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Isolation and Characterization of Biochemical Properties of DNA Methyltransferase *Fau*IA Modifying the Second Cytosine in the Nonpalindromic Sequence 5′-CCCGC-3′

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Abstract—A gene encoding DNA methyltransferase (methylase) *Fau*IA of the restriction–modification system *Fau*I from *Flavobacterium aquatile* (recognizing sequence 5′-CCCGC-3′) was cloned in pJW vector. The latter was used for transformation of *E. coli* RRI cells followed by subsequent thermoinduction and biomass elaboration. Highly purified DNA methyltransferase *Fau*IA preparation was obtained using chromatography on different sorbents. The molecular mass of the isolated enzyme of about 39 kD corresponds to its theoretical value. The enzyme was characterized by temperature and pH optima of 33°C and pH 7.5, respectively. Methylation of a synthetic oligonucleotide by *Fau*IA methylase followed by its cleavage with various restrictases and analysis of the resultant restriction fragments revealed that *Fau*IA methylase modified the second cytosine residue in the sequence 5′-CCCGC-3′. Kinetic analysis revealed K_m and catalytic constant values of 0.16 μM and 0.05 min^{-1}, respectively.

Key words: *Flavobacterium aquatile*, DNA methyltransferases, restriction–modification systems, enzyme kinetics

DNA methyltransferases (methylases, M.) are site-specific enzymes, which can methylate DNA bases (adenine or cytosine) in a recognizable nucleotide sequence [1]. In prokaryotes, these enzymes are components of restriction–modification systems protecting cells against foreign DNA (e.g., bacteriophage DNA).

We previously found a *Flavobacterium aquatile* strain producing restriction endonuclease *Fau*I. This enzyme recognizes the nonpalindromic site 5′-CCCGC-3′ of DNA duplex and cleaves DNA at the position 4/6 versus this site [2]. We have cloned and studied the primary structure of *Fau*IA methylase [3] and elucidated the DNA sequence of the whole operon containing genes encoding the *Fau*I restriction–modification system. We have also demonstrated unusual order of these genes, which has not been found in DNA sequences encoding components of other restriction–modification systems [4].

Most of restriction–modification systems recognizing nonpalindromic sites (IIS subtype) include either two DNA methyltransferases (each of which modifies one of two strands at the recognition site) [5, 6] or just one DNA methyltransferase, which consists of two domains responsible for methylation of the two strands at the recognition site [7].

Analysis of amino acid sequences of *Fau*I methylases revealed that both methylases of this system belong to the class of C5-DNA methyltransferases [3, 4] and, consequently, they methylate cytosine residues at C5 position.

In the present study, we have purified *Fau*IA methylase and investigated substrate specificity, temperature and pH optima, and also the kinetic behavior of this enzyme.

MATERIALS AND METHODS

Strains and reagents. Bacterial strains *Flavobacterium aquatile* I and *Escherichia coli* RRI were from the SibEnzyme collection (Novosibirsk, Russia) and New England Biolabs (USA), respectively. The following reagents and components of cultivation media were used in this study: Tris (Promega, USA); acrylamide, *bis*-acryl-

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amide, ampicillin, protein markers for electrophoresis (Helicon, Russia); EDTA (Fluka, Switzerland); agarose (Hybaid-AGS, Germany); glycerol, 2-mercaptoethanol, dithiothreitol (DTT), Triton X-100 (ICN, USA); lysozyme (Serva, Germany); S-adenosyl-L-methionine (SAM) (New England Biolabs); [^{3}H-CH^3]SAM (Amersham, England); phenylmethylsulfonyl fluoride (PMSF) (Sigma, USA); phosphocellulose P-11 (Whatman, England); hydroxyapatite, heparin-Sepharose (BioRad, USA); Sephacryl S-200, tryptone, yeast extract (Organotechnie, France); enzymes and DNA preparations (SibEnzyme). Other chemicals of "chemically pure" grade were produced by various Russian suppliers.

