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Substrate Specificity and Biochemical Properties of M3.BstF5I DNA Methyltransferase from the BstF5I Restriction–Modification System

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Abstract—Optimal conditions for DNA methylation by the M3.BstF5I enzyme from *Bacillus stearothermophilus* and kinetic parameters of λ phage DNA modification and that of a number of oligonucleotide substrates are established. Comparison of M1.BstF5I and M3.BstF5I kinetic parameters revealed that with similar temperature optima and affinity for DNA, M3.BstF5I has nearly fourfold lower turnover number (0.24 min^{-1}) and modifies the hemimethylated recognition site with lower efficiency under optimal conditions than the unmethylated one. In contrast to another three methylases of the BstF5I restriction–modification system, the M3.BstF5I enzyme is able to optionally modify the noncanonical 5′-GGATC-3′ DNA sequence with a rate more than one order of magnitude lower than the methylation rate of the canonical 5′-GGATG-3′ recognition site.

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Specific DNA methyltransferases (M, methylases) catalyze transfer of the methyl group from S-adenosyl-L-methionine (SAM) to adenine or cytosine amino groups or to the cytosine C5 atom. Most currently known DNA methylases of procaryotic organisms are involved in so-called restriction–modification systems (RM systems). RM system restrictases are able to hydrolyze DNA, preventing replication of alien DNA in bacterial cells. Modifying the same recognition site as restrictases, DNA methylases provide protection against hydrolysis of DNA of the host cell.

The unique BstF5I RM system from *Bacillus stearothermophilus* F5 includes four site-specific DNA methyltransferases. The M1.BstF5I and M3.BstF5I enzymes modify adenine in the 5′-GGATG-3′ DNA sequence, while M2.BstF5I and M4.BstF5I modify adenine in 5′-CATCC-3′ on the complementary DNA chain. Earlier, we studied properties of M2.BstF5I and M4.BstF5I DNA methyltransferases [1], some properties of M1.BstF5I DNA methyltransferase [2], as well as describing M3.BstF5I gene cloning and determination of the base modified by this enzyme [3]. DNA methyltransferases participate in gene transcription regulation, replication, and reparation [4] and are important for pathogenicity [5].

The most studied systems are second type RM systems which are united by a number of common features: only Mg^{2+} ions are required for exhibition of enzymatic activity of restriction endonucleases, and hydrolysis occurs inside or near the recognition site [6]. A palindromic DNA sequence is recognized in most known RM systems. In this case, a single DNA methyltransferase modifying both recognition site chains using SAM as methyl group donor is enough for host DNA protection. In this large group of RM systems, the IIS subgroup in which a non-palindromic DNA sequence is the recognition site is modified. In this case, two methyltransferases, one of which recognizes the top chain and the other the bottom chain are required for bacterial DNA protection

Abbreviations: DTT, dithiothreitol; HAP, hydroxyapatite; M, methylase; RM systems, restriction–modification systems; SAM, S-adenosyl-L-methionine.

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[6]. In several cases, methylation of both chains of a non-palindromic sequence by single protein is also possible, which is explained by the appearance of two separate domains responsible for recognition of an endogenous chain as, for instance, in the case of FokI methylase, *N*- and *C*-terminal domains of which methylate different chains [7-9]. There are also intermediate RM systems with confluent symmetric methylase recognition site (e.g. 5′-GASTC-3′) and non-palindromic sequence of restrictase recognition (5′-GAGTC-3′), which is one of the variants of the recognition site of the methylase [10].

We have earlier revealed two methylase pairs having similar substrate specificity in the BstF5I RM system [1-3, 11]. BstF5I restriction endonuclease recognizes non-palindromic 5′-GGATG-3′ DNA sequence. M1.BstF5I and M3.BstF5I recognize and methylate adenine in the top chain of the given recognition sequence, while M2.BstF5I and M4.BstF5I modifies adenine in the bottom chain [12].

The goal of the present work was to study substrate specificity and biochemical and kinetic properties of M3.BstF5I compared with the properties of M1.BstF5I methylase [3] and the *N*-terminal domain of FokI methylase [9] having similar recognition sites.

MATERIALS AND METHODS

Reagents. *Bacillus stearothermophilus* F5 strain was from the microorganism collection of Sibenzyme (Russia), and *Escherichia coli* RRI strain was from New England Biolabs (USA). The following reagents and nutrient medium components were used: Tris (Promega, USA); acrylamide, *bis*-acrylamide, KH_2PO_4, $NaHCO_3$, and ampicillin (Helicon, Russia); EDTA (Fluka AG, Switzerland); agarose (Hybaid-AGS, Germany); dithiothreitol (DTT) and Triton X-100 (ICN, USA); lysozyme (Serva, Germany); [^{3}H-CH_3]SAM (Amersham, England); phenylmethylsulfonyl fluoride (PMSF) (Sigma, USA); phosphocellulose P11 (Whatman, England); hydroxyapatite (HAP) and heparin-Sepharose (BioRad, USA); Sephacryl S-200, tryptone, and yeast extract (Organotechnie, France); enzymes and DNA preparations (Sibenzyme, Russia); marker kit of protein molecular masses containing BSA (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), and trypsin inhibitor (21.5 kDa) (Helicon, Russia). Other reagents were produced in Russia and qualified as chemically pure.

