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# STIMULATION BY POLYAMINES OF ENZYMATIC METHYLATION OF TWO ADJACENT ADENINES NEAR THE 3' END OF 16S RIBOSOMAL RNA OF ESCHERICHIA COLI

Kazuei Igarashi, Kazuo Kishida and Seiyu Hirose

Faculty of Pharmaceutical Sciences, Chiba University,
Yayoi-cho, Chiba 260, Japan

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SUMMARY: Effect of polyamines on the methylation of adenine in 16S rRNA was examined using the purified methylating enzyme. When 23S core particles were used as substrate, the activity was stimulated by $Mg^{2+}$, $Ca^{2+}$ and monovalent cations. Even in the presence of optimal concentrations of $Mg^{2+}$ and $NH_4^+$, the addition of 1 mM spermidine stimulated the methylation approximately 1.7-fold. When 30S ribosomal subunits were used as substrate, the rate of methylation was 20% of that of the methylation of 23S core particles. The activity was not influenced significantly by $Mg^{2+}$, $Ca^{2+}$ or monovalent cations. The addition of spermidine inhibited the methylation.

The universal presence of two dimethylated adenines near the 3' end of the RNA of the smaller ribosomal subunits (1-5) suggests an important role of this modification in protein biosynthesis. Several years ago, Helser et al. (6,7) isolated a kasugamycin-resistant mutant of Escherichia coli which specifically lacked the methyl groups on the two neighboring adenines. They showed that the mutant does not produce an active methylase responsible for the methylation of these sites.

We have recently shown the decrease in methylation of adenine of 16S ribosomal RNA in a polyamine-requiring mutant of E. coli grown in the absence of polyamines (8). In this communication, we have purified an enzyme which methylates two adjacent adenines near the 3' end of 16S rRNA and examined some of its properties,

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especially the effect of polyamines on the activity. Recently, Poldermans et al. (9) have also purified the methylating enzyme from E. coli Q13.

## MATERIALS AND METHODS

Materials — Escherichia coli washed ribosomes, 30S and 50S ribosomal subunits, and crude initiation factors were prepared as described previously (8). The preparation of 23S core particles was carried out according to the procedure of Traub et al. (10). A kasugamycin-resistant mutant (TPR201) of E. coli was kindly supplied by Dr. J. E. Davies.

Assay for methylase activity — The assay of methylation of 23S core particles was performed by the method of Thammana and Held (11) with some modifications. The standard reaction mixture (0.05 ml) containing 50 mM Tris-HCl (pH 7.8), 15 mM KCl, 50 mM magnesium acetate, 1 mM EDTA, 1 mM dithiothreitol, 0.5 $A_{260}$ unit of 23S core particles from kasugamycin-resistant mutant of E. coli, 60 units of the purified methylase, and 0.4 μCi of S-adenosyl-L-[methyl-$^3$H]methionine (specific activity, 200 μCi/μmol, Radiochemical Centre, Amersham), was incubated at 37° C for 10 min. The reaction was stopped by adding 5 μl of 10 mg/ml bovine serum albumin and 1 ml of 5% trichloroacetic acid. The precipitate was collected and washed with 5% trichloroacetic acid, and the radioactivity was assayed with a liquid scintillation spectrometer. One unit of enzyme activity was defined as the amount of enzyme which caused the attachment of 1 pmol of methyl group to 23S core particles under our experimental conditions. When 30S ribosomal subunits (0.5 $A_{260}$ unit) were used as substrate, 90 units of the methylase were added to the reaction mixture.