Biomass production for *Fau*IA methylase elaboration. The gene encoding *Fau*IA methylase was cloned into pJW vector [8] by *Bam*HI and *Fau*NDI restriction sites under thermoinducible promoter of phage λ. *E. coli* RRI strain was used for thermoinducible enzyme elaboration. Cells transformed with the M.FauIA-pJW plasmid were seeded into liquid LB cultivation medium containing tryptone (10 g/liter), yeast extract (5 g/liter), NaCl (5 g/liter), pH 7.0-7.3, and ampicillin (100 μg/ml). Biomass was produced in retorts at 30°C using a thermostatted shaker (180 rpm) up to optical density A_{550} = 0.8-0.9, and then thermoinduction was carried out at 42°C for 4 h. Cells were harvested by centrifugation at 3000*g* and stored at –20°C. The presence of the inducible protein was verified by electrophoresis in polyacrylamide gel (PAGE) under denaturing conditions. Aliquots of cell lysate were applied onto SDS-polyacrylamide gel, and electrophoresis was carried out using the standard protocol [9]. Figure 1 shows the electrophoregram of cell lysate of *E. coli* RRI strain containing plasmid M.FauIA-pJW. Thermoinduction resulted in appearance of a protein band of about 39 kD. This corresponds to theoretical molecular mass (38,956 daltons) calculated using the open reading frame nucleotide sequence [4].

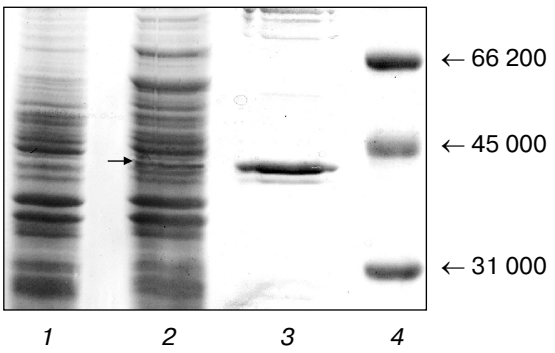

Fig. 1. Electrophoregram of the cell extract of *E. coli* RRI cells carrying vector M.FauIA-pJW and the final enzyme preparation. Lanes: *1*) before thermoinduction; *2*) after thermoinduction (the arrow shows the position corresponding to *Fau*IA methylase); *3*) final enzyme preparation; *4*) molecular mass protein markers (numbers on the right show molecular masses of the marker proteins).

Isolation of *Fau*IA methylase. All steps were carried out at 4°C. Biomass (12 g) was suspended in 35 ml of buffer A (10 mM Tris-HCl, pH 7.5 at 25°C, 10 mM β-mercaptoethanol, 0.1 mM EDTA, 5% glycerol) containing 0.3 mg/ml lysozyme for 40 min. This cell suspension was then sonicated using a Soniprep 150 sonicator (MSE, England) and ice bath. The sonication treatment (amplitude of 22-24 μm) included six periods for 30 sec followed by 1-min intervals. After 1 min of such treatment, Triton X-100 was added to final concentration 0.1%. Cell debris was removed by centrifugation at 15,000 rpm for 30 min at 4°C using a J2-21 centrifuge (Beckman, USA).

The resultant supernatant was mixed with protease inhibitor PMSF, sodium chloride (up to 0.2 M concentration), and 20 ml phosphocellulose P11 equilibrated with buffer A containing 50 mM NaCl. The mixture stirred for 1 h was applied onto a column (10 cm × 20 mm), which was then washed with 40 ml of buffer A containing 0.2 M NaCl. An absorbed material was eluted using a liner gradient of NaCl (0.2-0.9 M) in buffer A (total volume of 200 ml); 50 fractions (4 ml each) were collected.