Cultivation of M3.BstF5I biomass. Preparation of pJW-MF5-3 recombinant plasmid by cloning of the M3.BstF5I DNA methyltransferase gene into expression vector pJW was described earlier [3]. *Escherichia coli* RRI cells were transformed by the pJW-MF5-3 plasmid. A separate colony was seeded into a vial containing 100 ml of liquid LB nutrient 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). The culture was grown with mixing of the medium on a shake-flask propagator at 30°C and 150 rpm overnight. Then 0.5-liter flasks with 200 ml of fresh LB medium and ampicillin were inoculated with the culture. The cells were cultivated with mixing on the shake-flask propagator at 30°C and 180 rpm up to an absorption $A_{550} = 0.8$-0.9. Then the cells were thermo-induced at 42°C for 4 h. The cells were collected using a J2-21 centrifuge (Beckman, USA) at 3000 rpm and 4°C. The biomass was kept at −20°C.

Isolation of M3.BstF5I enzyme. All of the isolation procedures were carried out at 4°C. The following reagents were used in the isolation process at 25°C: buffer A containing 10 mM Tris-HCl (pH 7.5), 10 mM β-mercaptoethanol, 7 mM EDTA, 5% glycerol; buffer P containing 10 mM KH_2PO_4 (pH 7.3), 10 mM β-mercaptoethanol, 7 mM EDTA, 5% glycerol, 0.2 M NaCl; buffer C containing 10 mM Tris-HCl (pH 7.5), 10 mM β-mercaptoethanol, 7 mM EDTA, 0.1 M NaCl, 50% glycerol.

The biomass of *E. coli* cells (9.5 g) containing M3.BstF5I was suspended in 40 ml of buffer A containing lysozyme (0.4 mg/ml) using a magnetic stirrer for 30 min. The suspension was treated with ultrasound using Soniprep 150 disintegrator (MSE, England) eight times for 40 sec with 1 min intervals for cooling. Triton X-100 was added up to 0.1% concentration during the process of cell disintegration. Cell debris was separated by centrifuging at 15,000 rpm for 30 min using the J2-21 centrifuge. Proteinase inhibitor PMSF was added to the supernatant up to 1 μM concentration as well as 200 mM NaCl. Chromatographic isolation was carried out using P11 phosphocellulose, HAP, heparin-Sepharose, and Sephacryl S-200. Crude extract was applied on a column with phosphocellulose (1.6 × 20 cm, volume 20 ml) preliminarily equilibrated with buffer A containing 0.2 M NaCl. The column was washed using two volumes of the same buffer. The column was eluted using 200 ml of NaCl linear gradient from 0.2 to 0.9 M in buffer A, and fifty 4-ml fractions were collected. The fractions with maximum M3.BstF5I activity were combined and applied to a column with 4 ml of HAP preliminarily equilibrated with buffer P and washed with two volumes of buffer P. Then the column was eluted with 120 ml of potassium phosphate linear gradient (from 0.01 to 0.25 M) in buffer P. Forty 3-ml fractions were collected. The fractions with the target activity were diluted with three volumes of buffer A and applied on a column with 4 ml of heparin-Sepharose preliminarily equilibrated with buffer A. The column was washed with two volumes of buffer A containing 0.1 M NaCl. The enzyme was eluted with 100 ml of NaCl linear gradient (from 0.1 to 0.8 M) in buffer A. Forty 2-ml fractions were collected. The fractions containing M3.BstF5I were precipitated by 75% ammonium sulfate.

The precipitate was centrifuged at 12,000 rpm using the J2-21 centrifuge for 30 min and diluted in 4 ml of buffer A and applied on a column containing 120 ml of Sephacryl S-200 (2.0 × 80 cm). Gel filtration was carried out using 120 ml of buffer A containing 0.1% Triton X-100 as well as 0.3 M NaCl at the rate of 20 ml/h. The target fractions were diluted with two volumes of buffer A containing 0.05% sodium deoxycholate and rechromatographed on HAP. The fractions containing active M3.BstF5I were combined and concentrated against buffer C for 16 h. The M3.BstF5I preparation obtained was kept at –20°C.

Preparation of M1.BstF5I with protein concentration 100 μM was isolated as described earlier [2].

Examination of M3.BstF5I activity in fractions. M3.BstF5I activity in the chromatographic profile was determined by λ phage DNA protection from hydrolysis by BstF5I restriction endonuclease. For this goal, 2-μl aliquots were taken from the fractions and diluted with five volumes of SE buffer Y (33 mM Tris-acetate, pH 7.9, 10 mM magnesium acetate, 66 mM potassium acetate, 1 mM DTT), and 2 μl of the diluted solution was added to 20 μl of reaction mixture containing 1 μg λ phage DNA in SE buffer Y and 80 μM SAM. The mixture was incubated at 60°C for 15 min, then 1 μl of BstF5I restriction endonuclease preparation was added and kept at 60°C for 15 min more. Reaction products were separated in 1% agarose gel and treated with ethidium bromide solution and observed under UV light. The presence of foreign proteins in the fractions was determined by SDS-PAGE. For this goal, 25-μl aliquots from the fractions were applied to the gel, and electrophoresis was carried out by standard methods [13].

