

L. P. Shershneva, T. V. Venkstern and A. A. Bayev

Institute of Molecular Biology, Academy of Sciences of the USSR, Moscow, USSR

Received 6 December 1973

ABSTRACT

The acceptor activity of normal E. coli $tRNA_f^{Met}$ was compared with that of a preparation with surplus methyl groups introduced by a crude methylase preparation from rat hepatoma. No changes in charging were detected when the aminoacylation was carried out in a homologous system. The data indicate that neither the surplus methyl groups by themselves, nor the eventual changes in spatial arrangement are essential for charging of E. coli $tRNA_f^{Met}$.

INTRODUCTION

Owing to the rapid progress in tRNA sequencing, the study of minor components has also advanced very rapidly in the last years. Many new minor nucleotides have been discovered, the structures of some complex minor components have been established and a great deal of work has been done in the field of biosynthesis of these compounds. But one of the most important questions, namely that concerning the role of minor components in tRNA structure and function still remains to be solved. The most usual minor components are the methylated derivatives of the four main heterocyclic bases. Their biosynthesis as well as their distribution pattern in individual tRNA molecules from the same, and from different organisms was studied in detail. In particular it is known that there are species differences in the total quantity and in the distribution pattern of methylated components, tRNAs from E. coli are for instance much poorer in these compounds than tRNAs from yeast and mammalian tissues. The species specificity results in the possibility of introducing in vitro surplus methyl groups into tRNAs from one source by enzymes from another one. In such a way tRNAs containing an excess of methyl groups as compared with the initial species can be obtained. The study of hypermethylated tRNAs of known primary structure is of interest, since it allows one to find the

exact position of methylation sites and then, carrying out functional studies, to draw conclusions on the role of methylated nucleotides in different tRNA functions.

Previously, when studying the methylation of yeast $tRNA_1^{Val}$ by liver and hepatoma enzymes, we described the incorporation of one methyl group into C_{49} localized in the additional loop of the molecule. The acceptor activity of such a molecule did not differ from that of the initial one[1]. Methylation of E. coli $tRNA_f^{Met}$ by the same enzyme preparations resulted in incorporation of approximately three methyl groups per molecule[2]. In this work we report the result of acceptor activity determination of E. coli $tRNA_f^{Met}$ having surplus methyl groups in its molecule.

MATERIALS AND METHODS

S-Adenosyl [Me-^{14}C] methionine [Me-^{14}C] SAM (54-58 Ci/mole), L-[Me-^{14}C] methionine (59 Ci/mole) and L-[Me-^{3}H] methionine (100 Ci/mole) were purchased from Amersham (England).

Preparation of the methylating enzyme[1], identification of the methylation sites[2] in E. coli $tRNA_f^{Met}$ and the isolation of the crude aminoacylating enzyme preparation[3] from E. coli were performed as previously described. After methylation [Me-^{14}C] $tRNA_f^{Met}$ was precipitated with cetyltrimethylammonium bromide (cetavlon), converted to the sodium salt and dissolved in water. Aliquots were withdrawn for $A_{260\ nm}$ and radioactivity determinations. Aliquots of [Me-^{14}C] $tRNA_f^{Met}$ were incubated with the aminoacyl-tRNA-synthetase preparation and L-[^{14}C]- or L-[^{3}H]-methionine in a reaction mixture the composition of which was kindly imparted to us by Dr. Kelmers; the only difference was the use of Tris-HCl instead of HEPES-buffer. The reaction mixture contained: Tris-HCl buffer pH 8·0-25 μmole; MgAc - 2·5 μmole; KCl - 2·5 μmole; ATP - 1 μmole; [^{3}H] or [^{14}C]methionine - 750 pmole; β-mercapto-ethanol - 2·5 μmole; E. coli $tRNA_f^{Met}$ - 0·04 $A_{260\ nm}$ units; synthetase - 0·1 mg protein. After incubation at 37° for 30 min the aminoacylated tRNA was collected by filtration through Millipore discs and washed with 75-100 ml of a 0·03% solution of cetavlon. The discs were dried and the radioactivity was counted in a liquid scintillation spectrometer (Intertechnique SL-40). The following samples were used as controls:

1. $tRNA_f^{Met}$ preincubated with the methylase preparation without [Me-^{14}C] SAM.
2. $tRNA_f^{Met}$ preincubated with [Me-^{14}C] SAM without methylase preparation.

3. $tRNA_f^{Met}$ preincubated in the buffer without any additions.

