# FRAGMENTED E. COLI METHIONINE $tRNA_f$ AS METHYL ACCEPTOR FOR RAT LIVER tRNA METHYLASE: ALTERATION OF THE SITE OF METHYLATION BY THE CONFORMATIONAL CHANGE OF tRNA STRUCTURE RESULTING FROM FRAGMENTATION

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SUMMARY: In vitro methylation of E. coli $tRNA^{fMet}$ fragments or their reconstituted molecule by rat liver methylase showed that (1) the whole tRNA molecule was not necessary for recognition by tRNA methylase, and (2) the extent and the site of methylation of fragments differed depending upon the type of fragment used. This indicates that the conformational structure of the methyl acceptor molecule is important for recognition by the tRNA methylase.

We previously reported that rat liver contains at least two species of methylase specific for the guanylate residue of tRNA (1). These two methylases recognize specific regions of different tRNA's arranged in a clover-leaf structure, in addition to a nucleotide sequence. For example, methylation of E. coli $tRNA^{fMet}$ by methylase II occurred at the guanylate residue at the 51st position from the 3'-OH end, yielding $N^2$-methylguanosine, which was located between the dihydrouridine-stem and the anticodon-stem.

Since various E. coli tRNA fragments are now available (2-4), it is of interest to investigate their abilities to act as methyl acceptors to study the specificity of tRNA methylase with respect to the conformation of the tRNA molecule in more detail. This paper is on the methylation of the E. coli $tRNA^{fMet}$ fragment and its reconstituted molecule in vitro with a rat liver methylase II preparation. It was found that the extent and the site of methylation of the fragment differed from that of intact $tRNA^{fMet}$. Namely three quarters of the initial molecule, obtained by cleavage at the dihydrouridine-loop of $tRNA^{fMet}$, could be methylated to some extent to yield $N^1$-methyladenylic acid at the 19th position from the 3'-OH end, while the adenylate residue of intact $tRNA^{fMet}$ was not methylated. When the molecule representing three quarters of the initial molecule was combined with the remaining quarter of the initial molecule from the 5'-OH end to form a complex, the extent of methylation

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was restored to that observed with intact $tRNA^{fMet}$. In this case, methylation occurred at two sites, yielding $N^1$-methyladenosine at the 19th position and $N^2$-methylguanosine at the 51st position from the 3'-OH end. These results clearly showed that the conformation of tRNA is essential for its recognition by tRNA methylase.

## MATERIALS and METHODS

A preparation of methylase II, specific for the guanylate residue of tRNA, was prepared from rat liver as described previously (1). Assay of tRNA methylase activity and large scale preparation of $^{14}C$-methylated tRNA were carried out as described previously (1). *E. coli* $tRNA^{fMet}$ and fragments of it, i.e. Fragments L and N, obtained by cleavage at the dihydrouridine-loop, were prepared as described in previous papers (3,5). The methods used for measurement of the radioactivity of the $^{14}C$-methylated product, digestion of the products with RNase $T_1$, pancreatic RNase, RNase $T_2$ and *E. coli* alkaline phosphomonoesterase, and sequence analysis of the resulting products were as described previously (1). Limited hydrolysis of oligonucleotide with polynucleotide phosphorylase to leave trinucleoside diphosphate was performed as reported by Madison *et al.* (6), except that *E. coli* polynucleotide phosphorylase was used instead of the micrococcal enzyme.

RNase $T_1$ and RNase $T_2$ were purchased from Sankyo Co. Ltd. *E. coli* alkaline phosphomonoesterase and bovine pancreatic RNase (1-A) were obtained from Worthington Biochemical Co.

## RESULTS

As seen in Table I, the extent of methylation of intact $tRNA^{fMet}$ by tRNA methylase II from rat liver (specific for the guanylate residue) under optimal conditions was approximately one mole of methyl group per mole of tRNA, assuming that 1 O.D. unit of the tRNA is equal to 1.66 mμmoles (7). Fragment L (three quarters of the initial molecule from the 3'-OH end) was methylated to 5 % of the extent of intact $tRNA^{fMet}$ under the same conditions. Fragment N (a quarter of the initial molecule from the 5'-OH end) was not methylated at

Table I. Extent of $^{14}C$-methyl incorporation into intact *E. coli* $tRNA^{fMet}$ and its fragments by rat liver tRNA methylase.