Determinations of number of methyl groups incorporated into DNA. For carrying out the methylation reaction, reaction mixture containing 100 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT, and 0.2 mg/ml BSA was used. Tritium-labeled SAM was used.

When determining dependence of M3.BstF5I activity on pH, 100 mM citric acid/sodium citrate at pH 4.0-6.5, 100 mM Tris-HCl at pH 7.0-9.5, and 100 mM sodium carbonate/sodium bicarbonate at pH 10.0-11.0 were used [14].

When determining the dependence of enzyme activity on temperature and pH and reaction rate dependence on concentration of one of the substrates, reaction times were chosen under conditions so that the concentration of methylation reaction products (concentration of methylated DNA sequences) would not be more than 10% of the initial concentration of methylation sites.

The methylation reaction was stopped by adding the aliquot on a DE-81 filter (Whatman), which was three times washed with 0.02 M sodium hydrocarbonate (each time for 20 min) and then by water. After drying the filter, the included tritium tag was determined by measuring radioactivity using a Mark III scintillation counter.

Statistical processing of the results. Parameters of functions corresponding to kinetic models were estimated by regression analysis using Origin 5.0 software (Microcal, USA). Catalytic constant and Michaelis constant were determined using regression analysis of the Michaelis function describing experiment data using χ^2 value minimization (http://www.microcal.com).

RESULTS AND DISCUSSION

Isolation of M3.BstF5I enzyme. After thermal induction of protein synthesis, the recombinant protein was isolated from *E. coli* biomass as described in "Materials and Methods".

The results of M3.BstF5I activity determination after the first chromatography on P11 phosphocellulose and quantitative and qualitative estimation of target protein purity using SDS-PAGE are presented in Fig. 1.

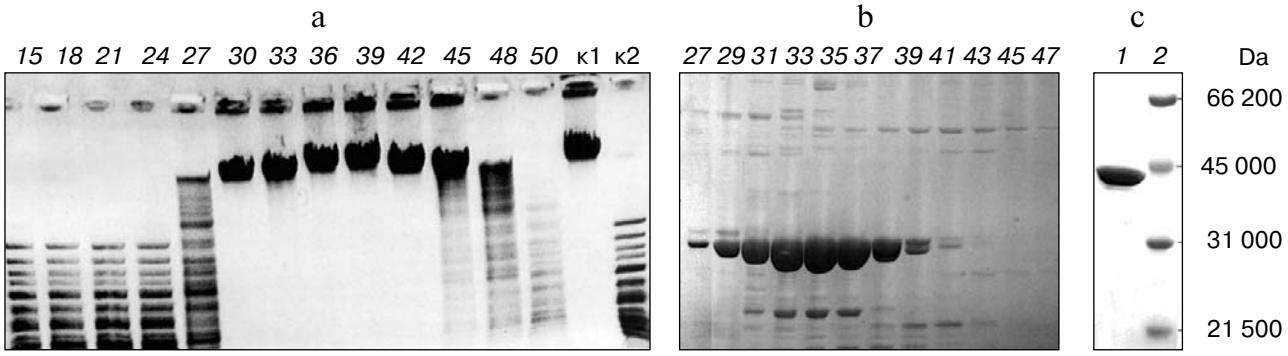

Fig. 1. Analysis of activity (a) and M3.BstF5I protein purity (b) after the first stage of chromatography on P11 phosphocellulose and in the final preparation (c). a) Electrophoregram of M3.BstF5I chromatography profile. Lane numbers correspond to the fraction numbers; *30-42*, methylase activity peak; k1, initial reaction mixture; k2, λ phage DNA treated with BstF5I restriction endonuclease. b) Testing of M3.BstF5I chromatography profile for protein (10% SDS-PAGE); lane numbers correspond to the fraction numbers. c) Electrophoregram (10% SDS-PAGE) of the final preparation of M3.BstF5I (*1*) and molecular mass marker (*2*).