The three control tRNA preparations were also precipitated with cetavlon and converted to the sodium salt. In experiments with [^{14}C] methionine the degree of aminoacylation was determined by subtracting the radioactivity of methylated tRNA ([Me-^{14}C] $tRNA_f^{Met}$) from the radioactivity of methylated and aminoacylated tRNA (^{14}C-methionyl-[Me-^{14}C] $tRNA_f^{Met}$). In experiments with [^{3}H] methionine the degree of aminoacylation was estimated by comparison of [^{14}C]- and [^{3}H]-methionine incorporation (double label count).

The digestion of tRNA with guanylo-RNase and the fractionation of the digest was carried out as described earlier[1] on cellulose thin layers on glass or quartz plates. The quartz plate was kindly supplied by Dr. V. D. Axelrod.

Contact prints were made from the quartz plate. Autoradiographs were prepared by placing the same plate on a RM-1 X-Ray film (USSR) with sensitivity of 300 reversed Röntgens. Exposure lasted for 9 days when the activity of the material put on the plate was 400,000 cpm.

RESULTS AND DISCUSSION

After methylation with an enzyme preparation from rat hepatoma, three sites of methylation were revealed in E. coli $tRNA_f^{Met}$[2]. In fig. 1a a contact print of a guanylo-RNase digest of $tRNA_f^{Met}$ is shown; the fractionation was carried out on a quartz plate covered with a cellulose FND (Filtrak, GDR) thin layer in the solvent systems previously used for fingerprinting ribonuclease digests of yeast valine tRNA on paper[4, 5]. Fig. 1b is a autoradiograph made from the same plate. As one can see the radioactivity is localized in three spots. The three oligonucleotides were identified previously[2]: spot 1 corresponds to the tetranucleotide C-U-C-Gp and its radioactivity is due to conversion of G to m^2G. Spot 2 corresponds to the tetranucleotide m^7G-U-C-Gp and the label was found to be present in m^5C. Spot 3 corresponds to the decanucleotide T-ψ-C-A-A-A-U-C-C-Gp and the radioactivity is due to the presence of m^1A, occupying the fifth position from the 5'-end of the oligonucleotide. Fig. 2 represents the 'clover leaf' pattern of E. coli $tRNA_f^{Met}$, the labelled nucleotides being marked with asterisks.

The maximal incorporation of methyl groups into $tRNA_f^{Met}$ amounted to 2·5-3 mole per mole $tRNA_f^{Met}$[2]. Unfortunately, due to a low activity of hepatoma enzymes, in the experiments with subsequent determination of acceptor activity the incorporation

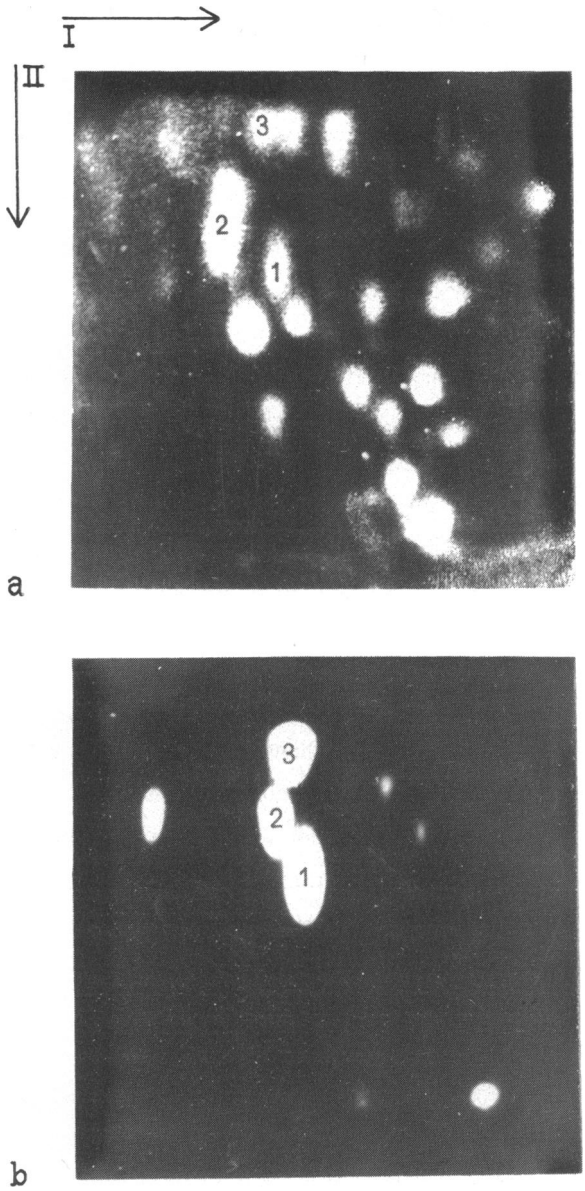

Figure 1
Fingerprint of a guanylo-ribonuclease digest of E. coli $tRNA_f^{Met}$ on a cellulose thin layer.
a. A contact print I. Isobutyric acid - 0·5 N NH_4OH (10:6), pH 3.7. II. Tert. Butanol-0·075 N HCOOH (1:1), pH 4·8. b. Autoradiograph. 1. C-U-C-m^2Gp 2. m^7G-U-m^5C-Gp 3. T-ψ-C-A-m^1A-A-U-C-C-Gp.

