The mechanism of action of tRNA methylases studied with immobilized tRNAs

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ABSTRACT

Each of the individual tRNAs immobilized on aminohydroxy-butyl-cellulose (ABC) through their oxidized 3'-termini binds affinitively all methylases present in the enzyme extract irrespective of whether this tRNA will be involved in the following step of methylation or not. These data allow to suggest that (a) the formation of a methylase-tRNA complex and the catalytic act of methylation are indeed autonomous processes and (b) the first step of interaction between tRNAs and tRNA methylases is rather unspecific and consists in the recognition of the whole class of tRNA molecules.

As has been shown earlier in our laboratory by studying the methylation of certain individual tRNAs taken separately and in pairs, the formation of a tRNA methylase* - tRNA complex and the methylation of tRNA are autonomous processes. Moreover, the first step in the interaction between tRNA methylases and tRNAs is not specific: apparently, methylases can recognize such features in the tRNA structure that are typical of the whole class of these molecules /1, 2/. These findings have been supported in this work for tRNAs immobilized on ABC /3/. The results obtained suggest that each of the individual tRNAs attached to cellulose through their oxidized CCA termini binds affinitively all methylases which are present in the enzyme extract irrespective of whether this tRNA will be involved in the following step of methylation or not. Indeed, the spectrum of methylases eluted from individual immobilized tRNAs hardly differs from that in the original enzyme prepe-

*S-adenosyl-L-methionine - tRNA methyltransferase (EC 2.1.1)

ration taken for sorption. No correlation can be established between the ability of various individual tRNAs to bind methylases, on the one hand, and to be methylated by corresponding methylases, on the other.

MATERIALS AND METHODS

The total yeast tRNA and methyl-deficient tRNA from a methionine auxotroph *E. coli* K₁₂ 58-161 RC^{rel} grown on a medium without methionine were prepared using the phenol extraction procedure. The preparations of individual yeast tRNA^{Ser} and tRNA^{Phe} were a gift from Prof. H.G. Zechau (West Germany); tRNA^{Met} and tRNA^{Glu} of *E. coli*, from Prof. A.D. Kelmers (USA); and yeast tRNA^{Ile}, from Prof. F. Cramer (West Germany). Yeast tRNA^{Val}₁ was isolated from the total preparation of yeast tRNA in this laboratory /4/.

ABC was synthesized in the laboratory of Dr. R.M. Khomutov /3/. Oxidized and nonoxidized tRNAs were immobilized on ABC as described elsewhere /5/.

S-Adenosyl-L-[¹⁴CH₃]-methionine (Adomet) with a specific activity of 54 mCi/mmcle was purchased from the Radiochemical Centre (Amersham, England) and used as a source of methyl groups.

tRNA methylases were isolated from rat nephroma and hepatoma as described in /1/. The protein fraction was precipitated from 105 000 g of the corresponding supernatant by adding to it (NH₄)₂SO₄ to a 60% saturation; the precipitate was then dissolved in the initial volume of 10 mM Tris-HCl which contained 1 mM EDTA, 1 mM DTT, and 0.3 M NH₄CH₃COO, pH 7.5 (buffer A). This original enzyme preparation contained about 4 mg of protein per 1 ml. Methylases were adsorbed from this enzyme solution by tRNA immobilized on ABC through its oxidized 3'-end. The suspension was stirred at 0°C for 30 min, the cellulose was recovered by centrifugation and washed with buffer A. The bound proteins were eluted with a small volume of 1 M NaCl in buffer A. All of the experiments were performed also on columns packed with the same sorbent.

The overall methylase activity of the original enzyme solution and of eluates from total and individual tRNAs immobilized on ABC was assayed by incubating 150 μ l aliquots of appropriate protein solutions at 37°C for 30 min in 1.5 ml of the reaction mixture containing 0.003 mM Adomet and 1 A₂₆₀ unit of the E.coli total methyl-deficient tRNA in buffer A. Once the incubation was over, 2x100 μ l of the incubation mixture were applied onto paper filters, the unreacted Adomet was washed out, and the radioactivity of tRNA was determined in a scintillation counter /6/. In order to determine the activity of individual methylases, methylated tRNA was precipitated with cetavlon, converted to its sodium salt, and hydrolyzed with 1M HCl at 100°C for 30 min; purine bases and pyrimidine nucleotides obtained thereupon were fractionated by means of thin-layer chromatography on cellulose /7/. Distribution of the label among individual methylated components was determined for the original enzyme preparation and for eluates from immobilized tRNAs. Comparison of these data provided information concerning the sorption of the corresponding tRNA methylases on various tRNAs immobilized on ABC.

