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ACCELERATED PUBLICATION

Interaction of Murine Dnmt3a with DNA Containing O^6-Methylguanine

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Abstract—O^6-Methylguanine (O^{6}meG) is one of the most toxic, mutagenic, and carcinogenic lesions caused by the interaction of DNA with several catabolism products as well as with environmental methylating agents. Carcinogenic impact of O^{6}meG can be conditioned not only by its mutagenic properties but also by alteration in enzymatic methylation of the C5 carbon atom of cytosine residue in CpG sequences. In this study, the effect of O^{6}meG on DNA methylation by the catalytic domain of murine DNA methyltransferase (MTase) Dnmt3a (Dnmt3a-CD) is assessed. Damaged DNA duplexes cooperatively bind with Dnmt3a-CD, and O^{6}meG changes the stability of enzyme–substrate complexes. Kinetic analysis of the methylation reaction revealed that O^{6}meG varies the ratio of productive and nonproductive enzyme–substrate complexes and, depending on localization in substrate, causes decrease or increase in DNA methylation. Dnmt3a-CD is less sensitive to the presence of O^{6}meG in DNA substrate than procaryotic MTase SssI recognizing CpG.

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Key words: cooperative binding, murine DNA methyltransferase Dnmt3a, O^6-methylguanine, nonproductive complex

Living organisms are always under the influence of carcinogenic and mutagenic substances able to damage DNA and other cell components [1, 2]. Harmful impact can be caused by environmental pollutants (present in food, tobacco smoke, etc.) or by metabolic intermediates as well as by a result of various pathologies including inflammatory processes [1-3]. One such process is DNA alkylation in eukaryotic cells, which takes place both during exogenous and endogenous impact of active alkylating particles and is mostly directed to the N7 nitrogen atom of guanine [4-6]. *In vivo*, slight alkylation of the O6 oxygen atom of guanine residue is observed. However, among products of DNA damage with methylating agents, O^6-methylguanine (O^{6}meG) is one of the most toxic, mutagenic, and carcinogenic [4, 7-9]. The cytotoxicity of O^{6}meG is not yet sufficiently studied. However, the presence of O^{6}meG in DNA had been shown to inhibit the activity of NF-κB transcription factor, significantly decrease efficiency of transcription performed by human RNA polymerase II, poison human topoisomerase I, and induce apoptosis [10-13].

Carcinogenic impact of O^{6}meG was previously thought to be connected just with its mutagenic activity [6-8]. During DNA replication, O^{6}meG can pair with thymine, which is structurally similar to the Watson–Crick G·C pair [14], leading to G·C→A·T mutation in human and animal cells [15-17]. In cancer cells, this mutation is often coupled with activation of oncogenes and inactivation of tumor suppressor genes [8, 16]. However, oncogene mutations are not observed in all types of cancer induced by substances methylating guanine residue in DNA [4]. We suppose that O^{6}meG affects other processes in cells, one of which can be enzymatic DNA methylation.

Enzymatic DNA methylation is one of the most important epigenetic processes involved in genome imprinting, inactivation of X chromosome, regulation of gene transcription, maintenance of genome integrity, etc. [18, 19]. In animal and human cells, it is performed by DNA methyltransferases (MTases) Dnmt1, Dnmt3a, and Dnmt3b, which transfer a methyl group from S-adenosyl-$_L$-methionine (AdoMet) cofactor to the C5 carbon atom of cytosine residue in CpG sequences [20]. Dnmt1 main-

Abbreviations: AdoHcy, S-adenosyl-L-homocysteine; AdoMet, S-adenosyl-L-methionine; Dnmt3a-CD, catalytic domain of DNA methyltransferase Dnmt3a; FAM, 6(5)-carboxyfluorescein; MTase, DNA methyltransferase; O^{6}meG, O^6-methylguanine; 8-oxoG, 7,8-dihydro-8-oxoguanine.