Fractions exhibiting maximal DNA methyltransferase activity (Fig. 2) were applied at the volume of 5 ml onto a hydroxyapatite column (10 cm × 20 mm) equilibrated with buffer B (10 mM KH_2PO_4, pH 7.2 at 25°C, 0.1 mM EDTA, 10 mM β-mercaptoethanol, 5% glycerol, 0.2 M NaCl). The rate of sample application onto the column was 30 ml/h. The column was washed with two volumes of buffer B. Proteins retained on the column were eluted with a linear gradient of KH_2PO_4 (10-300 mM) in buffer B in the volume of 120 ml at the elution rate 30 ml/h; 50 fractions (2.4 ml each) were collected. The enzyme was eluted at 100-155 mM KH_2PO_4. The most active fractions were pooled and dialyzed against 100 ml of the concentrating buffer containing 50% glycerol, 10 mM Tris-HCl (pH 7.5 at 25°C), 10 mM β-mercaptoethanol, 0.1 mM EDTA, and 0.2 M NaCl, under constant stirring for 16 h using a magnetic stirrer. This preparation was concentrated and then layered onto Sephacryl S-200 column (70 cm × 16 mm) equilibrated with buffer A containing 0.3 M NaCl, 7% glycerol, and 14 mM β-mercaptoethanol. Proteins were eluted with 30 ml of the same buffer at the rate 6 ml/h; 20 fractions (1.5 ml each) were collected.

Fractions containing methylase activity were pooled and applied onto a hydroxyapatite column (volume of 4 ml) equilibrated with buffer B; the rate of sample application was 30 ml/h. The column was washed with two volumes of buffer B. Proteins were eluted with a linear gradient of KH_2PO_4 (10-300 mM) in buffer B (total elution volume of 100 ml); 40 fractions (2.5 ml each) were collected.

Fractions containing DNA methyltransferase activity were pooled and dialyzed against 100 ml of the concentrating buffer for 10 h at 4°C with stirring.

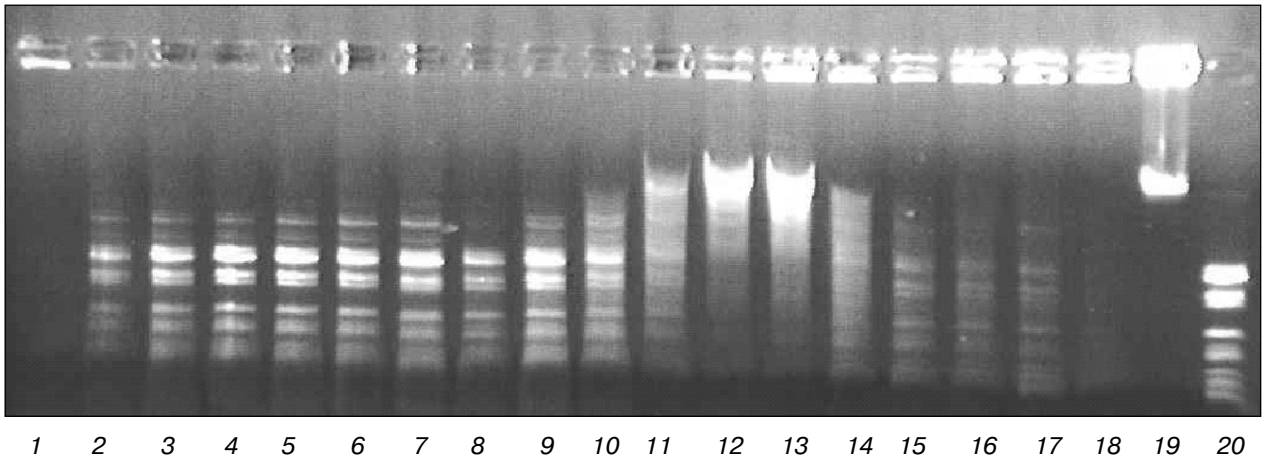

Fig. 2. Catalytic activity in fractions obtained after the first chromatographic step of methylase purification. (Electrophoregram of samples in 1% agarose.) Methylase activity was evaluated by protection of phage λ DNA incubated with fraction aliquots against hydrolysis by restriction endonuclease *Fau*I. Lanes: *1*) free volume; *2-18*) each third fraction starting from fraction 1 (highest methylase activities are in lanes *12* and *13*); *19*) initial incubation medium without added fraction aliquots and restriction endonuclease; *20*) hydrolyzate of non-methylated phage λ DNA obtained after incubation with restriction endonuclease *Fau*I.

