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Recognition of individual procaryotic and eucaryotic transfer-ribonucleic acids by B. subtilis adenine-1-methyltransferase specific for the dihydrouridine loop

H.Kersten, R.Raettig, J.Weissenbach+ and G.Dirheimer+

Institut für Physiologische Chemie, Wasserturmstrasse 5, 8520 Erlangen, GFR and
+Inst. Biol. Mol. et Cell du C.N.R.S., 15, rue Descartes and Fac. Pharm., Univ. Louis Pasteur, Strasbourg, France

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ABSTRACT

Bulk tRNA from *yeast* and *Rat liver* can be methylated *in vitro* with S-adenosylmethionine and *B. subtilis* extracts. The sole product formed is 1-methyladenosine (m^1A). This tRNA (adenine-1) methyltransferase converts quantitatively the 3'-terminal adenosine-residue in the dihydrouridine-loop of $tRNA^{Thr}$ and $tRNA^{Tyr}$ from *yeast* into m^1A. Out of 16 eucaryotic tRNAs with known sequences 6 accepted methyl groups, all at a molar ratio of 1. These tRNAs have in common an unpaired adenosine-residue at the specific site in the sequence Py-A-A^+-G-G-C-m_2^2G. Out of 12 tRNAs from *E. coli* 6 served as specific substrates. These *E. coli* tRNAs also have an unpaired adenosine-residue at the 3'-end of the D-loop. Besides restrictions in primary structure intact secondary and tertiary structure is important for recognition of the specific tRNAs by the enzyme.

INTRODUCTION

S-adenosylmethionine-dependent tRNA methyltransferases are specific for a given position in a tRNA molecule. They transfer methyl groups from S-adenosylmethionine (SAM) to particular nucleosides at specific sites (1).

Recently we have found a SAM dependent tRNA (adenine-1) methyltransferase in *B. subtilis* that methylates in eucaryotic tRNA a 3'-terminal adenosine-residue of the D-loop (2). This specific position has not been described before as a common methylation site in tRNA (3). The modified nucleoside m^1A is present in *B. subtilis* $tRNA^{Tyr}$ (4) the sequence of which is currently being investigated in our laboratories. The sequence of $tRNA^{Tyr}$ from *B. stearothermophilus* is now well established (5) and shows that m^1A is indeed present in tRNA from a *gram-positive* microorganism as a 3'-terminal residue of the D-loop. Thus the procaryotic tRNA (adenine-1) methyltransferase recognizes the same position in $tRNA^{Tyr}$ and $tRNA^{Thr}$ from *yeast* (2). The enzyme also methylates bulk tRNA from *gram-negative E. coli* (6). The study of the structural requirements in tRNA for recognition by a

specific tRNA-methyltransferase has been hampered in past, mainly, because of the lack of sufficiently characterized substrates. The tRNA (adenine-1) methyltransferase from *B. subtilis* seems to be a suitable enzyme for such investigations for the following reasons: (i) The enzyme transfers methyl groups *in vitro* from SAM to several specific tRNAs from *yeast* and from *E. coli*, the sequences of which are known; (ii) the enzyme has been shown to be highly specific because it converts in $tRNA^{Tyr}$ and $tRNA^{Thr}$ from *yeast* the specific adenosine-residue quantitatively to m^1A.

Out of 28 eucaryotic and procaryotic tRNAs tested, 12 accepted one mol of methyl groups. All these tRNAs had an unpaired 3'-terminal adenosine-residue in the D-loop. Further restrictions in primary, secondary and tertiary structures of the tRNA molecule in order to become recognized by this specific enzyme are described.