Purification of the methylase from E. coli Q13 — The methylase was purified from a crude initiation factor preparation (1 M $NH_4Cl$ ribosomal wash) of E. coli Q13. The enzyme activity was precipitated between 0 and 50% saturation with ammonium sulfate. The precipitate was dissolved in Buffer I (20 mM Tris-HCl, pH 7.4, 7 mM 2-mercaptoethanol, and 10% glycerol) containing 100 mM KCl, and dialyzed against the same buffer. After addition of an equal volume of Buffer I, the solution was applied to a column of DEAE-Sephadex A-50 (2.7 × 17.5 cm) previously equilibrated with Buffer I containing 50 mM KCl. The column was washed with the same buffer, and the enzyme was eluted by a linear gradient of 50 to 200 mM KCl, in Buffer I. The active fractions were pooled, concentrated by ultrafiltration, and dialyzed against Buffer II (30 mM Tris-HCl, pH 8.0, 0.2 mM dithiothreitol, 0.1 mM EDTA, and 10% glycerol) containing 400 mM KCl. The enzyme solution was applied to a column of Affi-Gel Blue (1.4 × 3 cm, Bio-Rad) previously equilibrated with Buffer II containing 400 mM KCl. After the column was washed with the same buffer, the methylase was eluted with Buffer II containing 1 M KCl. Triton X-100 was immediately added to make a final concentration of 0.04%, and the solution was concentrated by ultrafiltration. The enzyme solution was then applied to a column of Sephacryl S-200 (3.5 × 60 cm) previously equilibrated with Buffer III (50 mM Tris-HCl, pH 8.0, 1 mM dithiothreito, 200 mM KCl, 0.1 mM EDTA, and 0.04% Triton X-100). The enzyme was eluted with the same buffer (5 ml fractions) and the active fractions (fraction 60-65) were concentrated and stored at -80° C until used.

## RESULTS

Purification and properties of the methylase — The results of a typical purification of the methylase is summarized in Table 1. The enzyme was purified approximately 500-fold with an yield of about 14%. One major band was observed by polyacrylamide gel electrophoresis (Fig. 1). The molecular weight of the methylase was 30 000 as estimated by SDS-gel electrophoresis and 29 000 as estimated by Sephacryl S-200 gel filtration.

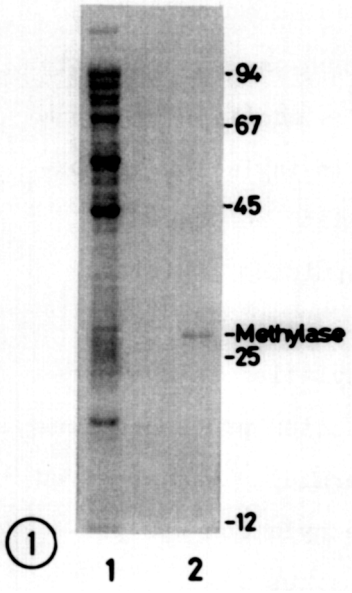
When 23S core particles were used as substrate, the addition of $Mg^{2+}$ or $Ca^{2+}$ up to 5 mM to the reaction mixture containing 0.5 mM $Mg^{2+}$ stimulated the methylation, but $Mn^{2+}$ inhibited the reaction (Fig. 2A). Monovalent cations ($Na^+$, $K^+$ and $NH_4^+$) also stimulated the methylation (Fig. 2B), and the optimal concentration of $Na^+$, $K^+$ and $NH_4^+$ was 25, 25 and 75 mM, respectively, although the reaction mixture contained 15 mM $K^+$.

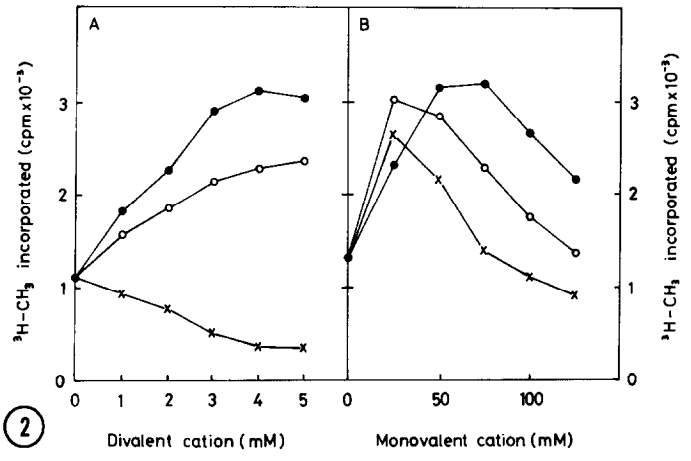
When 30S subunits were used as substrate, the activity was not influenced significantly by the addition of $Mg^{2+}$, $Ca^{2+}$ or monovalent cations. The rate of methylation of 30S subunits was approximately 20% of that of the methylation of 23S core particles.