reached only 2 methyl groups per mole tRNA; hence incorporation into each nucleotide amounted to about 60% of the maximal level.

The data in table 1 show the results of acceptor activity determination. Each figure is a mean value of two independent experiments, each run in duplicate. As it was mentioned, for acceptor activity determination we used [^{14}C]- and [^{3}H]-methionine; therefore the methionine incorporation was determined by two independent tests. These two tests gave coinciding results: it was shown that the fall in acceptor activity in both sets of experiments did not exceed 9-12%. These values cannot be regarded as significant, especially since a decrease in acceptor activity

TABLE 1

Acceptor activity of methylated E. coli $tRNA_f^{Met}$

tRNA was methylated with an enzyme preparation from rat hepatoma[1]. Three control samples were run in parallel; one of them was devoid of [Me-^{14}C]SAM, the second of the enzyme, the third contained $tRNA_f^{Met}$ in the buffer solution without any additions. After incubation for 6 hours $tRNA_f^{Met}$ was precipitated from all samples with cetavlon, converted to the sodium salt and dissolved in water. Aliquots were withdrawn from each sample for methyl group incorporation and $A_{260\ nm}$ assay. Acceptor activity of each tRNA sample was assayed with [^{14}C]- and [^{3}H]methionine as described in the text.

$tRNA_f^{Met}$ sample	Acceptor activity			
	[^{14}C]methionine incorporation*		[^{3}H]methionine incorporation*	
	cpm/nmole tRNA(x 10^{-3})	%	cpm/nmole tRNA(x 10^{-3})	%
Initial	73.5	100	21.4	100
Preincubated in the whole methylation system	68.8	93.5	19.2	89.7
Preincubated in the methylation system without [Me-^{14}C]SAM	68.5	93.2	20.8	97.2
Preincubated in the methylation system without enzyme preparation	65.5	89.2	21.1	98.5
Preincubated in the buffer solution without any addition	73.0	99.2	21.2	99.0

* Count efficiency for [^{14}C] on Millipore discs in toluene was 50%, for [^{3}H] - 16%.