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ly maintains methylation, while Dnmt3a and Dnmt3b perform *de novo* methylation. Numerous investigations show the interaction of all three enzymes *in vivo* is necessary in all stages of establishment and maintenance of normal DNA methylation [21-23]. The fact that disorders of epigenetic mechanisms is connected with development of numerous chronic diseases including cancer, cardiovascular diseases, diabetes, and obesity has been proved many times recently [24]. It was previously supposed that change in normal pattern of DNA methylation could follow from lesions of heterocyclic bases in DNA [25]. Treatment of human cells with active methylating agent (*N*-methyl-*N*-nitrosourea), also leading to O^6meG formation, was shown to decrease DNA methylation [26]. It can be supposed that disturbance of the DNA methylation pattern caused by O^6meG can play a significant role in carcinogenesis. There is almost no data about the impact of O^6meG on DNA methylation by MTases other than Dnmt1. Introduction of O^6meG to the enzyme recognition site was shown to cause significant decrease [27] and blocking of DNA methylation [28].

The goal of this study was to examine the impact of O^6meG on functional properties of the catalytic domain of Dnmt3a (Dnmt3a-CD). It is shown that the character of the impact of O^6meG on the DNA methylation level depends on the lesion localization relative to the Dnmt3a-CD recognition site and target cytosine and seems to be due to variation in ratio of productive and nonproductive enzyme–substrate complexes.

MATERIALS AND METHODS

Reagents. For this study, AdoHcy (S-adenosyl-L-homocysteine) was purchased from Sigma and [CH_3-^{3}H]AdoMet (S-adenosyl-L-methionine) (77 Ci/mmol, 13 μM) was obtained from Amersham Biosciences. Oligodeoxyribonucleotides (Table 1) were synthesized by Sintol (Moscow) and purified in polyacrylamide gel. The fluorescein label (6-carboxyfluorescein, FAM) was introduced at the 5′-end of the oligodeoxyribonucleotides by means of an aminoalkyl linker containing six methyl groups. Oligodeoxyribonucleotide concentrations were estimated spectrophotometrically [29]. The following buffers were used: buffer A (20 mM Hepes-NaOH, pH 7.5, 100 mM KCl, 1 mM EDTA, 0.2 mM dithiothreitol (DTT), and 10% (v/v) glycerol); buffer B (10 mM Tris-HCl, pH 7.9, 50 mM NaCl, 1 mM DTT, and 0.1 mg/ml BSA).

Purification of Dnmt3a-CD and M.SssI. Dnmt3a-CD and SssI MTases as derivatives containing a His_6 tag on N-terminal were purified as described in [30]. Purity of enzyme preparations was determined using electrophoresis in 12.5% SDS-polyacrylamide gel and was >90%. Protein concentrations determined by the Bradford method were considered as concentrations of MTases monomer form. Active concentration of M.SssI was determined by the fluorescence polarization method as described in [31].

Dnmt3a-CD binding with DNA. Dnmt3a-CD binding with DNA (10 mM) in the presence of AdoHcy (0.1 mM) was examined by fluorescence polarization by direct titration of FAM-labeled oligodeoxyribonucleotide duplexes by Dnmt3a-CD as described in [30]. The signals were measured using a Cary Eclipse spectrofluorimeter (Varian). Fluorescence polarization value, P, was determined according to the equation:

$$P = (I_v - GI_h)/(I_v + I_h),$$

where I_v and I_h are vertical and horizontal components of irradiated light, respectively; G is the instrumental correc-

Table 1. Oligodeoxyribonucleotide sequences*

Designation	Sequence
GCGC (or GCGCTCTC)	5'-CTGAATACTACTTGCGCTCTCTAACCTGAT
CGCG	5'-ATCAGGTTAGAGAGCGCAAGTAGTATTCAG
CGMG	5'-ATCAGGTTAGAGAGMGCAAGTAGTATTCAG
fGCGC (or fGCGCTCTC)	5'-FAM-CTGAATACTACTTGCGCTCTCTAACCTGAT
CGMGf	5'-FAM-ATCAGGTTAGAGAGMGCAAGTAGTATTCAG
XCGC	5'-CTGAATACTACTTXCGCTCTCTAACCTGAT
GCXC	5'-CTGAATACTACTTGCXCTCTCTAACCTGAT
CXMG	5'-ATCAGGTTAGAGAGMXCAAGTAGTATTCAG
CGMGAGAX	5'-ATCAGGTTAGAXAGAGMGCAAGTAGTATTCAG

* M, 5-methylcytosine; X, O^6meG; FAM (f), 6-carboxyfluorescein.

tion factor. The experimental data are presented as P as a function of Dnmt3a-CD total concentration. Curves of titration of DNA duplexes by MTase were reproduced at least three times and analyzed according to the following Scheme:

$$E \xleftrightarrow{K_{d1}} ES \xleftrightarrow{K_{d2}} E_2S,$$

where E is Dnmt3a-CD homodimer, S is FAM-labeled DNA duplex, and ES and E_2S are enzyme–substrate complexes.