Assay of DNA methyltransferase activity. Methylase activity in chromatographic fractions was evaluated by protection of phage λ DNA incubated with fraction aliquots against hydrolysis by restriction endonuclease *Fau*I. The aliquots of 2 μl were added to 10 μl of the reaction medium containing 0.5 μg phage λ DNA in SE buffer No. 5 (Y) (33 mM Tris-acetate, pH 7.9, 10 mM magnesium acetate, 66 mM potassium acetate, 1 mM DTT) and 160 μM SAM. The mixture was incubated for 30 min at 37°C, and after addition of 1 μl of restriction endonuclease *Fau*I preparation, it was incubated for 30 min at 55°C. Reaction products were separated in 1% agarose gel, stained with ethidium bromide, and analyzed under UV light. Figure 2 shows results of testing of fractions obtained after the first purification step (on phosphocellulose) on protection of phage λ DNA hydrolysis by restriction endonuclease *Fau*I.

The presence of protein contaminations in fractions was determined by PAGE under denaturing conditions. Aliquots of 25 μl were applied onto SDS-polyacrylamide gel, and electrophoresis was carried out according to standard protocol [9].

Assay of methyl group incorporation into DNA. Methylation reaction was carried out in reaction mixture containing 100 mM Tris-HCl with fixed pH, 1 mM EDTA, 1 mM DTT, and BSA (0.2 mg/ml) using tritiated SAM.

Studying temperature and pH dependences of enzyme activity and its kinetic behavior the reaction time was selected by accumulation of products, which did not exceed 10%.

The methylation reaction was stopped by addition of an aliquot on a DE81 filter (Whatman), which was washed three times with 0.02 M $NaHCO_3$ (each time for 20 min) and finally with water. The filter was dried, and radioactivity was counted using a Mark III scintillation counter (Nuclear Chicago).

Catalytic and Michaelis constants were calculated by regression analysis using Origin 5.0 software.

RESULTS AND DISCUSSION

Purification of *Fau*IA methylase. The protocol of *Fau*IA methylase purification included four sequential chromatographic stages: chromatography on phosphocellulose P-11, hydroxyapatite, and Sephacryl S-200 and finally repeated chromatography on hydroxyapatite. During purification, fractions exhibiting the highest catalytic activity were pooled and concentrated after the last stage by dialysis against the storage buffer. The final preparation of *Fau*IA methylase consisted of 2.5 ml with protein concentration 0.73 mg/ml. Its purity was verified by electrophoresis in 10% polyacrylamide gel under denaturing conditions (Fig. 1).

The molecular mass of the purified enzyme was about 39 kD. This corresponds to molecular mass calculated from the amino acid sequence of *Fau*IA methylase.

Temperature dependence of *Fau*IA activity. This was evaluated by the level of phage λ DNA methylation during 10 min of the reaction. The temperature optimum was observed at 33°C, and further temperature increase was accompanied by a sharp decrease in enzymatic activity (Fig. 3a). For example, at 43°C the enzyme activity was three orders of magnitude less than that observed at the optimal temperature. The temperature optimum of *Fau*IA