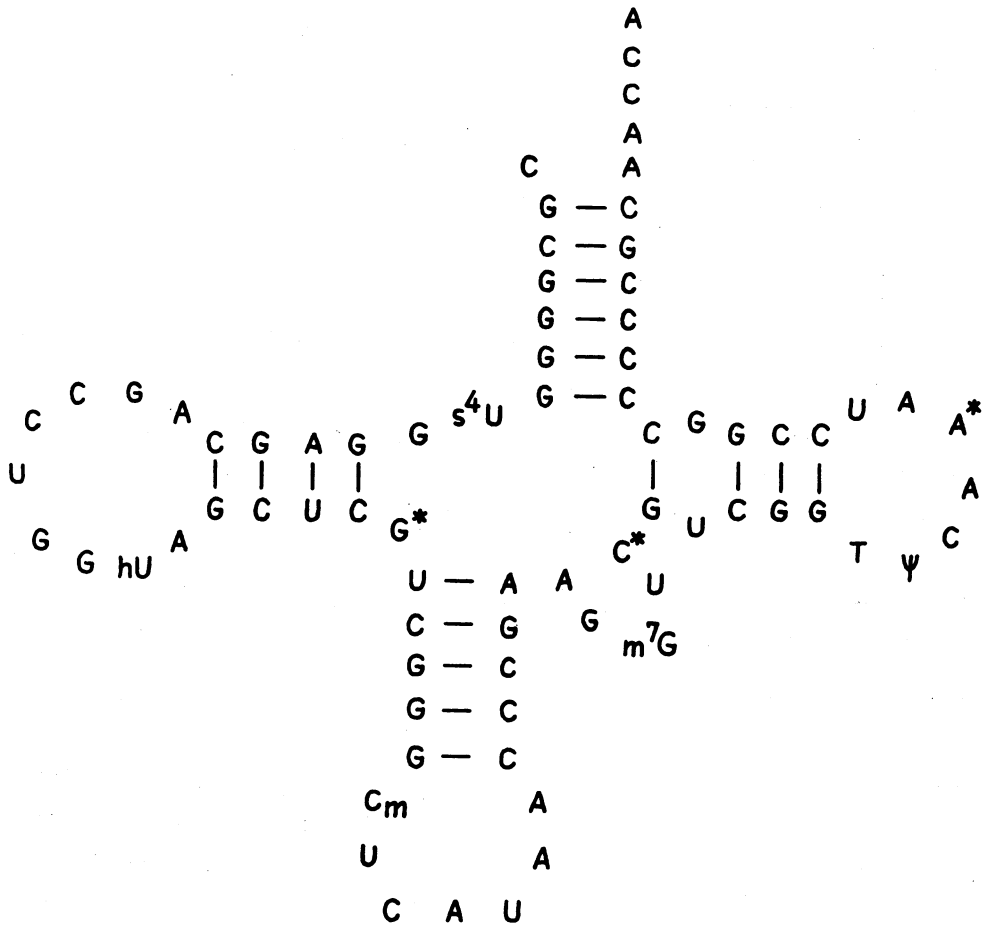

Figure 2
$tRNA_f^{Met}$ of E. coli in the 'clover leaf' pattern according to Dube et al. [16]
Asterisks indicate the nucleotides methylated in vitro by hepatoma enzymes.

of the same order of magnitude is observed in the two control samples. In the latter ones no methylation could proceed since one of them was devoid of the enzyme preparation, whereas [Me-^{14}C]SAM lacked in the other. The acceptor activity of $tRNA_f^{Met}$ incubated 6 hours in the buffer solution without any additions remained practically unchanged.

The results obtained show that methylation of three different nucleotides of $tRNA_f^{Met}$ does not influence the aminoacylation reaction. Obviously the three methylatable nucleotides localized in single stranded regions of the molecule are not involved in the recognition site. But the prerequisite of such a conclusion, is that the methylation of a nitrogen base in general can abolish its recognition ability. On the other hand one can assume that the incorporation at least of these methyl groups does not change considerably the conformation of the molecule: this conclusion follows from the consideration of the data presented here and that of our previous experiments[1,2], according to which the activity of tRNA-methylases depends on the tertiary structure of the molecule.

Modification of nitrogen bases often changes their ability to form hydrogen bonded base pairs. This question was extensively studied by Michelson et al[6-8]. These authors have found that N-methylation of purine bases exerts a profound effect on the physical properties of the respective nucleotides. For instance, poly(m_2^2G) as well as poly(m^2G) does not react with poly(C). Since the methylated amino group is somewhat distant from the actual hydrogen-bonding site, the change in hydrogen-bonding ability may be attributed to the steric effect of the methyl- and dimethyl-amino groups[6]. Poly(m^1G) also does not form complexes with poly(C)[7]. It is likely that in this case as well, the methyl group exhibits a profound steric hindrance, since according to the accepted concept three hydrogen bonds are involved in the double-stranded helical structure of poly(G)·poly(C) and only one of them, namely that between N^1 of guanine and N^1 of cytosine, is eliminated. On the other hand, poly(m^7G) does interact with poly(C), although the stability of the complex formed is much lower than in the case of unmodified G[8]. According to the same authors[8] the stability of complexes involving polypyrimidine nucleotides is markedly affected by substitution at C^5.

The shortcoming of our experiments is that the degree of methylation, as it was already mentioned, was not high: the total incorporation amounted to only 2 moles of methyl groups per 1 mole tRNA. Since the label is distributed between three

sites the degree of methylation of each nucleotide can be on average not more than 2/3 of the maximum level. However the fall in acceptor activity, if any, should be noticeable even at such a low level of methylation. We shall try to fractionate methylated $tRNA_f^{Met}$ according to the degree of methylation and to examine the acceptor activity of the completely methylated molecules, but we do not expect to get any different results. A more detailed acceptor activity assay, that is the determination of K_m and V_{max}, might reveal a difference between the fully methylated and non-methylated molecules. One cannot exclude that a change in acceptor activity will be detectable when carrying out aminoacylation with an enzyme from another source; according to Thiebe and Zachau[9] aminoacylation in heterologous systems is much more sensitive to tertiary structure changes than that in homologous ones.

