RESTORATION OF AMINOACYLATION ACTIVITY OF UNDERMETHYLATED TRANSFER RNA BY IN VITRO METHYLATION[1]

Lee Shugart, B. H. Chastain, G. David Novelli, and M. P. Stulberg
Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee

Received March 28, 1968

Undermethylated tRNA isolated from a RC^{rel} mutant of Escherichia coli has been examined to determine whether its capacity to accept amino acids was comparable to that of normal tRNA from the same organism. Previously, little, if any differences have been reported (see Littauer and Milbauer, 1965; Borek and Srinivasan, 1966). In this communication we report the restoration of aminoacylation activity of undermethylated tRNA by in vitro methylation. In a mixture of undermethylated tRNAs[2] from E. coli, we found that for four amino acids tested (Phe, Leu, Tyr, and His), the corresponding tRNAs showed decreased aminoacylation activities when compared to normal tRNA. Upon in vitro methylation of the undermethylated tRNAs with methylases free of RNase, an increase in the aminoacylation activities occurred. With two tRNA species (Phe and His), complete restoration of aminoacylation activity was observed.

METHODS AND PROCEDURES

The procedures for the isolation and assay of purified mixed normal and undermethylated tRNAs were as previously described by Shugart et al. (1968).

Purification of tRNA methylases. A cell-free extract was obtained from E. coli cells that had been treated as described by Neu and Heppel (1964) for the release of RNase. The tRNA methylating enzymes were purified through the $(NH_4)_2SO_4$ I step

---

[1]Research jointly sponsored by the National Institute of General Medical Sciences of the National Institutes of Health and by the U. S. Atomic Energy Commission under contract with the Union Carbide Corporation.

[2]Mixed undermethylated tRNAs are those tRNAs isolated from an RC^{rel} mutant of E. coli after methionine starvation; i.e., a mixture of both normal and undermethylated species of tRNA (Mandel and Borek, 1961).

according to the procedure of Hurwitz et al. (1964), and residual nucleases and nucleic acids were removed by chromatography on DEAE-cellulose. Pooled enzyme fractions were precipitated with $(NH_4)_2SO_4$, and passed through Sephadex G-75.

In vitro methylation. Twenty millimicromoles of [^{14}C] CH_3-S-adenosylmethionine (SAM) (47.5 μc/μmole — New England Nuclear) were added to a reaction mixture containing 40 μmoles of Tris-HCl buffer, pH 8.9; 16 μmoles methionine; 8 μmoles β-mercaptoethanol; 4 μmoles $MgCl_2$; 1.0 A_{260} units of tRNA; 1.2 mg of enzyme (methylases) protein; and 100 μ l of glycerol in a final volume of 1 ml. The reaction mixture was incubated at 30° for several hours and aliquots were removed at various times for the determination of methyl group incorporation into tRNA (Shugart et al., 1968). In those experiments where the tRNAs were to be recovered for subsequent analysis, a methylation reaction control (lacking SAM) was also included. This control indicated changes in the aminoacylation activities of the tRNA for reasons other than methylation.

Recovery of tRNA after in vitro methylation. The temperature of the methylation reaction mixture was lowered to 4°, and 1/2 volume of 1% cetyltrimethylammonium bromide (Ralph and Bellamy, 1964) was added. The tRNA was recovered from the mixture by filtration through Millipore filters. After the filter was washed with 70% ethanol in 0.1 M sodium acetate and 95% ethanol, the tRNA was dissolved in 0.01 M $MgCl_2$, reprecipitated with ethanol, and then dissolved in 0.01 M $MgCl_2$. Approximately 90% of the initial tRNA (A_{260} units) was recovered by this procedure.

Thin-layer chromatography. The distribution of methylated bases in tRNA was determined by two dimensional thin-layer chromatography (Bjork and Svensson, 1967) of acid hydrolyzates of tRNA.

RESULTS

The kinetics of in vitro methyl group incorporation into mixed undermethylated tRNAs are shown in Figure 1. The terminal adenosine content (Uziel et al., 1968) of this tRNA was 1250 ± 50 μμmoles per A_{260} unit. Normal, fully methylated tRNA does not accept methyl groups in this reaction. Since approximately half of the tRNA of this mixture is fully methylated, the undermethylated tRNA is methyl deficient (on the average) by 2.2 methyl groups per tRNA molecule. Hayashi et al. (1966) have reported that E. coli tRNA contains 2.3 methylated residues per molecule.

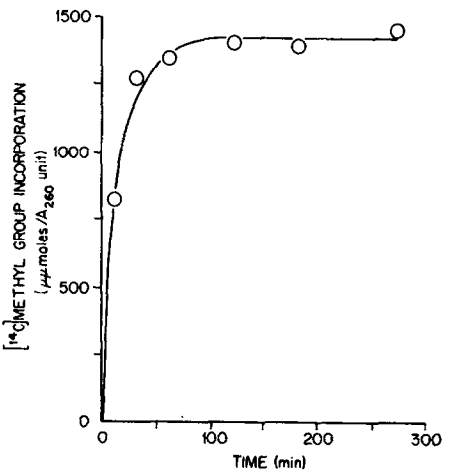

Fig. 1. Kinetics of in vitro methyl group incorporation into mixed undermethylated tRNAs.