| Methyl acceptor | $^{14}C$-Methyl incorporated, μμmole/tube | | |
|---|---|---|---|
| | Amount of tRNA of fragments added (O.D. unit/tube) | | |
| | 0.025 | 0.05 | 0.1 |
| Intact $tRNA^{fMet}$ | 46.8 | 96.5 | 168.2 |
| Fragment L | 4.0 | 7.1 | 11.5 |
| Fragment N | 0 | 0 | 0 |
| Fragment L + N | 46.8 | 85.5 | 157.2 |

The reaction mixture contained 10 μmoles of Tris-HCl (pH 8.0), 1 μmole of $MgCl_2$, 1 μmole of reduced glutathione, 4 mμmoles of $^{14}C$-methyl labeled S-adenosyl-L-methionine (specific activity, 30 C/M), 0.7 mg of the methylase II and tRNA or its fragments as specified in a final volume of 0.1 ml. In the case of Fragment L + N, Fragment L was mixed with Fragment N in a ratio of 3 to 1 by absorbance before the assay.

all. However, the reconstituted molecule (Fragment L + N) was methylated by methylase II to the same extent as native $tRNA^{fMet}$.

To characterize the structure of the $^{14}C$-methylated nucleotide in the fragments, each reaction product was extensively hydrolyzed by RNase $T_2$, and the digest was analyzed by Dowex 1 column chromatography as described previously (1). As shown in Fig. 1, with intact $tRNA^{fMet}$, radioactivity was eluted close to guanosine 3'-phosphate used as marker, as reported in the previous paper (1). On the contrary, 90 % of the radioactivity of Fragment L, and 40 % of the radioactivity of the reconstituted molecule was not absorbed on the column. This clearly indicates that some of the $^{14}C$-methylated nucleotide in the fragments is entirely different from the $N^2$-methylguanylic acid formed in intact $tRNA^{fMet}$ (1).

Evidence that the newly formed $^{14}C$-methylated product in the fragments is $N^1$-methyladenosine was obtained as follows. A reconstituted preparation

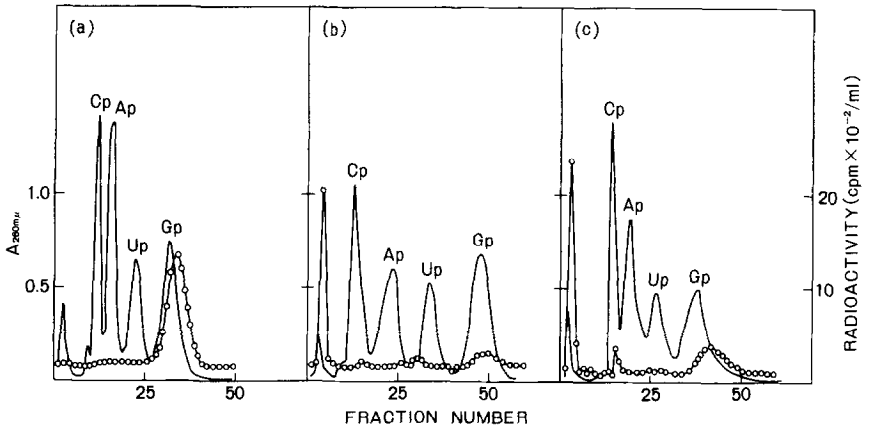


Fig. 1. Dowex 1 column chromatography of RNase $T_2$ digests of $^{14}C$-methylated *E. coli* $tRNA^{fMet}$ (a), Fragment L (b), and Fragment L + N (c). ————, UV absorbance; —o—o—, radioactivity; $^{14}C$-Methylated products were mixed with 20 O.D. units of unfractionated *E. coli* tRNA and hydrolyzed by RNase $T_2$.

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of L + N was subjected to $^{14}C$-methylation and then digestion with pancreatic RNase. As shown in Fig. 2, DEAE-Sephadex A-25 column chromatography of the digest gave two main radioactive peaks eluted between the di- and trinucleotide fractions. The second radioactive peak, eluted in front of the trinucleotide fraction was not observed in a digest of intact $tRNA^{fMet}$. This peak shifted to the tetranucleotide fraction when chromatography was carried out after the digest had been heated at 100 °C for 1 hr in alkali (pH 9.0) (Fig. 2d). This shift of the elution position of the radioactive oligonucleotide upon alkaline treatment is probably because the oligonucleotide

contains an $N^1$-methyladenylic acid residue, since $N^1$-methyladenylic acid has a positive charge which is lost on its conversion to $N^6$-methyladenylic acid by alkaline treatment (8). Similar results were obtained with the $^{14}C$-methylated Fragment L, but these results are not shown.