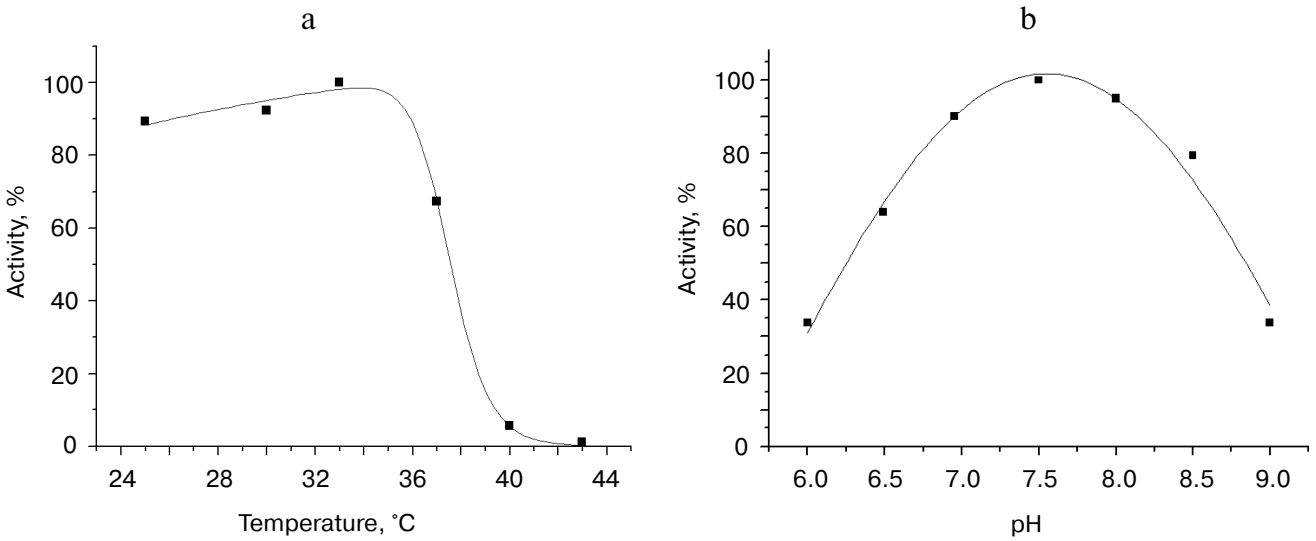

Fig. 3. Dependence of the rate of phage λ DNA methylation by *Fau*IA (enzyme concentration, 900 nM; phage λ DNA concentration calculated by sites 5′-CCCGC-3′, 500 nM; SAM concentration, 5 μM): a) on temperature (reaction time 10 min); b) on pH (reaction time 15 min).

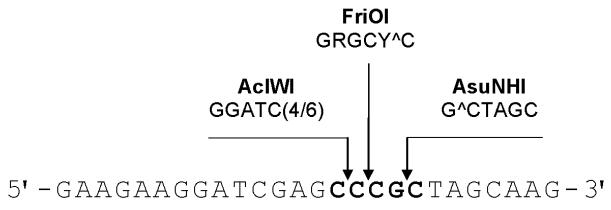

Fig. 4. Structure of synthetic oligonucleotide used for determination of methylated nucleotide residue. The recognition site for enzymes of the *Fau*I restriction–modification system is shown in bold. The arrows show position for the oligonucleotide cleavage by the restriction endonucleases shown above.

methylase sharply differs from that of restriction endonuclease *Fau*IA, which is about 55°C. This is the only example demonstrating such significant difference (by more than 20°C) between methylase and restrictase enzymes of the same restriction–modification system. This indicates that the *Fau*I restriction–modification system is unique. All subsequent experiments were carried out at 33°C.

The pH dependence of *Fau*IA methylase activity. The highest enzyme activity was detected within the range of pH from 7.0 to 8.5. This corresponds to usual pH optimum found for DNA methyltransferases; pH optimum for this particular enzyme was 7.5 (Fig. 3b). All subsequent experiments were carried out at pH 7.5.

Determination of the methylated base in the recognition site. In subsequent experiments, a synthetic oligonucleotide duplex containing site 5′-CCCGC-3′ was synthesized. Figure 4 shows one of the complementary strands of this duplex.