Dissociation constant values K_{d1} and K_{d2} were estimated by processing of binding curves using the SCIENTIST software (MacroMath) and system of equations (1)-(5) [30]:

$$P = P_0 + (P_{max} - P_0)(0.5[ES] + [E_2S])/[S]_0, \quad (1)$$

$$[E][S]/[ES] = K_{d1}, \quad (2)$$

$$[E][ES]/[E_2S] = K_{d2}, \quad (3)$$

$$[E] + [ES] + 2[E_2S] = [E]_0, \quad (4)$$

$$[S] + [ES] + [E_2S] = [S]_0, \quad (5)$$

where P_0 and P_{max} are fluorescence polarization values of free and bound DNA, respectively; $[E]_0$ is total concentration of Dnmt3a-CD; [S] and $[S]_0$ are concentrations of free and total DNA.

This data processing takes into account the decrease in free DNA and enzyme concentrations during complex formation. Equation (1) assumes that P value of the E_2S complex is two times higher than that of ES, and that the volume of E_2 is nearly two times larger than the volume of E. Equations (2) and (3) are expressions for corresponding K_d values. Equations (4) and (5) describe mass conservation for Dnmt3a-CD and DNA, respectively.

DNA methylation by MTases Dnmt3a-CD and SssI. DNA methylation by Dnmt3a-CD was monitored by measuring the amount of tritium incorporated during transfer of methyl groups from $[CH_3$-$^3H]$AdoMet to cytosine residues. The reaction was performed at 37°C in buffer A in the case of Dnmt3a-CD and in buffer B for M.SssI. Kinetics of DNA methylation by Dnmt3a-CD were examined under conditions where the DNA concentration was 1.7 times greater than that of the enzyme as well as under conditions where the enzyme concentration was 10 times greater than that of DNA ("single turnover" conditions) as described in [30]. In the first case, the reaction mixture contained 1.5 μM DNA, 0.88 μM Dnmt3a-CD, and 2 μM $[CH_3$-$^3H]$AdoMet. Reaction mixture aliquots were taken from 1 to 15 min starting from the beginning of the reaction and applied to DE81 ion-exchanging filters (Whatman), which were treated as described in [32]. Initial rate values of the methylation reaction (v_o) were estimated from the linear region slope of DNA methylation dependence on time.

In the second case, the reaction mixture contained 300 nM DNA, 3 μM Dnmt3a-CD, and 1.2 μM $[CH_3$-$^3H]$AdoMet. Reaction mixture aliquots were withdrawn at times from 4 to 90 min. Values of rate constants of the methylation reaction (k_{st}) were estimated assuming pseudo-first order of the reaction (under conditions of saturation by DNA and AdoMet) according to Eq. (6) [33]:

$$[MS] = [MS]_f\,(1 - e^{-k_{st}t}), \quad (6)$$

where [MS] and $[MS]_f$ are concentrations of reaction products (methylated DNA) at moment t and at the ending point of the reaction, respectively.

Methylation of DNA duplexes by MTase SssI was carried out under steady-state conditions as described in [30]. Reaction mixture aliquots containing 500 nM DNA, 20 nM M.SssI, and 1.2 μM $[CH_3$-$^3H]$AdoMet were withdrawn at times from 1 to 3.5 min, and v_o values were estimated similarly to that for Dnmt3a-CD.

RESULTS

To estimate the impact of O^{6}meG on functional properties of Dnmt3a-CD, binding to 30-mer DNA duplexes containing O^{6}meG and their methylation by Dnmt3a-CD were studied. Dnmt3a-CD can introduce methyl groups into unmethylated DNA as well as into DNA containing a methyl group in one of the strands (hemimethylated DNA). Hemimethylated DNA duplexes were used to estimate the impact of O^{6}meG (X) on methylation of a particular DNA strand. A damaged base was introduced into one of the strands of hemimethylated substrate analogs into the Dnmt3a-CD recognition site (G**C**XC/C**GM**G, G**C**GC/C**XM**G), at the 5′-side of recognition site (X**C**GC/C**GM**G), or four nucleotide residues from it (G**C**GCTCTC/C**GM**GAGAX) (Table 2).