To assay the ability of various tRNAs to be methylated and to compare it with their capacity to bind tRNA methylases, total and individual tRNAs were methylated in solution under the above conditions; thereby the total incorporation was determined as well as the distribution of the label among individual methylated components.

In order to compare dissolved and immobilized tRNA methylation the following set of experiments was performed. The reaction mixtures containing 2 mg protein (original enzyme preparation from hepatoma or nephroma), 0.01 mM Adomet and 4 A₂₆₀ units of tRNA - dissolved or bound to ABC - in 1 ml buffer A were incubated for 2.5 and 18 hr at 37° under continuous stirring. The dissolved tRNA was precipitated with cetavlon, converted to the sodium salt and hydrolyzed with 1 M HCl by 100°C for 30 min. [¹⁴CH₃] tRNA-ABC was recovered by centrifugation and washed with water, 2M NaCl, ethanol and hydrolyzed for 1 min with 1M HCl at 100°C. After centrifugation an aliquot of the supernatant was taken for A₂₆₀ and

radioactivity determination. The remaining part of the supernatant was hydrolyzed for another 30 min in 1 M HCl at 100°C. The hydrolyzates were fractionated on cellulose thin layers /7/ and the radioactivity determined in individual spots.

In order to identify the sites of methylation ABC- $-\left[^{14}\text{CH}_3\right]\text{tRNA}$ was digested with RNAase T₁. After centrifugation on aliquot of the supernatant was fractionated by twodimensional thin layer chromatography /11/. The label distribution on the fingerprints was compared with that previously obtained in experiments with $\text{tRNA}_{1}^{\text{Val}}$ digested in solution /11/.

RESULTS

The total preparations of yeast and bacterial tRNAs immobilized on ABC bind all methylases that are present in the original enzyme preparation (Table 1). Since the total preparation comprises all types of tRNA, and consequently the substrates for virtually all methylases, it is not surprising that all these undergo sorption. According to the data obtained with immobilized individual tRNAs (Table 1), all of them (with an exception of bacterial $\text{tRNA}_2^{\text{Glu}}$) "trap" all methylases in the enzyme solution irrespective of which of these methylases catalyses their methylation.

As follows from the comparison of the data given in Tables 1 and 2, no correlation exists between the binding of methylases by individual tRNAs and their ability to be methylated. For instance, no m^2G formation can be established in Val, Ser, fMet and Ile tRNAs although each of them has a G residue in position 10 from the 5'-end of the molecule, which is the usual location of m^2G in tRNAs. Therefore, they can be regarded as potential substrates for m^2G methylase. However, G_{10} residues of these tRNAs do not accept methyl groups either in vivo or, according to our experiments, in vitro. Nevertheless, the enzyme producing m^2G effectively binds to all of the immobilized individual tRNAs (Table 1); its ratio in the corresponding eluates is nearly the same as in the original enzyme preparation, that is about half of the total methylase activity.

Table 1.

Production of methylated components in methyldeficient tRNA of *E. coli* by methylases of rat nephroma recovered from tRNAs immobilized on ABC.

Incorporation of $[^{14}\text{CH}_3]$, in cpm, within 30 min in 0.1 A_{260} unit tRNA.

Control = nonoxidized total yeast tRNA immobilized on ABC.

Enzyme preparation	Label incorporation Total	Distribution among methylated components formed					
		$m^5\text{C}$	$m^1\text{G}$	$m^7\text{G}$	$m^1\text{A}$	$m^2_2\text{G}$	$m^2\text{G}$
Original	2220	110	240	40	400	280	1150
Recovered from tRNA bound to ABC							
Control	24	-	-	-	-	-	-
Ser	650	50	20	50	220	40	270
Val	692	25	47	30	70	80	440
Glu	110	26	6	7	37	7	39
Phe	662	60	28	36	60	78	380
Original	4510	350	300	240	1060	310	2250
Recovered from tRNA bound to ABC							
Control	60	-	-	-	-	-	-
Total yeast	1100	130	80	110	60	40	680
Total <i>E. coli</i>	1420	130	100	140	180	60	810
fMet	840	80	80	140	60	20	460
Ile	1440	320	50	80	80	80	830

An interesting result has been obtained with $m^2_2\text{G}$ -methylase and yeast $\text{tRNA}_1^{\text{Val}}$ which cannot be, and in fact is not, a substrate for this methylase (Table 2) since it contains an A rather than a G residue at position 27 from the 5'-terminus of the molecule (the usual location of $m^2_2\text{G}$ in a tRNA molecule). However, $\text{tRNA}_1^{\text{Val}}$ binds this methylase contained in the enzyme

Table 2.