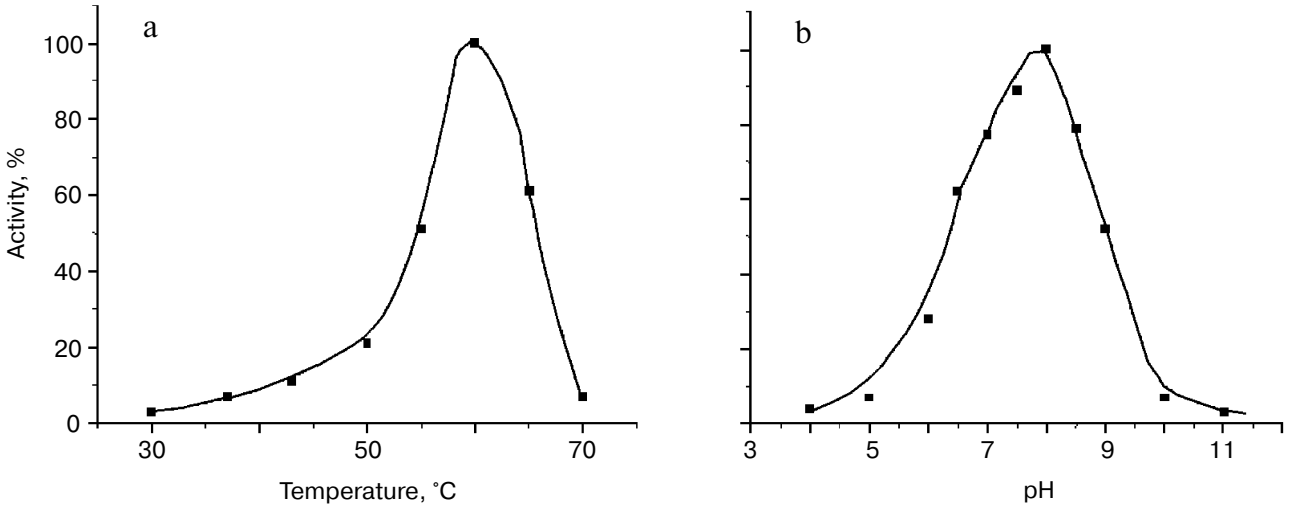

Fig. 2. Dependence of methylation rate of λ phage DNA by M3.BstF5I enzyme on temperature (a) and pH (b). DNA concentrations in terms of 5′-GGATG-3′ sites – 1.3 μM, SAM – 5 μM, and M3.BstF5I – 120 nM. Reaction time 15 min.

The enzyme was eluted in fractions from 30 to 42 of the chromatographic profile, which corresponds to 0.45-0.65 M NaCl concentration. In later stages of isolation, the target protein was eluted at 82-195 mM potassium phosphate concentrations from HAP and at 0.52-0.66 M NaCl from heparin-Sepharose. After repeated chromatography on HAP, the enzyme was eluted at 170-210 mM potassium phosphate concentration.

As a result, 10.5 mg of highly purified M3.BstF5I preparation was obtained from 9.5 g of biomass. Electrophoretic mobility of the protein (Fig. 1c) corresponds to the primary structure data (41.1 kDa), and the estimated molar concentration is 36 μM.

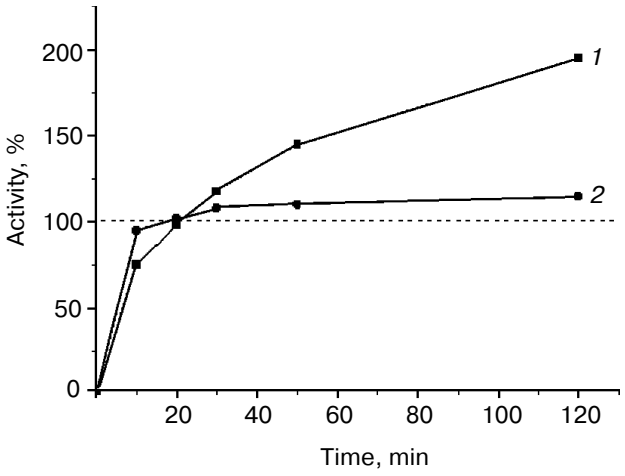

Fig. 3. Dependence of number of methyl groups incorporated into DNA on time: *1*) M1.BstF5I, 100 nM; *2*) M3.BstF5I, 100 nM. Concentration of λ phage DNA in terms of 5′-GGATG-3′ sites – 100 nM, SAM – 5 μM.

Dependence of M3.BstF5I activity on temperature. As seen from Fig. 2a, maximal activity for M3.BstF5I is observed at 60°C, which is close to the 55°C temperature optimum for M1.BstF5I [2]. Temperature optima for both of the enzymes correspond to that of BstF5 restrictase and M2.BstF5I and M4.BstF5I DNA methyltransferases [1]. Repeated experiments did not confirm an activity optimum of M3.BstF5I enzyme at low temperature observed earlier [2].

Dependence of enzyme activity on pH. The influence of pH on enzyme activity was studied at 60°C (Fig. 2b). The enzyme exhibits the highest activity in the range of pH 7.0-8.5, which corresponds to the usual pH optimum for DNA methyltransferases.

All further activity measurements were carried out at 60°C, pH 8.0. Activity of M3.BstF5I reaches its maximum in the absence of Na^+ and K^+ (like that of M.AluI, Dam *E. coli*, M.HhaI, and M.HpaII enzymes [15]) and it decreases with increase in salt concentration (data not presented).

Examination of M3.BstF5 substrate specificity. Data of methylation of λ phage DNA by M3.BstF5I and M1.BstF5I enzymes under suitable reaction conditions are presented in Fig. 3. The concentration of 5′-GGATG-3′ sites in λ phage DNA is 100 nM (dotted line). Comparison of the curves shows that M3.BstF5I methylase modifies one site relatively rapidly followed by additional DNA modification with significantly lower rate. This seems to be connected with the fact that M3.BstF5I, in contrast to M1.BstF5I, modifies not only the 5′-GGATG-3′ site, but also different non-canonical sites as well.