Initial rates of methylation of DNA duplexes containing O^{6}meG by Dnmt3a-CD. Initial rates (v_o) of methylation of DNA duplexes containing O^{6}meG were estimated using the Dnmt3a catalytic domain under conditions where the DNA concentration was 1.7 times greater than that of the enzyme (Fig. 1), and values of relative initial methylation rates (v^{rel}) were estimated (Table 2). Introduction of O^{6}meG into the recognition site of (G**C**XC/C**GM**G) target strand led to 3.2-fold decrease in methylation rate compared with undamaged G**C**GC/C**GM**G substrate. At the same time, v_o values for duplexes containing O^{6}meG near a CpG site (X**C**GC/C**GM**G) and at the recognition site opposite to the target cytosine (G**C**GC/C**XM**G) were 2 and 3 times larger, respectively, than v_o value of G**C**GC/C**GM**G. Replacement of a dis-

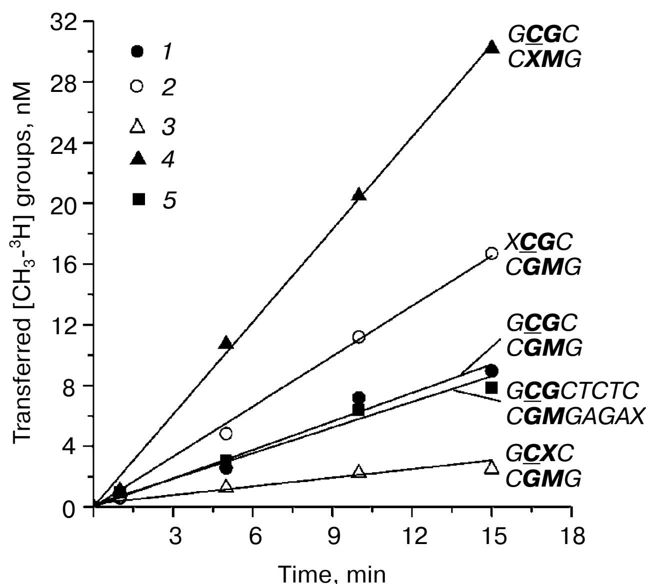

Fig. 1. Methylation of DNA duplexes by Dnmt3a-CD. C_{DNA}, 1.5 μM; $C_{Dnmt3a\text{-}CD}$, 0.9 μM; $C_{[CH_3\text{-}^3H]AdoMet}$, 1.5 μM. *1*) G**CG**C/C**GM**G; *2*) X**CG**C/C**GM**G; *3*) G**CX**C/C**GM**G; *4*) G**CG**C/C**XM**G; *5*) G**CG**CTCTC/C**GM**GAGAX.

tant guanine residue with O[6]meG (G**CG**CTCTC/C**GM**GAGAX) had almost no influence on the ability of Dnmt3a-CD to methylate cytosine in the CpG site.

Dnmt3a-CD binding to DNA duplexes containing O[6]meG. When O[6]meG is introduced, change in DNA methylation rate might be connected with change in Dnmt3a-CD affinity with damaged DNA. To test this conjecture, binding of FAM-labeled DNA duplexes containing O[6]meG to Dnmt3a-CD was examined by fluorescence polarization (Fig. 2). An experiment was carried out in the presence of AdoHcy cofactor analog, which promotes formation of a specific complex between DNA and C5-MTases [34] and prevents cytosine deamination [35]. Binding isotherms for fG**CG**C/C**GM**G, fG**CG**C/C**XM**G, and fG**CG**CTCTC/C**GM**GAGAX duplexes were similar to hyperbolic curves commonly observed when proteins bind with DNA in 1 : 1 ratio. However, curves for duplexes G**CX**C/C**GM**Gf and X**CG**C/C**GM**Gf had a definite sigmoid shape indicating positive cooperativity. A similar picture, when Dnmt3a-CD bound with DNA containing 7,8-dihydro-8-oxoguanine (8-oxoG), was observed previously [30]. A model of consecutive binding of two Dnmt3a-CD dimers to 30-mer DNA duplex (Scheme) was used for K_d estimation for complexes between Dnmt3a-CD and O[6]meG-DNA as well as in case of complexes between the enzyme and 8-oxoG-DNA [30]. At that time, a complex between one DNA molecule and two Dnmt3a-CD dimers forms [30]. The assumption that Dnmt3a-CD dimer is a binding protein unit conforms to mutation analysis data showing that Dnmt3a-CD monomer does not possess catalytic activity and is not able to bind DNA [36, 37]. As opposed to the Hill model, in common use for analyzing data on cooperative processes, the model used (see Scheme and Eqs. (1)-(5)) allows taking into consideration a decrease in equilibrium concentrations of free DNA and the enzyme during complex formation. According to this model, K_{d1} and K_{d2} values were estimated (Table 2). For X**CG**C/C**GM**Gf and G**CX**C/C**GM**Gf duplexes, K_{d1} was at least 10 times larger than K_{d2}, which indicates lowering of ES complex to stoi-