Effect of polyamines on the methylation — Effect of spermidine on the methylation was examined using 23S core particles as substrate (Fig. 3A). The addition of 1 mM spermidine stimulated

Table 1. Purification of the methylase from 100 g of *E. coli* Q13.

| Step | Protein (mg) | Total activity (units) | Specific activity (units/mg protein) | Yield (%) |
|---|---|---|---|---|
| 1. Ribosomal wash | 286.5 | 37600 | 131 | 100 |
| 2. Ammonium sulfate | 196.8 | 37000 | 188 | 98 |
| 3. DEAE-Sephadex A-50 | 16.7 | 24400 | 1461 | 65 |
| 4. Affi-Gel Blue | 1.2 | 8400 | 7000 | 22 |
| 5. Sephacryl S-200 | 0.08 | 5200 | 65000 | 14 |



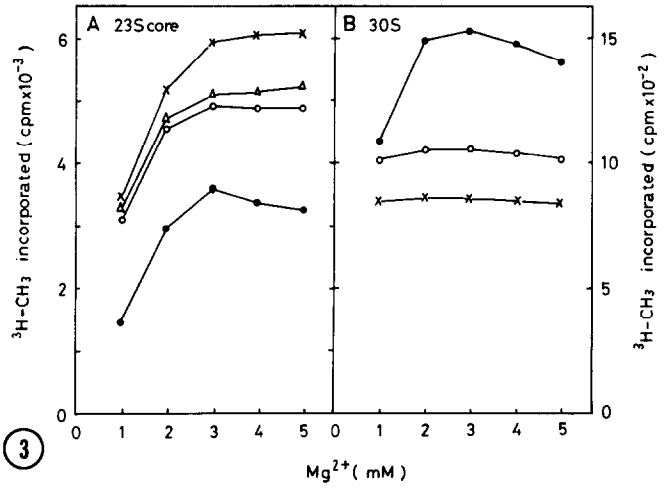




Fig. 1. SDS-gel electrophoresis of the methylase. SDS-gel electrophoresis was carried out according to the procedure of Laemmli (12). 1, 17 μg of 1 M $NH_4Cl$ ribosomal wash; 2, 0.3 μg of the purified methylase. The markers used were cytochrome c (Mr. 12 400), α-chymotrypsinogen (Mr. 25 000), ovalbumin (Mr. 45 000), bovine serum albumin (Mr. 67 000), and phosphorylase a (Mr. 94 000).

Fig. 2. Effect of cations on the methylation of 23S core particles. A, The reaction mixture contained 0.5 mM $Mg^{2+}$ and divalent cations as specified in the figure. ●, $Mg^{2+}$; ○, $Ca^{2+}$; ×, $Mn^{2+}$. B, The reaction mixture contained 15 mM $K^+$ and monovalent cations as specified in the figure. ●, $NH_4^+$; ○, $K^+$; ×, $Na^+$.

Fig. 3. Effect of spermidine on the methylation. The assays were carried out under standard conditions except that $Mg^{2+}$ concentration was varied and spermidine was added as specified in the figure. A, 23S core particles; B, 30S subunits. ●—●, no addition; ○—○, 0.5 mM spermidine; ×—×, 1 mM spermidine; △—△, 1.5 mM spermidine.

the methylation approximately 1.7-fold. The stimulation degree by 1 mM spermidine increased up to 2.0-fold when the concentration of $NH_4^+$ was decreased from 50 mM to 10 mM. The addition of 2.5 mM putrescine and 0.1 mM spermine stimulated the methylation approximately 1.3-fold and 1.6-fold, respectively (data not shown). When 30S subunits were used as substrate, spermidine inhibited the methylation (Fig. 3B).