To study possible non-canonical DNA methylation by M3.BstF5I, enzyme hydrolysis of pF5-32 plasmid obtained by M3.BstF5I gene incorporation into pMTL22 vector [2] and subsequent transformation of cells of *E. coli*

JM119 strain deficient in the dam-methylase gene was investigated using different enzymes. The results of pF5-32 plasmid hydrolysis by various restrictases are presented in Fig. 4. Methylation by M3.BstF5I *in vivo* blocks BstF5I restrictase hydrolysis of plasmid DNA (Fig. 4, lane *2*). Restriction endonuclease Kzo9I recognizing the 5′-GATC-3′ sequence and insensitive to methylated adenine in the recognition site hydrolyzes DNA completely (Fig. 4, lane *3*). At the same time, an extra fragment of 1.7-1.8 kb length (Fig. 4, lane *4*) appears when DNA is hydrolyzed by BstKTI, which also recognizes the 5′-GATC-3′ sequence but does not cleave it if there are two methylated adenines in the recognition site [16]. According to the primary structure of pF5-32 plasmid, a DNA fragment of 1792 kb length is formed when hydrolysis of a single 5′-GGATCC-3′ sequence is blocked. This sequence will be methylated by M3.BstF5I *in vivo* if, besides non-canonical 5′-GGATG-3′ recognition site, it modifies the 5′-GGATC-3′ nucleotide sequence as well. In this case, we observe that methylation of 5′-GGATC-3′ sites leads to 5′-GGATCC-3′ sequence modification by both chains and blocking of hydrolysis by *Bst*KTI restrictase.

Of interest, non-canonical methylation is also observed for several other methylases and does not seem to be an exception. The possibility of methylation by enzymes non-canonically is shown for both methylases modifying palindromic recognition sites (e.g. EcoDam and T4Dam methylases with canonical 5′-GATC-3′ site [17, 18], HaeIII with canonical 5′-GGCC-3′ site [19], EcoRI with canonical 5′-GAATTC-3′ site [20]), and enzymes with non-palindromic recognition sequences (e.g. FauI methylase with canonical 5′-CCCGC-3′ site [21]). However, the *N*-terminal domain of FokI methylase, with which M3.BstF5I is the most homologous and has similar recognition site 5′-GGATG-3′ [2, 3], also exhibits "star" activity [9].

Examination and comparison of stationary kinetic parameters of M1.BstF5I and M3.BstF5I. Kinetic parameters of DNA methylation by M1.BstF5I and M3.BstF5I were examined on both high-polymer substrate (λ phage DNA) and synthetic oligonucleotides.

Phage λ DNA hydrolysis by *Vsp*I restrictase (recognition site ATTAAT) was preliminarily carried out to obtain more concentrated solution of the phage DNA and lower its viscosity. All of the kinetic parameters on high-molecular-weight DNA were examined on the resulting mixture of λ phage DNA fragments. In our estimations, concentration of 5′-GGATG-3′ methylation sites was determined by multiplication of molar concentration of λ phage DNA by a number of 5′-GGATG-3′ sites in a single DNA molecule (150 sites).

Dependence of M1.BstF5I and M3.BstF5I activity on concentration of SAM and λ phage DNA is presented in Fig. 5. As seen, the experimental points correspond well to the Michaelis curve [22]. Kinetic parameters (Michaelis constant, catalytic constant, specificity coefficient) estimated as described in "Materials and Methods" are presented in Table 1. The catalytic constant and Michaelis constant values estimated by SAM for M1.BstF5I appeared to be nearly 2 and 5 times less in magnitude than that described earlier [22].

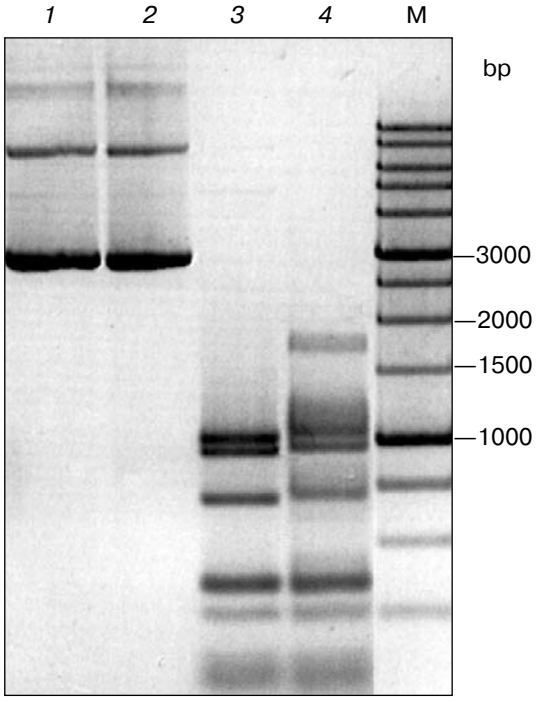

Fig. 4. Restriction analysis of pF5-32 plasmid: *1*) pF5-32; *2-4*) pF5-32 treated by *Bst*F5I, *Kzo*9I, and *Bst*KTI restrictases, relatively. M, marker.

Comparison of M1.BstF5I and M3.BstF5I kinetic parameters revealed that M3.BstF5I has nearly fourfold lower turnover number with similar temperature optima and under optimal conditions. At the same time, the value of Michaelis constant of λ phage DNA was similar for both of the enzymes under optimal conditions and was equal to 0.11 μM, and the value of SAM Michaelis constant for M3.BstF5I appeared to be nearly two times higher in magnitude.

Analysis of kinetic parameters of M3.BstF5I modification reaction on oligonucleotide duplexes. To study the kinetic parameters of synthetic duplex methylation, the following complementary oligonucleotides were used:

1) 5′-CGGCCAGC**GGATG**CGGGAGCG,

2) 5′-CGCTCCCG**CATCC**GCTGGCCG,

3) 5′-CGGCCAGC**GG(m6A)TG**CGGGAGCG,

4) 5′-CGCTCCCG**C(m6A)TCC**GCTGGCCG,

where m6A is N6-methyl adenine.