Table 2. Parameters of interaction of Dnmt3a-CD and M.SssI with DNA duplexes containing O[6]meG

DNA duplex*	K_{d1} (nM)	K_{d2} (nM)	$K_{d1}K_{d2}$ (nM^2)	v^{rel} (%)**	k_{st} (h^{-1})	R (%)***	v^{rel} (%)**
	Dnmt3a-CD						M.SssI
G**CG**C C**GM**G	12 ± 1	5 ± 2	60	100	2.5 ± 0.1	25 ± 2	100
X**CG**C C**GM**G	K_{d1} >> K_{d2}		4400 ± 700	198 ± 17	2.7 ± 0.3	33 ± 2	101 ± 14
G**CX**C C**GM**G	K_{d1} >> K_{d2}		8000 ± 1200	31 ± 8	2.1 ± 0.2	7.8 ± 0.1	4 ± 1
G**CG**C C**XM**G	27 ± 1	3.0 ± 1.2	81	330 ± 38	5.3 ± 1.7	25 ± 6	67 ± 6
G**CG**CTCTC C**GM**GAGAX	2.9 ± 0.2	3 ± 1	9	92 ± 11	2.6 ± 0.4	22 ± 2	105 ± 18

* Designations of oligodeoxyribonucleotides are as in Table 1. Dnmt3a-CD and M.SssI recognition sites are in bold type, the target cytosine is underlined.

** Relative methylation rate. Rate of G**CG**C/C**GM**G duplex methylation (0.7 nM/min for Dnmt3a-CD and 11 nM/min for M.SssI) is taken as 100%.

*** Ratio between methylated DNA at the end of the reaction and total DNA in the reaction mixture (Fig. 3).

chiometrically negligible quantities and prevents estimating definite values of individual constants. In these cases, however, values of the $K_{d1}K_{d2}$ product were calculated with high accuracy (Table 2). From the calculations it follows that K_{d1} and K_{d2} values depend on each other. This suggests that Dnmt3a-CD binding to X**C****G**C/C**GM**Gf and G**CX**C/C**GM**Gf has apparent cooperative character. For fG**CG**C/C**GM**G substrate and duplexes containing O^6meG in a methylated strand (fG**CG**C/C**XM**G and fG**CG**CTCTC/C**GM**GAGAX), K_{d1} was not more than 9 times larger than K_{d2}. This also indicates that Dnmt3a-CD binding to the given substrate has apparent cooperative character because in the case of independent binding of two enzyme dimers to DNA the macroscopic ratio of constants (K_{d2}/K_{d1}) would be equal to 4 [38]. Thus, during formation of enzyme–substrate complex, two Dnmt3a-CD dimers sequentially bind to different sites of 30-mer DNA duplex.

Introduction of O^6meG into already methylated DNA strand (G**CG**C/C**XM**G and G**CG**CTCTC/C**GM**GAGAX) leads to 1.3-fold increase and 6.7-fold decrease in $K_{d1}K_{d2}$ value. In case of duplexes containing damaged guanine in the methylated strand of recognition site (G**CX**C/C**GM**Gf) or at the 5′-side from it (X**CG**C/C**GM**Gf), $K_{d1}K_{d2}$ increased 73- and 130-fold. Thus, introduction of O^6meG into DNA affects stability of enzyme–substrate complex but it does not explain variations in initial rates of the methylation reaction.