MATERIALS AND METHODS

Isolation, *in vitro* methylation and analysis of transfer RNA:

The sources of purified tRNAs from *Saccharomyces cerevisiae, Torulopsis utilis, Rat liver* and *Escherichia coli* are indicated in Table II and III. $tRNA^{Thr}$ and $tRNA^{Leu}$ from *yeast* were purified by counter current distribution (7), followed by chromatography on Sepharose 4B. The purification of *E. coli* $tRNA^{Ser}$, $tRNA_1^{Leu}$, $tRNA_2^{Leu}$ and $tRNA^{Gln}$ was started from enriched tRNA pools, kindly donated by Boehringer, Mannheim. The following methods were used for purification: BD-cellulose chromatography at pH 7.5 in the presence of Mg^{2+}; BD-cellulose chromatography at pH 6.0 in the absence of Mg^{2+} (8) and RPC-5 columns at pH 4.5 (Mg^{2+} present) (8,9).

The purity of a preparation of a specific tRNA was checked by nucleoside analysis and by assays in aminoacylation capacity (10). The *yeast* tRNAs were at least 90%, those from *E. coli* for at least 60% pure. The methyl group acceptor-capacity was always referred to the amino acid acceptor-capacity of a particular tRNA (see Table II and III).

Enzyme extracts from *B. subtilis* were prepared as described previously (2), S-100 fractions from *yeast* and from *E. coli* were obtained by the same procedure.

Conditions for tRNA methylation and analysis of the methylated products were described previously (2). Denatured *yeast* $tRNA_3^{Leu}$ was methylated at 20° instead at $37^{\circ}C$ as the other tRNAs.

Denaturation and renaturation of *yeast* $tRNA_3^{Leu}$:

Conditions for partial or complete denaturation of *yeast* $tRNA_3^{Leu}$ and for renaturation of partially or completely denatured $tRNA_3^{Leu}$ are shown in Table I. After the first heating cycle (denaturation) under conditions similar to those described by Lindhal et al. (11) the assays were chilled in ice, aliquots were withdrawn and analyzed. To the remaining sample 0.2 M $MgAc_2$ solution was added to give a final concentration of 19 mM and the renaturation heating cycle was started. The percentage of denatured $tRNA_3^{Leu}$ within the total population of each sample was determined by aminoacylation as described below.

Table I: Denaturation and renaturation of *yeast* $tRNA_3^{Leu}$

	percentage of denaturation		
	30%	60%	90%
denaturation conditions			
tRNA A_{260}/ml	10	10	10
Hepes pH 6.9 mM	5	10	10
Na_2 EDTA mM	0.5	1	2
heating time (60°C) min	5	8	10
renaturation conditions			
$MgAc_2$ 19 mM heating time (60°C) min	2	5	5

Dimerisation of *E. coli* $tRNA^{Tyr}$:

Dimers of *E. coli* $tRNA^{Tyr}$ were prepared and characterized by gel-filtration as described by Yang et al. (9).

Aminoacylation:

The conditions for aminoacylation were as described by Vold (10). *E. coli* tRNAs were aminoacylated with homologous S-100 preparations, tRNAs from *yeast* as well as tRNAs from *Rat liver* were aminoacylated with the S-100 fraction from *yeast*.

RESULTS

1. Methylation of heterologous tRNAs with *B. subtilis* enzymes:

Upon methylation of specific $tRNA^{Thr}$ and $tRNA^{Tyr}$ from *yeast* with *B. subtilis* cell-free extracts and S-adenosylmethionine, exclusively

1-methyladenosine is produced. The site of this methylated nucleoside within the tRNA sequence was shown to be the 3'-terminal adenosine-residue of the D-loop (2).

Bulk tRNA from *S. cerevisiae, T. utilis, Rat liver* and *E. coli* were now methylated *in vitro* with ^{14}C-methyl-S-adenosylmethionine and *B. subtilis* extracts. The analyses of nucleosides showed that only one product, 1-methyladenosine, was formed in these heterologous transmethylation reactions (results are not shown). Specific tRNAs of known sequences were purified from *yeast, Rat liver* and *E. coli* and tested as possible methyl group acceptors for the *B. subtilis* enzyme (Table II and III). The methyl group acceptor-capacity was always referred to the amino acid acceptor-capacity. Out of 16 eucaryotic tRNAs only 6 accepted methyl groups, all at a molar ratio of 1. These tRNAs have in common the sequence Py-A-A-G-G-C-m_2^2G in the D-loop, D-stem region. However tRNA^{Ile} from *T. utilis* was not methylated although the heptanucleotide sequence was present in the same site.