Fig. 4 shows the time course of the methylation in the presence or absence of 1 mM spermidine. The reaction proceeded linearly until 10 min and the stimulation by spermidine was observed in all incubation times tested. Then, the methylation was performed by changing the concentration of substrates (Fig. 5). The maximum velocity of the methylation in the presence of 1 mM spermidine was higher than that in the absence of spermidine.

## DISCUSSION

Although the physiological role of the methylation of 16S rRNA at the two adenines near the 3' end is still not clear, it is of interest that the methylation was stimulated by polyamines

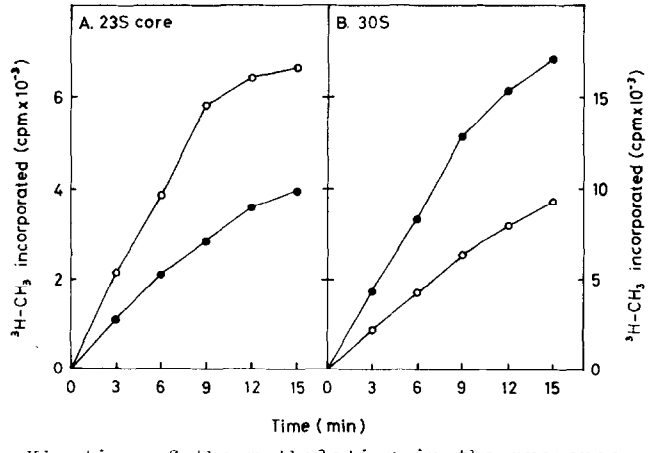



Fig. 4. Kinetics of the methylation in the presence or absence of spermidine. Standard reaction mixture with (○) and without (●) the addition of 1 mM spermidine was incubated for various periods. A, 23S core particles; B, 30S subunits.

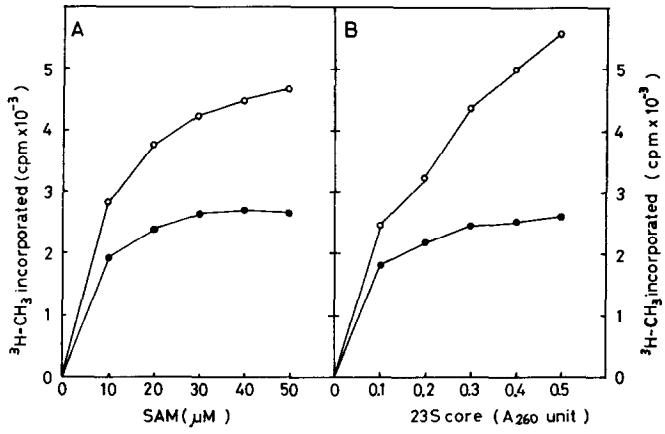



Fig. 5. Effect of substrate concentrations on spermidine stimulation of the methylation of 23S core particles. The assays were carried out under standard conditions except that the reaction mixture contained various amounts of S-adenosylmethionine (A) or 23S core particles (B) as specified in the figure. ● , no addition; ○ , 1 mM spermidine.

when 23S core particles were used as substrate. We have previously proposed the following hypothesis (8). Polyamines stabilize a conformation of 23S core particles in which the methylation of adenine in 16S rRNA easily takes place. In consequence of the stimulation of the methylation of adenine in 16S rRNA, the assembly of some kinds of split proteins to 23S core particles may be stimulated. The results shown in this paper fit well the hypothesis which we have proposed (8). Since the methylation occurs at an intermediate stage and is inhibited at a late stage of assembly (11), the idea that the methylation stimulates the maturation of 30S subunits by the stimulation of the binding of some kinds of split proteins to 23S core particles seems to be quite reasonable. We have recently found that the content of S1 protein in 30S particles reconstituted from 23S core particles and split proteins from kasugamycin-resistant mutant of E. coli was increased by the previous treatment of the 23S core particles with the methylase (manuscript in preparation).

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