Integral kinetics of methylation of DNA duplexes containing O^6meG by Dnmt3a-CD. Further, under conditions of single turnover (under 10-fold excess of the enzyme over the substrate), the kinetics of DNA methylation by Dnmt3a-CD MTase was studied (Fig. 3). The chosen concentration of Dnmt3a-CD (3 µM) satisfied saturation conditions (Fig. 2) and must have promoted formation of a complex between all free DNA and the enzyme. Values of rate constants of the methylation reaction (k_{st}) were estimated using Eq. (6) (see "Materials and Methods"). For all duplexes, k_{st} values were almost the same, except for the G**CG**C/C**XM**G duplex for which k_{st} was 2.1 times larger compared with the undamaged substrate G**CG**C/C**GM**G (Table 2). However, yield of reaction product $[CH_3\text{-}^3H]$DNA, R, varied depending the position of the O^6meG. At the same time, even in case of undamaged substrate G**CG**C/C**GM**G, R value was only 25%. These results relate with data obtained previously when the impact of 8-oxoG on Dnmt3a-CD methylation was studied, where reaction product yield was up to 100% with neither modified substrate due to formation of nonproductive enzyme–substrate complexes [30]. In the studied system, Dnmt3a-CD seems to form stable, slowly dissociating nonproductive complexes with DNA substrates (Fig. 4). In the case of G**CX**C/C**GM**G duplex, in which O^6meG is located in the recognition site near to the target cytosine, R value decreased 3.2-fold in magnitude that corresponded to v_o decrease. And in the case of X**CG**C/C**GM**G duplex containing O^6meG near the recognition site, increase in v_o value with simultaneous 1.4-fold increase of R value was observed.

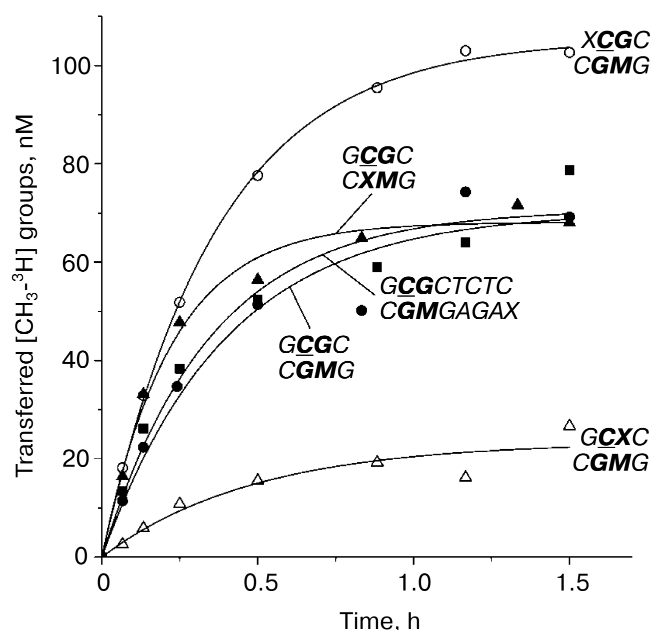

Fig. 3. Methylation of DNA duplexes by Dnmt3a-CD under "single turnover" conditions. C_{DNA}, 0.3 µM; $C_{Dnmt3a\text{-}CD}$, 3 µM; $C_{[CH_3\text{-}^3H]AdoMet}$, 1.2 µM. Solid lines represent theoretical curves obtained from experiment data processing by Eq. (6). Designations are as in Fig. 1.