Out of 12 tRNA species from *E. coli*, 6 were methylated specifically. All these tRNAs have an 3'-terminal adenosine residue in the D-loop, three of them the pentanucleotide sequence A-A-G-G-C. However tRNA$_1^{Leu}$ and tRNA$_2^{Leu}$ from *E. coli* differ in the pentanucleotide sequence in this region of the tRNA.

Some procaryotic and some eucaryotic tRNA preparations were methylated by the m^{1}A-forming enzyme to an extent less than 0.1 mol CH_3/mol tRNA. This formation of m^{1}A in tRNAs that lack the specific site is probably caused by a slight contamination of these tRNAs with a cognate tRNA (see discussion). Attempts to increase the methyl group acceptance of these tRNAs by varying the conditions of the reaction (Mg^{2+} concentration, pH) failed.

2. Methylation of dimers of *E. coli* tRNA^{Tyr}:

E. coli tRNA^{Tyr} can be converted into aggregates by heating at 50°C in buffer containing 0.5 M Na^+, but no Mg^{2+} (9). The main product of this reaction is a dimer which does not accept tyrosine in the usual charging assay. The aggregates can rapidly be dissociated by heating to temperatures above 65°C. Nuclear magnetic resonance investigations of both, the monomer and the dimer have revealed that they have different base pairing structures and alternate tertiary structures (12).

We attempted to methylate the dimers of tRNA^{Tyr} from *E. coli* in order

Table II: Methyl group acceptance of eucaryotic tRNAs upon incubation with enzyme extracts from *B. subtilis* and S-adenosyl-L-(Me-^{14}C) methionine

tRNA		amino acid acceptance pmol/A_{260}	methyl group acceptance mol CH_3/mol tRNA	sequence of the pentanucleotide in the D-loop / D-stem region
S. cerevisiae				
$tRNA_{1a,b}^{Thr}$	(a)		1	A-A^+-G-G-C
$tRNA^{Tyr}$	(a)		1	A-A^+-G-G-C
$tRNA_1^{Leu}$	(a)		1	A-A-G-G-C
$tRNA_3^{Leu}$	(a)		1	A-A-G-G-C
$tRNA_{1,2}^{Ser}$	(a)		1	A-A-G-G-C
$tRNA^{Asp}$	(a)		0	A-G-A-A-U
$tRNA_3^{Arg}$	(a)	1550	0.05	A-A-C-G-C
$tRNA^{Ile}$	(a)	1700	0.04	unknown
$tRNA^{Phe}$	(b)		0	A-G-A-G-C
$tRNA^{His}$	(a)		0	unknown
$tRNA^{Val}$	(a)		0	A-U-C-G-C
$tRNA^{Ala}$	(a)		0	A-G-C-G-C
$tRNA_2^{Arg}$	(a)		0	A-C-G-G-C
$tRNA^{Lys}$	(a)		0	A-G-A-G-C
T. utilis				
$tRNA^{Ile}$	(c)	1320	0	A-A-G-G-C
Rat liver				
$tRNA_1^{Ser}$	(d)	1450	0.8	A-A-G-G-C

(a) tRNAs from the Strasbourg group
(b) $tRNA^{Phe}$ kindly supplied by Boehringer, Mannheim
(c) $tRNA^{Ile}$ was a kind gift from Dr. Takemura
(d) $tRNA^{Ser}$ was a kind gift from Dr. Rogg, Basel
A^+: position of m^1A determined by sequence analysis (ref. 2)