TABLE I

MAXIMUM ACCEPTANCE OF AMINO ACIDS
BY MIXED UNDERMETHYLATED tRNAs
BEFORE AND AFTER IN VITRO METHYLATION[a]

tRNA	[^{14}C] Aminoacyl-tRNA (cpm/A_{260} unit x 10^{-3})
Before	
Undermethylated	32.5
Normal	52.2
After	
Undermethylated[b]	40.6
Normal	52.0

[a]Maximum charging performed with a total amino acid mixture (3 μc/μgram) New England Nuclear.

[b]Incorporated 1450 μμmoles [^{14}C] methyl groups per A_{260} units of undermethylated tRNA.

Quantitative data on the acceptance of a mixture of labeled amino acids into mixed undermethylated tRNA are shown in Table I. After maximum methylation, the amino acid acceptor activity of the undermethylated species has been increased by 25%.

A more detailed analysis of these materials before and after in vitro methylation for several individual aminoacylation activities is given in Table II. For the four amino acids tested, the corresponding tRNAs of the undermethylated material showed decreased aminoacylation activities when compared to normal tRNAs. After in vitro methylation, a change in these activities occurred. With two tRNA species (Phe and His), complete restoration of aminoacylation activity was observed, whereas the activity of $tRNA^{Leu}$ was only partially restored and that of $tRNA^{Tyr}$ was refractory to restoration.

TABLE II

AMINOACYLATION ACTIVITIES OF SPECIFIC tRNAs BEFORE AND AFTER IN VITRO METHYLATION OF MIXED UNDERMETHYLATED tRNAs

tRNA	[^{14}C]CH_3 groups incorporated ($\mu\mu$moles/A_{260})	Aminoacyl-tRNA formation ($\mu\mu$moles [^{14}C]Aminoacyl-tRNA/A_{260} unit)			
		Phenylalanyl-	Leucyl-	Histidyl-	Tyrosyl-
Before					
Undermethylated	1400[a]	20	73	9.7	33
Normal		35.2	163.4	30.5	58.4
After[b]					
Expt. 1 (-SAM)		23.2	70	10.7	30
(+SAM)	968	28.1	70	19.3	34.6
Expt. 2 (-SAM)		22.3	66		30
(+SAM)	1187	35.2	75.5		29
Expt. 3 (-SAM)		22.3	74	9.5	
(+SAM)	1351	35.8	82	28.9	

[a] Data from Figure 1.

[b] Each experiment represents recovered tRNA methylated in vitro to a different degree.

The distribution of in vitro incorporated methyl groups in the mixed undermethylated tRNAs, as analyzed by thin-layer chromatography, is shown in Table III. These data indicate that the methylase preparation used in these experiments caused incorporation of methyl groups into both purine and pyrimidine constituents. Approximately 90% of the radioactivity was isolated as a methylated uridine phosphate. This is in accord with the observations reported by Sarkar and Comb (1966).

TABLE III

THIN-LAYER CHROMATOGRAPHIC ANALYSIS
OF THE DISTRIBUTION OF IN VITRO INCORPORATED
METHYL GROUPS INTO MIXED UNDERMETHYLATED tRNAs

Constituent	Radioactivity[a] (cpm)	Distribution (%)
6-methylamino purine	88	0.6
7-methyl guanine	478	3.0
1-methyl guanine	216	1.3
Methylated cytidine phosphates	635	3.9
Methylated uridine phosphates	14915	91.3

[a] Applied 50 µliters (16,000 cpm) of acid hydrolyzed, mixed undermethylated tRNAs that had incorporated 1450 µµmoles [^{14}C] methyl groups per A_{260} unit.

DISCUSSION

We have shown that mixed undermethylated tRNAs isolated from an RC^{Rel} mutant of E. coli have decreased aminoacylation activities when compared to the mixed normal tRNAs from the same organism. These differences have been demonstrated in experiments with either a mixture of amino acids or several amino acids separately. Moreover, we have demonstrated that upon in vitro methylation, which results in the incorporation of methyl groups into the undermethylated tRNAs, the aminoacylation activities of some,

but not all tRNAs were fully restored. This may indicate that certain specific methylases were absent from our preparations.

REFERENCES

Bjork, G. R., and Svensson, I., Biochim. Biophys. Acta 138, 430 (1967).

Borek, E., and Srinivasan, P. A., Ann. Rev. Biochem. 35, 275 (1966).

Hayashi, Y., Osawa, S., and Miura, K., Biochim. Biophys. Acta 129, 519 (1966).

Hurwitz, J., Gold, M., and Anders, M., J. Biol. Chem. 239, 3462 (1964).

Littauer, U. Z., and Milbauer, R., Federation European Biochem. Soc. 4, 21 (1965).

Mandel, L. R., and Borek, E., Biochem. Biophys. Res. Commun. 4, 14 (1961).

Neu, H. C., and Heppel, L. A., Biochem. Biophys. Res. Commun. 14, 109 (1964).

Ralph, R. K., and Bellamy, A. R., Biochim. Biophys. Acta 87, 9 (1964).

Sarkar, N., and Comb, D. G., J. Mol. Biol. 17, 541 (1966).

Shugart, Lee, Novelli, G. David, and Stulberg, M. P., Biochim. Biophys. Acta 157 83 (1968).

Uziel, M., Koh, C., and Cohn, W. E., Anal. Biochem., in press (1968).