Methylation of various tRNAs by enzyme preparations
from rat nephromaIncorporation of [$^{14}\text{CH}_3$] in cpm within 30 min in 0.1 A₂₆₀
unit of tRNA

tRNA preparation	Label incorporation Total	Distribution among methylated components					
		m ⁵ C	m ¹ G	m ⁷ G	m ¹ A	m ² G	m ² G
Total bacterial	4300	250	330	180	950	430	2200
Total yeast	700	87	32	85	480	0	14
Val	400	160	48	24	125	0	0
Phe	360	280	0	0	80	0	0
Ser	1050	0	0	0	1020	0	0
Ile	0	0	0	0	0	0	0
fMet*	3320	870	120	0	1500	0	750
Glu	2000	300	8	8	1600	0	100

*As has been established earlier, m²G is formed in tRNA^{fMet}
in position 27 from the 5'-end of the molecule /8/.

extract so that it can be recovered, along with other methyl-
lases, in the eluate from AEC with immobilized tRNA^{Val}₁
(Table 1). This finding is consistent with the results of our
previous experiments which have shown that tRNA^{Val}₁ is a com-
petitive inhibitor for the methylation of G₂₇ in tRNA^{fMet} /2/.

Mention must also be made of the results obtained with
methylase that catalyses formation of m⁷G. Immobilized indivi-
dual tRNAs bind this enzyme as can be seen from the production
of m⁷G in the methyl-deficient tRNA by eluates from the
respective columns (Table 1). Experiments in which individual
tRNAs were methylated by the enzymes of rat nephroma show
that m⁷G is formed only in tRNA^{Val}₁ where G₄₇ is only partly

converted into m^7G in vivo and can therefore be additionally methylated in vitro (Table 2). *E. coli* tRNA^{fMet}, as well as yeast tRNA^{Phe}, has a variable loop five nucleotides long with m^7G in the middle. Though no m^7G is formed in these tRNAs, the binding of m^7G methylase is not surprising since these tRNAs are, in principle, a substrate for m^7G -methylase (methylation in vivo does take place). Production of a complex between m^7G methylase and yeast tRNA^{Ser} is of most interest (Table 1). The variable loop of this tRNA has an entirely different structure: it comprises 14 rather than 5 nucleotides, 8 of which are linked by hydrogen bonds. Therefore, not only this tRNA does not possess a guanine residue that can be methylated, but the structure of a region in which such a residue is normally located differs sharply from that of other tRNAs. Consequently, these lines of evidence suggest that the enzyme-substrate complex formation and the catalysis of methylation are autonomous processes and that the variable loop is not involved in the recognition of tRNAs by methylases. In the case of m^7G -methylase, these findings also show that the recognition site and the site for methylation are located at different positions of the molecule. It would be relevant to mention the result obtained with yeast tRNA^{Ile} which is not methylated either in vivo or in vitro (Table 2) by m^1G -, m^2G and m^7G -methylases, though one might expect it as this tRNA contains necessary nucleotides at appropriate positions. Nevertheless, this tRNA forms complexes with the respective methylases as readily as other individual tRNAs (Table 1).

Somewhat different results were obtained with *E. coli* tRNA₂^{Glu} whose structure has been the object of a number of studies /9, 10/. The data of Table 1 show that this tRNA poorly attaches guanyl-methylases. This evidence can be regarded as proof of a viewpoint according to which various tRNA molecules though having much in common can nevertheless be divided into several groups that differ more significantly in their conformation. Apparently, the tertiary structure of tRNA₂^{Glu} differs more significantly from that of other tRNAs, which is consistent with other experimental data obtained by us.

DISCUSSION

Immobilization of enzymes and substrates is a useful method for studying different aspects of the enzyme action. We used tRNAs bound to ABC for studying the mechanism of their interaction with methylases. The work with immobilized individual tRNAs proved to be particularly informative.

We have got evidence that the binding of methylases by tRNAs immobilized via their 3'-ends on ABC is due exclusively to a specific affinity effect. Nonoxidized tRNAs bound to ABC presumably via their non-hydrogen-bonded C residues /5/, do not bind methylases (Table 1).