Table III: Methyl group acceptance of procaryotic tRNAs upon incubation with enzyme extracts from *B. subtilis* and S-adenosyl-L-(Me-^{14}C) methionine

tRNA	amino acid acceptance pmol/A_{260}	methyl group acceptance mol CH_3/mol tRNA	sequence of the pentanucleotide in the D-loop / D-stem region
E. coli			
$tRNA_1^{Leu}$ (c)	1460	1	G-A-C-G-C
$tRNA_2^{Leu}$ (c)	1760	1	G-A-C-A-C
$tRNA_{1,2}^{Gln}$ (c)	1080	1	A-A-G-G-C
$tRNA_1^{Ser}$ (c)	1300	1	A-A-G-G-C
$tRNA_3^{Ser}$ (c)	1800	1	A-A-G-G-C
$tRNA^{Tyr}$ (b)		1	A-A-G-G-G
$tRNA_f^{Met}$ (b)	1700	0	A-G-C-U-C
$tRNA^{Gly}$ (b)	1000	0.096	A-G-A-A-C
$tRNA^{Lys}$ (b)	1100	0.02	A-G-A-G-C
$tRNA^{Phe}$ (b)	1100	0.07	A-G-A-G-C
$tRNA_2^{Glu}$ (b)	1200	0	A-G-G-A-C
$tRNA_1^{Val}$ (b)	1100	0.02	A-G-A-G-C

(b) tRNAs kindly supplied by Boehringer, Mannheim
(c) tRNAs from the Erlangen group

to find out as to whether changes in secondary and / or tertiary structure influence the capacity of this tRNA to become methylated by the tRNA (adenine-1) methyltransferase. Tyrosine acceptance of $tRNA^{Tyr}$ can be taken as a measure of the degree of aggregation, respectively dissociation. The results show that the dimers did not accept methyl groups (Table IV) whereas the monomers - after an aggregation and dissociation cycle - accepted tyrosine and methyl groups at a ratio of almost 1:1 as the native $tRNA^{Tyr}$. The maximal acceptor activities of the monomers after the complete cycle are lower than those of the native tRNA. This decrease is probably caused by nonenzymatic hydrolysis at high temperatures or by a nuclease contamination.

3. Methylation of denatured *yeast* $tRNA_3^{Leu}$:

Yeast $tRNA_3^{Leu}$ can be converted into a denatured conformation by heating

Table IV: Methylation of *E. coli* $tRNA^{Tyr}$.

	native	dimers	monomers [a]
pmol Tyr/A_{260}	908	0	540
pmol CH_3/A_{260}	850	0	450
mol CH_3/mol Tyr	0.94	-	0.83

The conditions for measuring tyrosine acceptance, methyl group acceptance and aggregation/dissociation are described in materials and methods.
[a] monomers obtained after aggregation and dissociation.

at 60°C in a buffer containing EDTA (11). The denatured $tRNA_3^{Leu}$ does not accept leucine and is characterized by an alternate base-pairing structure and tertiary structure. We have determined the methyl group acceptance of mixtures of native and denatured $tRNA_3^{Leu}$ of *yeast*. The mixtures with varying amounts of denatured tRNA were prepared by changing the EDTA-concentration and the heating time as shown in Table I. The percentage of native tRNA within a given population was determined by measuring the leucine acceptance.

The results (Fig. 1) show that there is a linear relationship between the amino acid and the methyl group acceptance capacities. We conclude from these data that the sequence Py-A-A^+-G-G-C-m_2^2G in the denatured form of $tRNA_3^{Leu}$ is not available for the (adenine-1) methyltransferase.

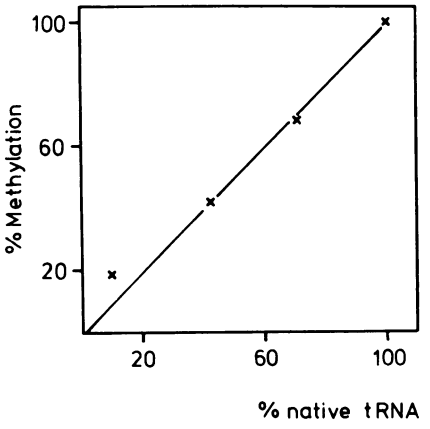

Fig. 1: Methylation of mixtures of native and denatured *yeast* $tRNA_3^{Leu}$. The percentage of native tRNA was determined from aminoacylation experiments.