The effect of nucleotide modification on functional properties of E. coli $tRNA_f^{Met}$ was extensively studied by Schulman[10-12]. In particular, it was shown that methylene-blue sensitized photooxidation of G_{46}, localized in the additional loop results in a loss of methionine acceptor activity[10]. On the other hand, modification by UV irradiation of U_{48} in the same region of the molecule does not change the acceptor activity of $tRNA_f^{Met}$[11]. Of interest is the work by Dube[13] who has shown that E. coli methionyl-tRNA synthetase protects three regions of the $tRNA_f^{Met}$ molecule and among them the additional loop, against digestion by T1 RNase. However, consideration of the accumulated data allows one to suggest that one can speak only about single nucleotides participating in the formation of the recognition site rather than about extensive nucleotide sequences. Three nucleotides of the additional loop namely nucleotides 47, 48 and 49 can be substituted or modified without loss of acceptor activity: this includes $m^7G \rightarrow A$ substitution in naturally occurring species, both of which are aminoacylated by the same enzyme. Conversion of U_{48} to H_2U_{48} and C_{49} to m^5C_{49} also does not lead to inactivation of the molecule.

Thus, only one from the four nucleotides studied in the additional loop, namely that located in position 46, is essential for $tRNA_f^{Met}$ recognition by its cognate enzyme. The data quoted present direct evidence that single nucleotides take part in the recognition reaction rather than long nucleotide sequences. The former ones participate probably in the formation of tRNA tertiary structure necessary for nucleic acid-protein interaction.

To-day an opinion exists that methylation of tRNA can be regarded as a kind of

'make-up' of the molecule not important for its functional properties. Our data seem to support this opinion. The experiments of Gefter et al.[14] can be interpreted in the same way: the suppressor $tRNA^{Tyr}$ of an E. coli mutant devoid of the ability to methylate U and containing tRNAs with the sequence U-ψ-C-Gp instead of T-ψ-C-Gp was active in protein biosynthesis. On the other hand, Kjellin-Straby[15] studying aminoacylation of tRNA from a mutant of S. cerevisiae, unable to synthesize m_2^2G, found that the amount of serine incorporated into tRNA is lower than in the case of the wild type. Aminoacylation with valine was at the normal level. The difference between the two aminoacids was suggested as being due to the presence of m_2^2G in $tRNA^{Ser}$ and its absence in $tRNA^{Val}$ of yeast. Therefore the inability to make m_2^2G affects only the acceptance of serine.

The experience with tRNAs show that the elucidation of the significance of the methyl group for tRNA function and structure has to be done with the use of individual tRNAs and must be accompanied by structural analysis. Only after accumulation of more data one will be able to draw conclusions about the function of methyl groups located in different parts of the molecule.

REFERENCES

1 Shershneva, L.P., Venkstern, T.V. and Bayev, A.A. (1973) Biochim. Biophys. Acta 294, 250-262
2 Shershneva, L.P., Venkstern, T.V. and Bayev, A.A. (1973) FEBS Letters 29, 132-134
3 Kelmers, A.D., Novelli, G.D. and Stulberg, M.P. (1965) J. Biol. Chem. 240, 3979-3983
4 Venkstern, T.V., Krutilina, A.I., Li.L., Axelrod, V.D., Mirzabekov, A.D. and Bayev, A.A. (1968) Mol. Biol. (USSR), 2, 394-407
5 Venkstern, T.V., Li, L., Krutilina, A.I., Axelrod, V.D., Mirzabekov, A.D. and Bayev, A.A. (1968) Mol. Biol. (USSR), 2, 597-611
6 Pochon, F. and Michelson, A.M. (1969) Biochim. Biophys. Acta, 182, 17-23
7 Pochon, F. and Michelson, A.M. (1967) Biochim. Biophys. Acta, 145, 321-327
8 Michelson, A.M. and Pochon, F. (1966) Biochim. Biophys. Acta, 114, 469-480
9 Thiebe, R. and Zachau, H.G. (1970) Biochim. Biophys. Acta, 217, 294-304
10 Schulman, L.H. (1971) J. Mol. Biol., 58, 117-131
11 Schulman, L.H. (1970) Proc. Natl. Acad. Sci. USA, 66, 507-514
12 Schulman, L.H. (1972) Proc. Natl. Acad. Sci. USA, 69, 3594-3597
13 Dube, S.K. (1973) Nature, 243, 103-105
14 Gefter, M., Yang, S. and Reinitz, R. (1973) EMBO Workshop on tRNA Structure and Function, Göteborg, Sweden
15 Kjellin-Stråby, K. (1973) Ninth Int. Congr. Biochem., Abstract Book, p. 160, Stockholm
16 Dube, S.K., Marcker, K.A., Clarke, B.F.C. and Cory, S. (1968) Nature, 218, 232