To confirm the structure of the methylated nucleotide, fractions of the radioactive oligonucleotide which shifted to the tetranucleotide fraction on alkaline treatment were pooled, desalted and completely hydrolyzed by RNase $T_2$. The $^{14}C$-methylated nucleotide thus formed was converted to the corresponding nucleoside by treatment with *E. coli* alkaline phosphomonoesterase. The resulting $^{14}C$-methylated nucleoside was analyzed by electrophoresis in triethylammonium bicarbonate buffer (pH 7.5). The position of radioactivity coincided with that of $N^6$-methyladenosine.

The location of the $N^1$-methyladenylic acid residue formed in the fragments was determined by analysis of the chromatographic profiles of digests of the $^{14}C$-methylated fragments with pancreatic RNase and RNase $T_1$ on DEAE-Sephadex A-25 column chromatography, as shown in Fig. 2. From the known primary sequence of *E. coli* $tRNA^{fMet}$ (9), the tetranucleotide fraction of the pancreatic RNase digest should consist solely of A-A-A-Up. Since the oligonucleotide containing $N^1$-methyladenosine described above was shifted to the tetranucleotide fraction after alkaline treatment, the adenylate residue(s) of A-A-A-Up must be methylated. The radioactive oligonucleotide containing $N^1$-methyladenosine found in digests of $^{14}C$-methylated L + N or L with RNase $T_1$ but not in digests of intact $tRNA^{fMet}$ was eluted in front of the fraction containing T-$\psi$-C-A-A-A-U-C-C-Gp (Fig. 2). These results support the conclusion reached from results on digests with pancreatic RNase.

To determine which adenylate residue of A-A-A-Up was actually methylated, the $^{14}C$-methylated tetranucleotide (Fig. 2d) was dephosphorylated by *E. coli* alkaline phosphomonoesterase and hydrolyzed by snake venom phosphodiesterase. The resulting nucleoside 5'-phosphate and nucleoside were analyzed by paper electrophoresis with triethylammonium bicarbonate buffer (pH 7.5). The radioactive component coincided with $N^6$-methyladenylic acid used as marker, but not with $N^6$-methyladenosine. This indicates that the 5'-terminal adenosine moiety of the tetranucleotide was not methylated. Another portion of the dephosphorylated radioactive tetranucleotide was digested with polynucleotide phosphorylase (6). Formation of trinucleoside diphosphate was confirmed by comparing the electrophoretic mobility of the radioactive product with that of an unlabeled authentic sample. The radioactive trinucleoside diphosphate was then completely hydrolyzed by RNase $T_2$, and the digest was again analyzed by paper electrophoresis. The position of radioactivity coincided with that of $N^6$-methyladenylic acid, but not with that of $N^6$-methyladenosine, demonstrat