DISCUSSION

A detailed analysis of the structural restriction in tRNA to be recognized by a specific tRNA methyltransferase requires an appropriate number of suitable,well defined substrates that accept molar amounts of methyl groups. In most cases when heterologous tRNAs had been used in specific transmethylation reactions the extents of methylation varied greatly (13-18). We have observed (2) that the methylation of *yeast* $tRNA^{Thr}$ and $tRNA^{Tyr}$ by a crude extract from *B. subtilis* gave only one product m^1A. The enzyme converts also in mature *E. coli* tRNA an adenosine-residue to m^1A. These findings offered the possibility to test several fully sequenced eucaryotic and procaryotic tRNAs as substrates in this reaction. Out of 28 individual tRNAs from *yeast, Rat liver* and *E. coli*, 12 accepted a molar amount of methyl groups, whereas all other tRNAs did not accept methyl groups. In all cases were a slight formation of m^1A (less than 0.1%) was found, the tRNA preparations were contaminated, as judged from amino-acylation experiments, respectively Boehringer specifications,with one or two cognate tRNAs. Since the extent of methylation e.g. the absolute number of methyl groups per unit of tRNA at infinite time and maximum protein concentration is a sensitive probe for the specificity of the methyltransferase we conclude, that the enzyme is highly specific for the adenosine-residue at the 3'-terminal of the D-loop in a slightly varying pentanucleotide sequence. For all eucaryotic tRNAs and three *E. coli* tRNAs this sequence was found to be $A\text{-}A^{+}\text{-}G\text{-}G\text{-}C$.

From a more profound comparison of the sequences of the tRNAs which have been analyzed for methyl group acceptance the following features can be considered as a necessary requirement of primary, secondary and tertiary structure of the substrate in order to become methylated: There must be an adenosine-residue at the target site, the 4^{th} position from the 3'-end of the D-stem. This adenosine-residue must not be base-paired, e.g. the D-stem must only have three base pairs. These criteria are fulfilled only by those tRNAs which have a purine-residue at position 13 from the 5'-end. $tRNA_3^{Arg}$ from *Saccharomyces cerevisiae* and $tRNA^{Ile}$ from *Torulopsis utilis* - although having an adenosine-residue at the target site - have a pyrimidine-residue at position 13 and do not become methylated.

The 5'-neighbouring nucleoside can be either a guanosine- or an adenosine-residue. Adenosine occurs exclusively in this position of the individual eucaryotic tRNAs. No tRNAs fulfilling the above mentioned re-

quirements and having a pyrimidine as 5'-neighbour of the target adenosine are known.

The 3'-neighbouring nucleoside was found to be either cytidine or -predominantly - guanosine, the eucaryotic tRNAs had exclusively G. No definite statement as to whether it is necessary that one of these nucleosides must be present is possible. tRNAs that fulfill the above mentioned requirements and have either an uridine or an adenosine as 3'-neighbour of the target adenosine are unknown at the present time.

The size of the extra loop does not seem to have any influence on the methyl group acceptance capacity of the substrates, since tRNAs with a small as well as tRNAs with a large extra loop accept molar amounts of methyl groups.

Nucleoside modifications in the D-stem and D-loop region, for example m^2G or ac^4C or Gm do not influence the methyl group acceptance capacity.

The primary structure requirement of a tRNA in order to become methylated by the *B. subtilis* tRNA (adenine-1) methyltransferase seem to be very limited, in fact they are restricted to only two positions within a sequence of 75 or more nucleotides.

A definite importance can be attributed to the native tertiary structure, whatever the native tertiary structure may be. This statement is based on the results presented for the methylation of denatured *yeast* $tRNA_3^{Leu}$ and of dimers of *E. coli* $tRNA^{Tyr}$.

During tRNA evolution in higher organisms e.g. *yeast* or *Rat liver* the primary structure which recognizes the tRNA (adenine-1) methyltransferase from *gram-positive* bacteria has been conserved. However the enzyme, sofar studied, seems to occur only in *gram-positive* microorganisms. The enzyme might influence specific as yet unknown functions of particular tRNAs in *gram-positive* microorganisms.

Work is now in progress to purify the enzyme and to study it's kinetic properties.

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