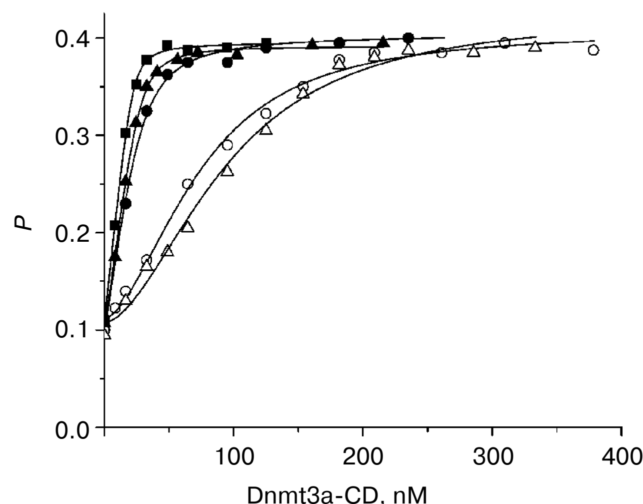

Fig. 2. Binding curves of fluorescein-labeled DNA duplexes (10 nM) with Dnmt3a-CD in the presence of AdoHcy (0.1 mM). P, fluorescence polarization. Solid lines represent theoretical curves obtained from experimental data processing by Eqs. (1)-(5). Designations are as in Fig. 1.

Methylation of DNA duplexes containing O^6meG by M.SssI. Procaryotic MTase SssI and Dnmt3a recognize

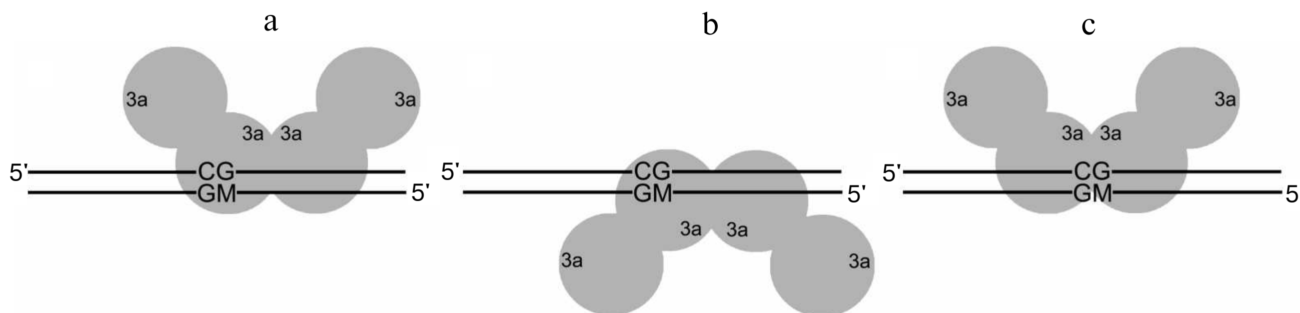

Fig. 4. Binding of Dnmt3a-CD to DNA in productive (a) and nonproductive (b and c) orientations.

the same DNA sequence [39]. To examine the impact of O^{6}meG on the DNA methylation by M.SssI, v_o values of methylation of DNA duplexes containing O^{6}meG were measured using the given enzyme under steady-state kinetic conditions (DNA concentration was 25 times higher than that of M.SssI) (Fig. 5 and Table 2). As it is clear from the figure, M.SssI is more sensitive to the presence of O^{6}meG in the recognition site of the enzyme. Thus, in case of G<u>C</u>**X**C/C**GM**G duplex containing damaged guanine residue in the recognition site at the 3′-side from the target cytosine, v_o value was 25 times smaller than that for G<u>**C**</u>**G**C/C**GM**G. Replacement of guanine residue in the recognition site opposite to the methylated cytosine (G<u>**C**</u>**G**C/C**XM**G) led to 1.5-fold decrease in v_o value. Introduction of O^{6}meG into the outer region of the enzyme recognition site had practically no influence on