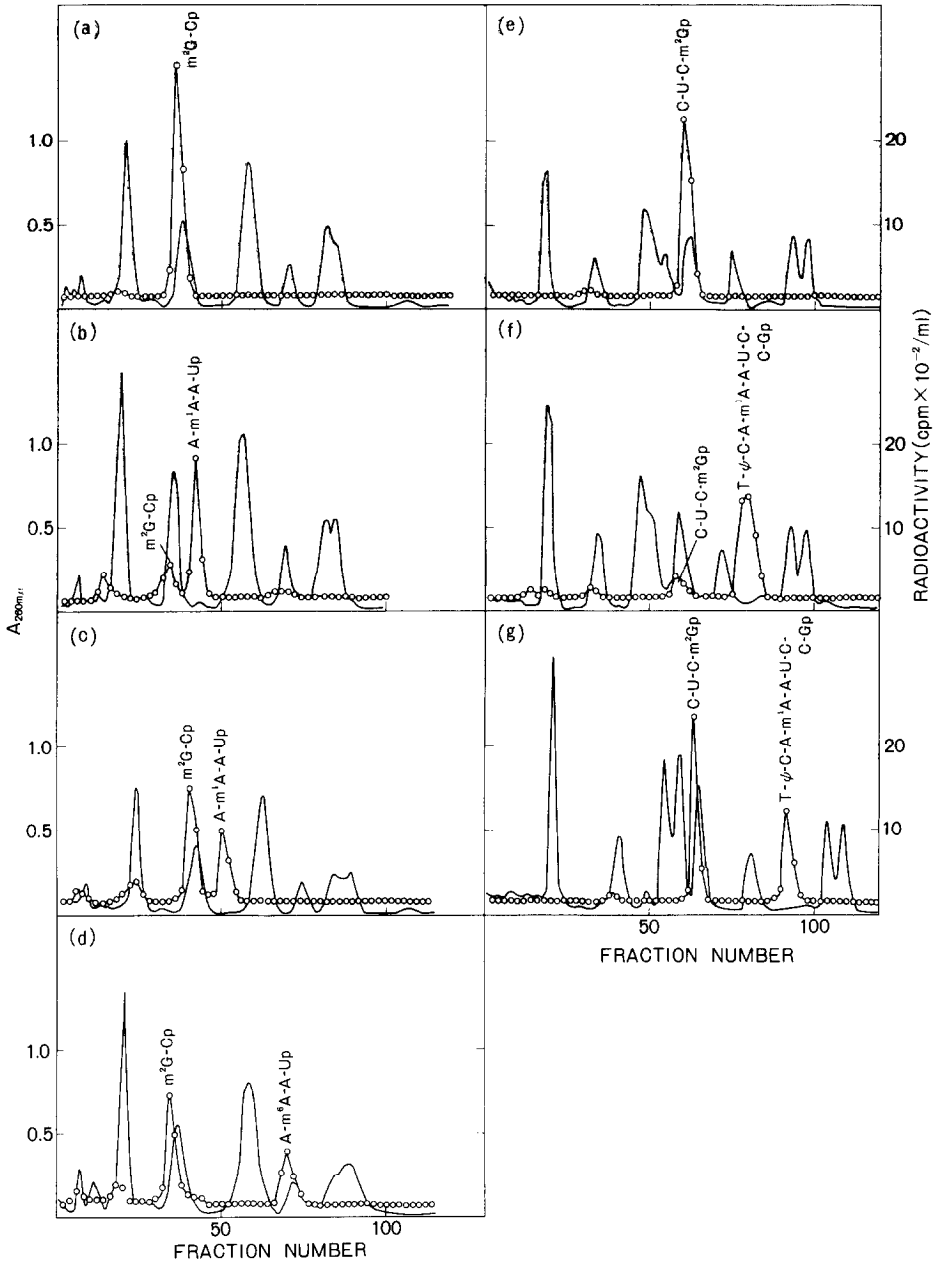


Fig. 2. DEAE-Sephadex A-25 column chromatography of $^{14}C$-methylated E. coli $tRNA^{fMet}$, Fragment L and Fragment L + N. a) pancreatic RNase digest of intact $tRNA^{fMet}$, b) pancreatic RNase digest of Fragment L, c) pancreatic RNase digest of Fragment L + N, d) pancreatic RNase digest of Fragment L + N after treatment with alkali, e) RNase $T_1$ digest of intact $tRNA^{fMet}$, f) RNase $T_1$ digest of Fragment L, g) RNase $T_1$ digest of Fragment L + N; ————, UV absorbance; —o—o—, radioactivity. $^{14}C$-Methylated products were mixed with 25 O.D. units of E. coli $tRNA^{fMet}$, and hydrolyzed by the RNase as specified. Chromatography was carried out as reported previously (1).

ing that $N^6$-methyladenosine was not located in the position next to 3'-terminal uridine. Therefore, it is concluded that the $^{14}C$-methylated tetranucleotide must be A-$m^1$A-A-Up. Thus the site of methylation of the adenylate residue in the molecule reconstituted from fragments of $tRNA^{fMet}$ was unambiguously determined as the 19th position from the 3'-OH end, located in the TψC-loop of the clover-leaf structure (9).

The $^{14}C$-methylated nucleotide in the first radioactive peak of a digest of Fragment L + N with pancreatic RNase or RNase $T_1$ (Fig. 2c and g) was examined. The radioactive oligonucleotide was hydrolyzed by RNase $T_2$, and then treated with E. coli alkaline phosphomonoesterase. The resulting $^{14}C$-methylated nucleoside was analyzed by paper chromatography as described previously (1). It was characterized as $N^2$-methylguanosine as in the case with intact $tRNA^{fMet}$. Since the radioactive $N^2$-methylguanosine of Fragment L + N appeared in the same fraction as that of intact $tRNA^{fMet}$, after digestion with either RNase $T_1$ or pancreatic RNase (Compare Fig. 2a with Fig. 2c, and Fig. 2e with Fig. 2g), it was concluded that the location of $N^2$-methylguanosine formed in Fragment L + N was exactly the same as that in intact $tRNA^{fMet}$, namely the 51st position from the 3'-OH end.