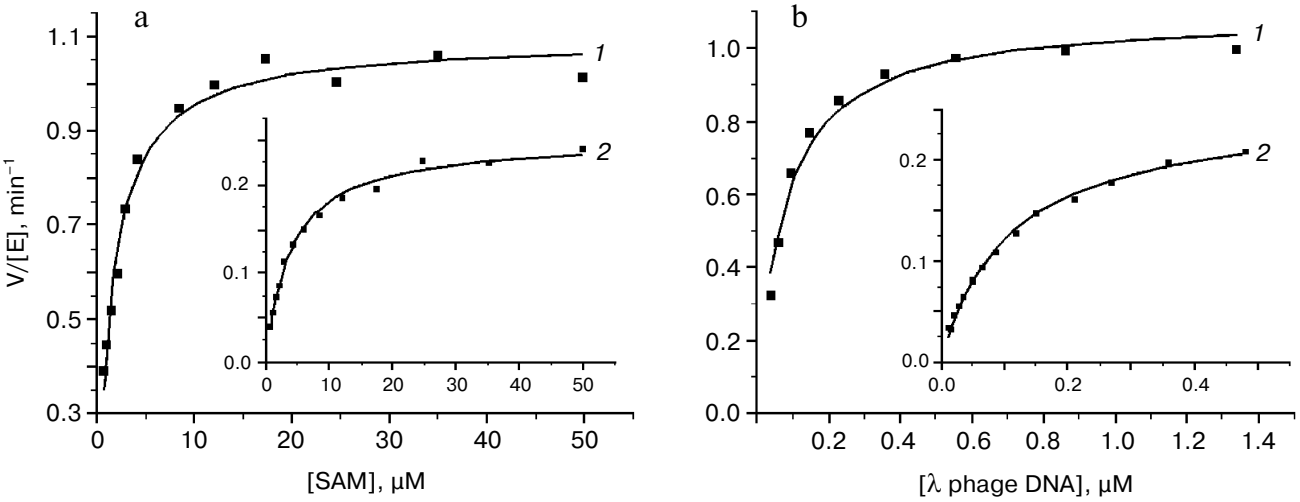

Fig. 5. Dependence of methylation rate of λ phage DNA on concentration of SAM (a) and restricts of λ phage DNA (b). *1*) M1.BstF5I; *2*) M3.BstF5I. Concentration of λ phage DNA in terms of 5′-GGATG-3′ sites – 1.3 μM, M1.BstF5I – 24.7 nM, and M3.BstF5I – 100 nM; reaction time 20 min.

The oligonucleotides with odd numbers are complementary to the oligonucleotides with even numbers. Oligonucleotides 1 and 3 as well as 2 and 4 differ from each other by N6-methyl adenine presence or absence. Dependence of methylation rate of oligonucleotide duplex 1/2 by M1.BstF5I and M3.BstF5I on SAM concentration is presented in Fig. 6. Dependence of methylation rate on concentration of unmethylated (1/2) and hemimethylated (1/4) duplexes is presented in Fig. 7. However, methylation of already modified duplex 3/2 does not occur (data not presented), which shows the absence of non-canonical methylation of duplex 1/2 by M3.BstF5I. Thus, use of oligonucleotide complexes 1/2 and 1/4 as methylation substrates reveals exclusively canonical M3.BstF5I methylation. Kinetic parameters estimated based on the presented dependences are shown in Table 1.

Methylation rates of high-polymer DNA and oligonucleotide substrates by M1.BstF5I and M3.BstF5I are different (Table 1). For high-molecular-weight substrate, kinetic constants and specificity coefficients for SAM and DNA are several times higher. Thus, both

Table 1. Kinetic parameters of reactions catalyzed by DNA methyltransferases recognizing the 5′-GGATG-3′ sequence

Enzyme (temperature)	Substrate	k_{cat}, min^{-1}	$K_{m\ DNA}$, μM	$K_{m\ SAM}$, μM
M1.BstF5I (55°C)*	λ phage DNA	0.98	0.11	1.47
	unmethylated duplex 1/2	0.328	0.229	0.682
	hemimethylated duplex 1/4	0.51	0.16	
M3.BstF5I (60°C)*	λ phage DNA	0.24	0.11	3.3
	unmethylated duplex 1/2	0.053	0.168	4.36
	hemimethylated duplex 1/4	0.032	0.09	
	5/6 duplex with non-canonical 5′-GGATC-3′ site	0.0026	3.3	–
M.FokI, *N*-terminal domain (37°C)**	λ phage DNA	0.20	0.0007	0.4
	unmodified duplex 21 bp	0.007	1.24	–
	hemimethylated duplex 21 bp	0.12	1.13	–

* Kinetic parameters estimated using hemimethylated oligonucleotide duplex 1/4 are shown.
** K_m value is shown for λ phage DNA in terms of molecular concentration of λ phage DNA but not 5′-GGATG-3′ sites.

enzymes modify λ phage DNA more effectively than short DNA substrates. Preferable methylation of high-molecular-weight DNA was also observed for other site-specific DNA methyltransferases. For EcoDam, EcaI, EcoRII, and MspI methyltransferases (Table 2), catalytic constant value for high-molecular-weight substrate is also nearly 3-7 times higher than for methylation of oligonucleotide duplexes [23-26].