Before determination of the methylated base, it was necessary to check that the enzyme methylates only one nucleotide in the duplex. So maximal methylation of 1 μM duplex was carried out. The concentration of methylated DNA was determined by radioactivity (in cpm) of the tritiated label incorporated into DNA. Results of this experiment are shown at Fig. 5. After 1-h incubation, the concentration of methylated DNA reached a plateau and its concentration was 1 μM. This corresponded to the duplex concentration in the reaction

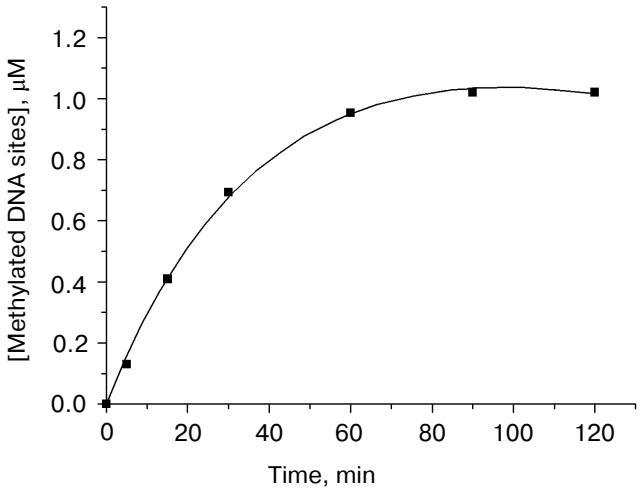

Fig. 5. Time dependence of concentration of methylated sites in duplex. Concentrations of *Fau*IA methylase, duplex, and SAM were 1.7, 1, and 5 μM, respectively.

Table 1. Radioactivity (in cpm) of tritiated restriction fragments obtained using restrictases *Acl*WI, *Fri*OI, and *Asu*NHI (the sequence recognized by *Fau*I restrictase is underlined)

Restrictases used for hydrolysis	Fragments obtained			
	Upper strand		Lower strand	
	restriction fragment sequence	cpm	restriction fragment sequence	cpm
*Fri*OI	5'-GAAGAAGGATCGAGCC-3' 5'-CGCTAGCAAG-3'	876 60	5'-CGATCCTTCTTC-3' 5'-CTTGCTAGCGGGCT-3'	85 55
*Asu*NHI	5'-GAAGAAGGATCGAGCCCG-3' 5'-CTAGCAAG-3'	980 121	5'-CTAGCGGGCTCGATCCTTCTTC-3' 5'-CTTG-3'	141 50
*Acl*WI	5'-GAAGAAGGATCGAGC-3' 5'-CCGCTAGCAAG-3'	58 830	5'-GGGCTCGATCCTTCTTC-3' 5'-CTTGCTAGC-3'	45 52

medium. Thus, the results indicate that only one nucleotide residue was methylated. This modified substrate was further used for determination of the methylated base.

The methylated oligonucleotide (after introduction of tritiated methyl group into duplex by *Fau*IA methylase) was separately cleaved by three restrictases—*Acl*WI, *Fri*OI, and *Asu*NHI. Fragments obtained after hydrolysis were separated by electrophoresis in 20% polyacrylamide gel. The presence of high urea concentration (8 M) provided denaturation of double stranded DNA. Resultant restriction fragments were eluted from the gel and transferred onto DE81 filters and radioactivity was measured. Table 1 shows results of assay of radioactivity for each restriction fragment.

These data suggest that *Fau*IA methylates the upper strand and the methyl group is incorporated into the second cytosine residues of the site 5′-CCCGC-3′.

Determination of standard kinetic parameters for *Fau*IA methylase. Standard kinetic parameters of DNA methylation reaction catalyzed by *Fau*IA were determined using the oligonucleotide duplex as a substrate; this was used for determination of the methylated base in the recognition site.

According to conditions of kinetic constant determination using the Michaelis–Menten equation, the dependence of methylation rate on concentration of one substrate was analyzed at saturating concentration of the other substrate. Figure 6a shows the dependence of methylation rate on duplex concentration.