## DISCUSSION

The formation of a complex of Fragments L and N was shown by thermal denaturation as reported previously (3). However, the conformational structure of the complex does not seem to be as tight as that of intact $tRNA^{fMet}$, since thermal denaturation occurred at a lower temperature than that of native $tRNA^{fMet}$. The inefficient binding of the reconstituted fragments to ribosomes also indicates that there must be some structural deformation (3). From these above results and those reported here, it seems likely that the conformational structure of the methyl acceptor molecule is important for recognition by the tRNA methylase. The reconstituted molecule might have a conformation partially deformed in such a way that the site of the adenylate residue at the 19th position becomes susceptible to methylation, while the guanylate residue at the 51st position can still be recognized by the tRNA methylase. On the other hand, the conformation of Fragment L uncombined to the complementary Fragment N is more drastically distorted so that the methylase is no longer able to recognize the site of methylation, the guanylate residue. Another important conclusion deduced from results on the methylation of the tRNA fragment is that the whole tRNA molecule is not necessary for recognition by tRNA methylase, since the Fragment L alone could act as a methyl acceptor to some extent for methylation of the adenylate residue.

Baguley and Staehelin reported the partial resolution of a methylase specific for the guanylate residue of tRNA from one specific for the adenylate

residue in a preparation from rat liver using E. coli methyl-deficient tRNA as substrate (8,10). Thus it seems likely that our preparation of methylase specific for the guanylate residue of tRNA was contaminated with the latter enzyme which participates in the methylation of the adenylate residue of the $tRNA^{fMet}$ fragment. In fact, in one experiment E. coli methyl-deficient tRNA or normal unfractionated tRNA was methylated by our methylase II preparation, and an RNase $T_2$ digest of the $^{14}C$-methylated products was analyzed by Dowex 1 column chromatography as described in the Results. Approximately 7 % of the radioactivity was eluted in the position of $N^1$-methyladenylic acid, together with most of the radioactivity in the guanylic acid fraction from the digest of $^{14}C$-methylated E. coli methyl-deficient tRNA, while only 2 % of the radioactivity was found in the $N^1$-methyladenylic acid fraction from $^{14}C$-methylated normal unfractionated tRNA. (Y. Kuchino and S. Nishimura, unpublished data). The conformation of methyl-deficient tRNA may be partially deformed in a similar fashion to that of the fragmented tRNA molecule, so that only slight methylation of the adenylate residue is possible.

There is some discrepancy between our results and those obtained by Staehelin and his coworkers. First, Baguley and Staehelin reported that the supernatant fraction of rat liver, obtained by high-speed centrifugation, or a partially purified enzyme preparation from rat liver methylated both the guanylate and the adenylate residue in E. coli methyl-deficient tRNA with equal efficiency. However we found that when E. coli methyl-deficient tRNA or normal unfractionated tRNA was methylated by the supernatant fraction of rat liver obtained by high-speed centrifugation at pH 8.0 in the presence of magnesium ion, only 12 % and 5 % of radioactivity, respectively, was counted as $N^1$-methyladenylic acid. Second, when the methylation reaction was carried out at pH 9.2 without magnesium ion in 0.4 M ammonium acetate buffer, as reported by Staehelin and his coworker, there was little methylation of E. coli $tRNA^{fMet}$ or bulk E. coli tRNA using our methylase II preparation (Y. Kuchino and S. Nishimura, unpublished data). The reason for these discrepancies is unknown, but might be due to a difference in the enzyme preparations and tRNA used.

It was previously shown that methylation of intact tRNA in vitro occurs at the identical positions to those where methylated nucleotide residues are present in native tRNA (1,11). In this connection, it is interesting to note that the adenylate residue which is methylated in the reconstituted fragments is located at exactly the same position as $N^1$-methyladenosine in $tRNA^{Tyr}$, $tRNA^{Val}$, $tRNA^{Phe}$ and $tRNA^{Ile}$ from yeast, $tRNA^{Ser}$ from rat liver, and $tRNA^{Phe}$ from wheat germ (see review by Zachau (12) for example). Similarly, methylation of the guanylate residue in the reconstituted fragments occurs in the

region where $\underline{N}^2$-dimethylguanosine was found in several tRNA's from yeast and wheat germ (12). These results indicate that tRNA methylases recognize strictly the same places in fragmented molecules as in intact tRNA.

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