Data on the properties of M3.BstF5I and other two DNA methyltransferases (M1.BstF5I [2] and M.FokI *N*-terminal domain [27, 28]) also having 5′-GGATG-3′ recognition site are presented in Table 1. Catalytic constants of λ phage DNA modification by the highly homologous enzymes M3.BstF5I and M.FokI *N*-terminal domain practically do not differ and are four times lower in magnitude than k_{cat} for M1.BstF5I. Values of $K_{m\ DNA}$ for all three enzymes are practically the same (0.1 μM). However, more significant differences in enzyme properties are observed when modifying oligonucleotide duplexes containing 5′-GGATG-3′. For M1.BstF5I, k_{cat} is six times higher than k_{cat} for M3.BstF5I and more than 40 times higher than for M.FokI *N*-terminal domain. Such significant difference in reaction rate of oligonucleotide duplex modification by the *N*-terminal domain can be connected with the structure of the given protein obtained by cleavage of M.FokI in half and addition of leader histidine peptide. Comparison of synthetically

Table 2. Catalytic constants of DNA methyltransferases that preferably methylate high-polymer DNA

DNA methyl-transferase	Substrate	Catalytic constant, min^{-1}	Source
EcoDam	plasmid DNA duplex	19.02 6.6	[23]
EcoRII	λ phage DNA duplex	0.042 0.006	[25]
MspI	λ phage DNA duplex	0.17 0.056	[26]
EcaI	λ phage DNA duplex	0.019 0.0047	[24]

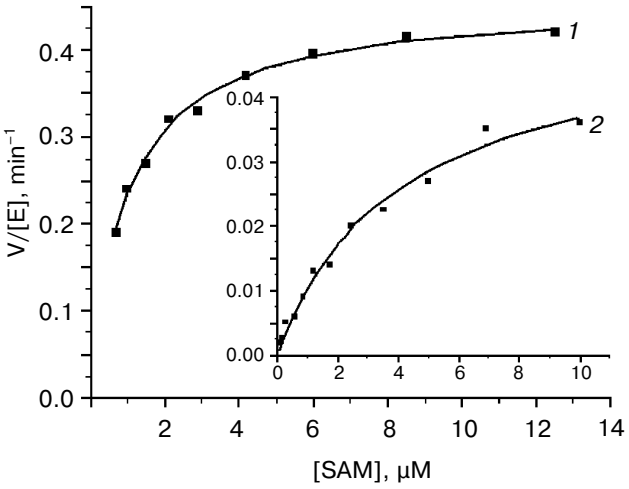

Fig. 6. Dependences of M1.BstF5I and M3.BstF5I methylation rates of oligonucleotide duplex 1/2 on SAM concentration: *1*) M1.BstF5I (24.7 nM); *2*) M3.BstF5I (28.7 nM). Oligonucleotide duplex concentration for M1.BstF5I – 5 μM, for M3.BstF5I – 7 μM; reaction time 20 min.

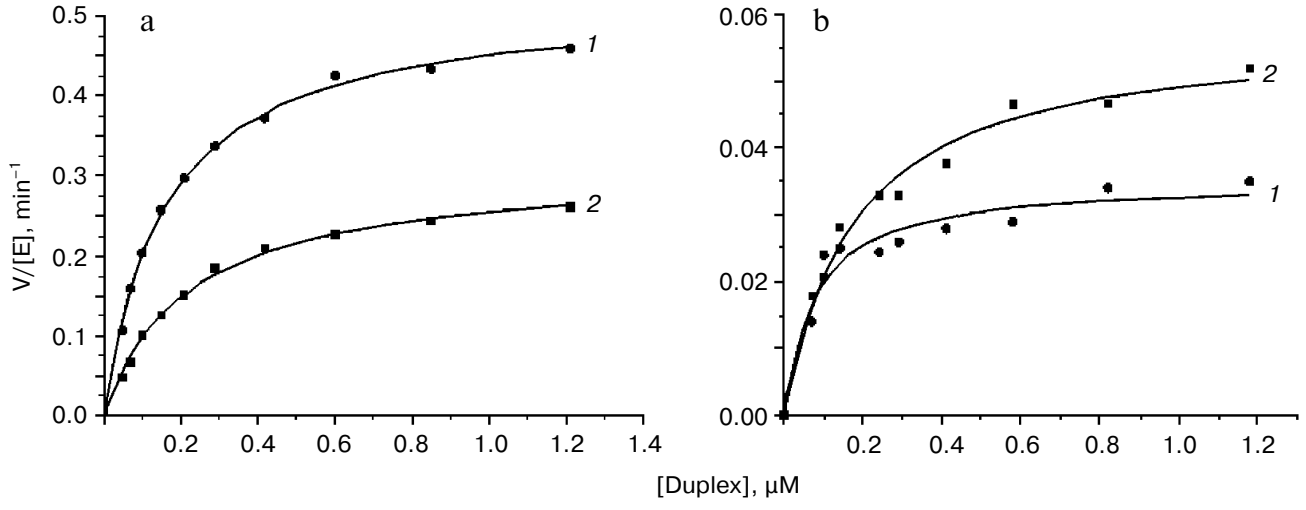

Fig. 7. Dependences of M1.BstF5I and M3.BstF5I methylation rates of unmethylated (1/2) and hemimethylated (1/4) oligonucleotide duplexes on DNA concentration. a) M1.BstF5I (24.7 nM); b) M3.BstF5I (28.7 nM). *1*) Hemimethylated duplex (1/4); *2*) unmethylated duplex (1/2). Concentration of SAM – 30 μM; reaction time 20 min.