One may suggest that the binding via the 3'-end has little, if any, effect on the tRNA tertiary structure; the latter is, as has been shown earlier /11/, the factor which determines the interaction of tRNAs with methylases.

On the contrary, the immobilization of non-oxidized tRNA on ABC most probably has a dramatic effect on the tRNA tertiary structure or leads to shielding of the sites essential for the binding of methylases. As a result no complex is formed between tRNAs bound in this manner and methylases (Table 1).

Results which have shown that immobilized *E. coli* tRNA^{Glu}₂ binds methylases very poorly and practically does not bind m¹G-, m⁷G- and m²G-methylases is also proof that the binding occurs not owing to a nonspecific polyelectrolytic, but to a highly specific affinity effect.

The most convincing evidence that the binding of tRNAs on ABC via their oxidized 3'-ends has little effect on their native structure, and that they bind methylases specifically, are experiments which have shown that productive enzyme-substrate complexes are formed thereupon. When the enzyme and all of the ingredients necessary for methylation are added to ABC with immobilized tRNAs methyl groups are incorporated into tRNA molecules (Table 3).

The rate of bound tRNA methylation is lower, but when the time of methylation is prolonged the same degree of methylation can be obtained with immobilized as with dissolved tRNAs (Table 3). These experiments were carried out with the total

Table 3.

Methylation of dissolved and ABC-bound tRNAs by methylases from rat hepatoma.

Incorporation of $[^{14}\text{CH}_3]$ in cpm in 0.1 A_{260} unit of tRNA. In order to get a higher degree of methylation the concentration of the ingredients in the reaction mixture was raised and the time of incubation prolonged.

tRNA preparation		Label incorporation of incubation	Total	Distribution among methylated components					
				m ⁵ C	m ¹ G	m ⁷ G	m ¹ A	m ² G	m ² G
Total methyl-deficient from E.coli	In solution	2.5 hr	7300	2700	300	300	3000	100	900
	Immobilized on ABC		1900	840	130	130	580	80	140
In solution	2400		2250	0	0	traces	0	0	
Immobilized on ABC	320		290	0	0	traces	0	0	
Val ¹ from yeast	In solution	18 hr	4000	3700	0	0	traces	0	0
	Immobilized on ABC		3600	3600	0	0	traces	0	0

E. coli tRNA as well as with valine specific yeast tRNA. Comparison of the label distribution among products of the acid digest of tRNAs methylated in the free and in the immobilized state has shown that the same methylated components are formed (Table 3). Analysis of T₁RNAase fingerprints of valine

tRNA has shown that the label is concentrated in the same oligonucleotides as it was earlier established for this tRNA methylated in solution /11/, that is the predominant reaction is the methylation of C₄₉ in the additional loop with the formation of m⁵C; some label is found also in m¹A in the TΨ-loop. That means that the specificity of the reaction is the same whether tRNA is in solution or immobilized on ABC.

These data suggest that the binding of tRNA via its oxidized 3'-end does not change essentially its conformation; the molecule preserves its capacity to bind methylases whereby a productive enzyme-substrate complex is formed. Therefore we believe it to be lawful to use this method for the study of the intimate mode of tRNA methylase-tRNA interaction. The same reasons allow us also to compare the sorption of methylases by immobilized tRNAs with the methylation of the corresponding tRNAs in solution.

Such a comparison (Tables 1 and 2) show that no correlation exists between the capacity of individual tRNAs to bind different methylases and their capacity to be methylated by them. Individual tRNAs "trap" practically all methylases from the enzyme solution irrespective of whether they can be methylated by them or not. The use of ABC with tRNAs immobilized via their oxidized 3'-ends has allowed to uncouple the two stages of the methylation reaction, namely, the enzyme-substrate complex formation and the catalytic action per se, to show their autonomy and to study them separately. The first step of interaction between tRNA methylases and tRNAs proved to be nonspecific and to consist in recognizing the whole class of tRNA molecules, most probably owing to their unique tertiary structure. The subsequent steps of interaction between tRNAs and methylases, on the contrary, must be highly specific to give the unique pattern of the methyl group distribution.

Experiments which have shown that productive substrate-enzyme complexes are formed between tRNAs immobilized via their oxidized 3'-ends and methylases affinitively bound to them allow us to suggest this model to be a valid one also for the study of the subsequent specific stages of tRNA

methylase - tRNA interaction. These investigations are in progress.

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