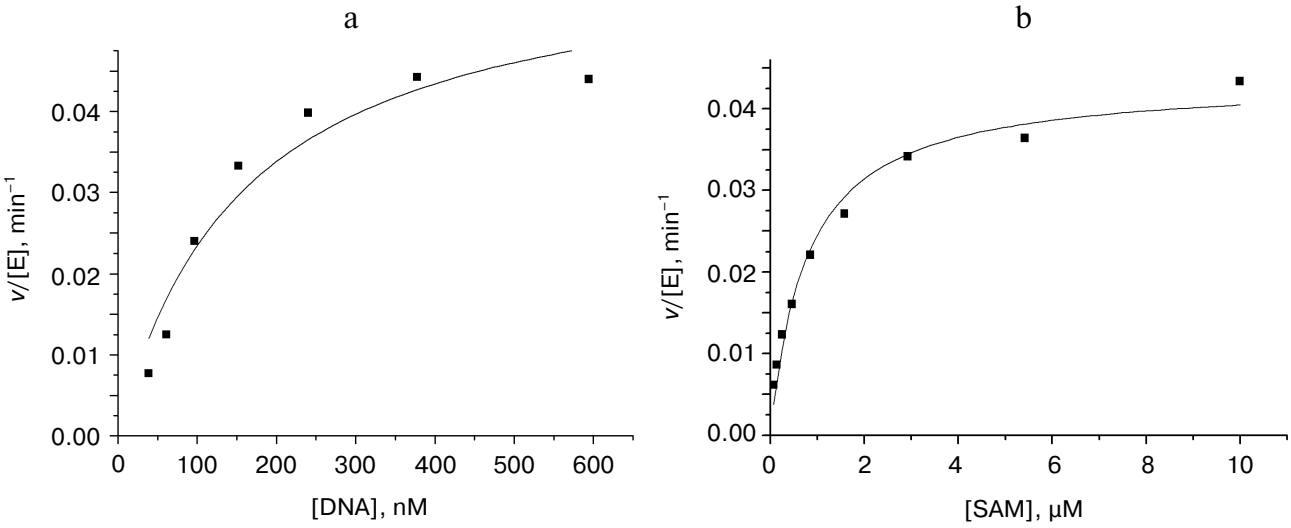

Fig. 6. Dependence of methylation rate by *Fau*IA: a) on DNA concentration (reaction time 50 min; concentrations of enzyme and SAM were 30 nM and 5 μM, respectively); b) on SAM concentration (reaction time 40 min; concentrations of enzyme and DNA were 200 nM and 1 μM, respectively).

Table 2. Kinetic parameters of prokaryotic C5-DNA methyltransferases (catalytic parameters were studied using oligonucleotide duplexes, except as specified separately)

DNA methyltransferase	k_{cat}, min^{-1}	$K_{m\ DNA}$, nM	$K_{m\ SAM}$, μM	Reference
*Fau*IA	0.052	168	0.776	Present study
*Msp*I	3.36	7.1	0.016	[10]
*Hha*I	5.1	4.0	0.16	[11]
*Eco*RII	0.36	15	—	[12]
*Eco*HK31I*	3.0	2.0	0.58	[13]

* Catalytic parameters were studied using pWM2372 plasmid.

Table 3. Catalytic parameters of prokaryotic N6-DNA methyltransferases

DNA methyltransferase	k_{cat}, min^{-1}	$K_{m\ DNA}$, nM	$K_{m\ SAM}$, μM	Reference
*Bam*HI	3.24	350	1.6	[16]
*Eco*Dam	6.6	55	2.6	[17]
*Eco*RI	7.2	2.4	0.12	[18]
*Fok*I, N-terminal domain	0.0007	1240	—	[14]
*Fok*I, C-terminal domain	0.024	230	—	[14]
*Bst*F5I-2	2.6	720	0.67	[15]
*Bst*F5I-4	0.99	1290	0.94	[15]

The kinetic constants calculated by regression analysis of these data were: $K_m = 0.17 \pm 0.04$ μM, and $k_{cat} = 0.050 \pm 0.005\ min^{-1}$.

For analysis of the dependence of DNA methylation rate on the concentration of the second substrate (SAM), we used the concentration of methylation sites of 1 μM. Figure 6b shows the dependence of methylation rate on SAM concentration. The Michaelis constant calculated on the basis of these data was 0.78 ± 0.08 μM.