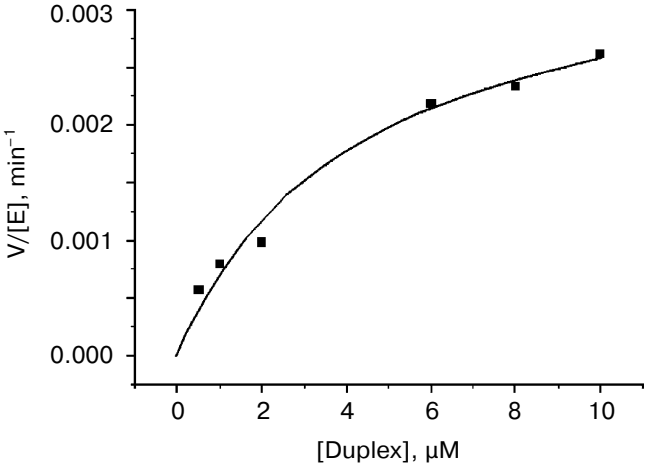

Fig. 8. Dependence of methylation rate on concentration of oligonucleotide duplex 5/6 with 5′-GGATG-3′ non-canonical site. Concentration of SAM – 10 μM, M3.BstF5I – 100 nM; reaction time 60 min.

obtained protein with highly homologous natural M3.BstF5I enzyme shows that constructed M.FokI *N*-terminal domain contains, besides histidines, an additional 20 amino acid residues on the *C*-terminal, five of which are lysines [27]. Thus, M.FokI *N*-terminal domain contains a significant number of redundant positively charged amino acid residues that seems to lead to nonspecific interaction with oligonucleotide phosphates and change in structure of the oligonucleotide due to its small size [27]. Since there are no significant changes in properties of lengthy λ phage DNA, required DNA helix conformation seems to remain constant, and k_{cat} does not change. At the same time, due to additional positively charged amino acid residues, $K_{m\ DNA}$ for duplex appears to be slightly better for M.FokI *N*-terminal domain compared with M1.BstF5I and M3.BstF5I enzymes.

Comparison of kinetic parameters of hemimethylated duplex modification (Fig. 7) reveals that, in the case of M1.BstF5I k_{cat} is higher compared with unmethylated oligonucleotide, while for M3.BstF5I k_{cat} is lower. As a result, for hemimethylated duplex modification, k_{cat} for M1.BstF5I is 16 times higher than k_{cat} for M3.BstF5I. At the same time, $K_{m\ DNA}$ values for the two enzymes differ by less than two times, which indicates preferential DNA methylation by M1.BstF5I enzyme after replication in the bacterial cell.

M3.BstF5I also methylates the non-canonical 5′-GGATC-3′ recognition site (Fig. 3). Therefore, we have established kinetic parameters for M3.BstF5I modification of oligonucleotide duplex containing 5′-GGATC-3′ sequence. The structure of the duplex is presented below (5′-GGATC-3′/5′-GATCC-3′ sequence is highlighted by boldface font):

5) 5′-CAGTTTA**GGATC**CATTTCAC-3′,

6) 3′-GTCAAAT**CCTAG**GTAAAGTG-5′.

Dependence of M3.BstF5I methylation rate on concentration of oligonucleotide duplex 5/6 is presented in Fig. 8, and kinetic parameters estimated based on the presented dependences are given in Table 1.

The catalytic constant of oligonucleotide duplex methylation with 5′-GGATC-3′ site is 20 times lower than that of the modification reaction of duplex with canonical site, while specificity coefficient of this reaction ($k_{cat}/K_{m\ DNA}$) is 400 times lower. As a result, 5′-GGATC-3′ site methylation by M3.BstF5I observed both *in vivo* (Fig. 3) and *in vitro* (Fig. 8) occurs with significantly lower efficiency compared with methylation of the 5′-GGATG-3′ canonical site.

Thus, compared with M3.BstF5I, M1.BstF5I is more active and specific towards hemimethylated DNA modification. It seems to be just the enzyme that participates in post-replicative DNA methylation. The role of M3.BstF5I in cell functioning remains unclear. The genes of M2.BstF5I and M3.BstF5I methylases are characterized by similar and lower GC-content values (29.1 and 29.0%, respectively) compared with that for M1.BstF5I and M4.BstF5I genes (32.3 and 32.2%, respectively) also possessing similar GC-content values. Along with the described earlier high homology of the second and third methylases with isospecific *C*- and *N*-domains of FokI methylase, correspondingly [2], this fact indicates the appearance of some extra functions for M2.BstF5I and M3.BstF5I methylase pair compared with M1.BstF5I and M4.BstF5I.

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