Comparison of our results with literature data shows that for most prokaryotic DNA methylases modifying cytosine residues at C5 position the catalytic constant is higher whereas K_m values for DNA and SAM are lower than for the DNA methylase investigated in this study (Table 2). In contrast to *Fau*IA all other DNA methylases recognize palindromic sites. Perhaps the asymmetric recognition site for *Fau*IA is the main reason underlying differences of kinetic parameters of these enzymes. Indeed, it is known that enzymes recognizing nonpalindromic sites (e.g., C- and N-terminal domains of *Fok*I methylase [14], methylases of *Bst*FI5 restriction–modification system [15]) are characterized by lower catalytic constants than enzymes modifying palindromic recognition sites (Table 3).

Most DNA methylases modifying palindromic sites are dimers; this apparently represents the molecular basis for higher catalytic constants of these enzymes compared with monomeric enzymes recognizing nonpalindromic sites. Interestingly, among all C5-cytosine DNA methylases listed in Table 2 the properties of *Eco*RII are the closest to those of *Fau*IA methylase; however, even in this case the catalytic constant of *Eco*RII is seven times higher than that for *Fau*IA. Since some evidence exists that *Eco*RII may function as a monomer [12], this may explain why it exhibits some properties closest to *Fau*IA among all methylases.

High $K_{m\ DNA}$ values for *Fau*IA compared with other DNA methylases are also related to the monomeric nature of this enzyme and asymmetric recognition site. Previously we also determined abnormally high $K_{m\ DNA}$ values for two enzymes of the *Bst*FI5 restriction–modification system [15]; these enzymes also recognize an asymmetric site.

Figure 7 shows the time dependence of methyl group incorporation into phage λ DNA catalyzed by *Fau*IA methylase. This enzyme catalyzes incorporation of methyl groups into DNA exceeding the concentration of 5′-CCCGC-3′ sites in the incubation medium. Taking

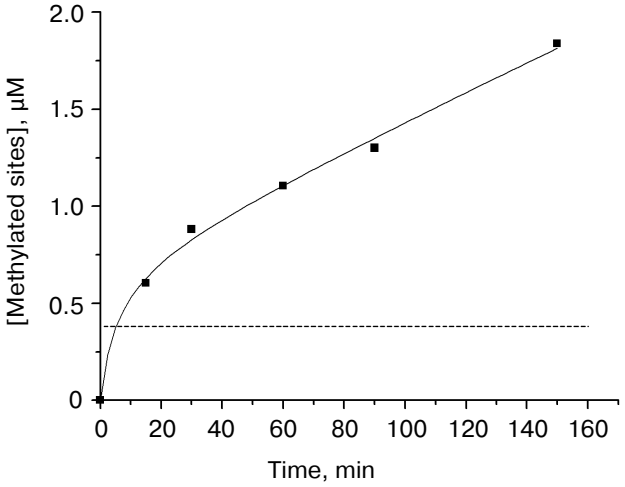

Fig. 7. Time dependence of phage λ DNA methylation by *Fau*IA (concentrations of phage λ 5′-CCCGC-3′ site, the enzyme, and SAM were 378 nM, 1.8 μM, and 5 μM, respectively). The broken line indicates the 5′-CCCGC-3′ site concentration in solution.

into consideration almost stoichiometric modification of the oligonucleotide duplex, this suggests additional methylation of phage λ DNA sites lacking in the structure of the synthetic oligonucleotide.

Methylation of non-canonic sites is poorly investigated. It has been demonstrated for DNA methylases of restriction–modification systems and also for methylases lacking corresponding restrictase. For example, EcoDam and T4Dam methylases usually methylating adenine in the 5′-GATC-3′ sequence can also modify adenine in the shorter sequence 5′-GAT-3′ at a rate which is somewhat lower than that observed during methylation of canonic sequences [19, 20]. Possibility of non-canonic methylation by enzymes of restriction–modification systems has been demonstrated not only for methylases modifying palindromic sites [21], but also for methylases recognizing nonpalindromic sequences [14]. Perhaps this is not a rare phenomenon and it might be found for many restriction–modification systems.

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