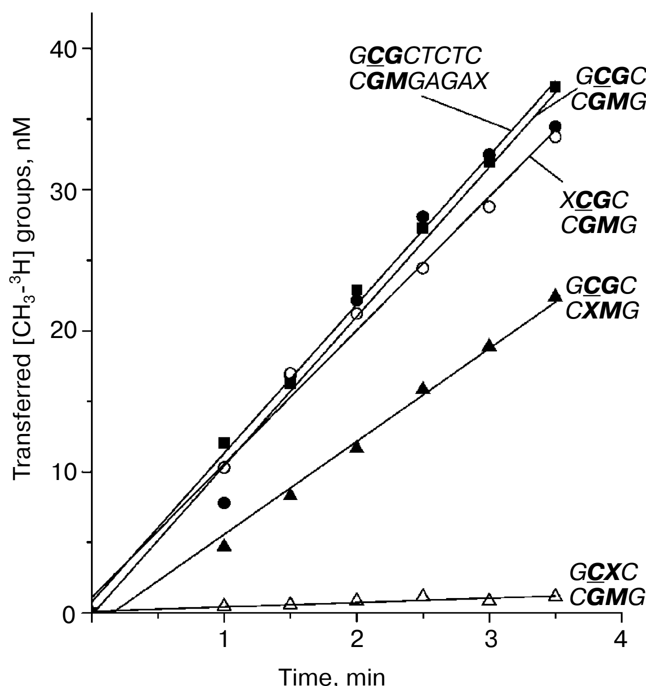

Fig. 5. Methylation of DNA duplexes by M.SssI. C_{DNA}, 0.5 μM; $C_{M.SssI}$, 20 nM; $C_{[CH_3\text{-}^3H]AdoMet}$, 1.2 μM. Designations are as in Fig. 1.

v_o values of corresponding DNA duplexes (X<u>**C**</u>**G**C/C**GM**G and G<u>**C**</u>**G**CTC/C**GM**GAGAX).

DISCUSSION

In the present work, it is shown that the appearance of very cytotoxic O^{6}meG in DNA influences the functioning of murine Dnmt3a-CD. The effect of O^{6}meG on the initial rate of methylation of 30-mer DNA duplexes catalyzed by murine Dnmt3a-CD MTase depends on the position of the damaged guanine residue relative to the CpG sequence and methylated cytosine. Introduction of O^{6}meG into the CpG recognition site lowers methylation rate of the target cytosine located near the O^{6}meG and raises methylation rate of the target cytosine opposed to the lesion. The presence of O^{6}meG near the recognition site in the methylated strand increases the DNA methylation level. The ability of O^{6}meG to stimulate DNA methylation opposite to the target cytosine correlates with data obtained for human Dnmt1 [27]. However, in contrast to Dnmt3a-CD, Dnmt1 loses its DNA methylation ability when O^{6}meG is in a CpG region near the methylated cytosine [27]. In the case of M.SssI, a procaryotic C5 MTase recognizing CpG, this position also appears to be the most critical (Table 2). The presented data indicate the importance of the guanine residue adjoined with the methylated cytosine for recognition of the CpG sequence by C5-MTases.

Studying a stage of Dnmt3-CD binding to 30-mer DNA duplexes containing and not containing O^{6}meG indicate cooperative character of the process. This observation conforms to recently obtained data about cooperative character of interaction between 146-mer DNA and Dnmt3a-CD/Dnmt3L (Dnmt3L is a regulatory factor) complex [37] and interaction between 208-mer DNA and full-size Dnmt3a [40]. The data (Fig. 2) were well described by a previously proposed model of sequential binding of two Dnmt3a-CD dimers with two different DNA sites (Scheme) [30]. The model was developed based on X-ray structure analysis data about homoassociation of Dnmt3a-CD or Dnmt3a-CD/Dnmt3L complex

[36]. As on the model, Dnmt3a-CD possesses practically identical affinity with both binding sites in the case of undamaged substrate (G**C̲G**C/C**GM**G) and DNA duplex containing a damaged guanine residue at the distance of four nucleotide residues from the recognition site (G**C̲G**CTC/C**GM**GAGAX) (Table 2). Introduction of O^{6}meG into the CpG site (G**C̲X**C/C**GM**G and G**C̲G**C/C**XM**G) or at the 5′-side from it (X**C̲G**C/C**GM**G) leads to reduction in Dnmt3a-CD affinity with the first binding site relative to the second ($K_{d1} > K_{d2}$) (Table 2). At the same time, both deceleration (G**C̲X**C/C**GM**G) and acceleration (X**C̲G**C/C**GM**G and G**C̲G**C/C**XM**G) of methylation was observed for the given substrates. It should be noted that the substrate concentration (1.5 μM) used for v_o value estimations (Fig. 1) was saturating in all cases. It follows that v_o variations caused by replacement of guanine residues with O^{6}meG in DNA are not due to alteration of Dnmt3a-CD affinity with damaged DNA.

Analysis of dependences of methylation level of DNA duplexes on time under single turnover conditions (Fig. 3) and *R* values (Table 2) allowed making suggestions about reasons for changes in v_o values. Dnmt3a-CD was shown to form stable nonproductive complexes with DNA duplexes containing O^{6}meG as well as with previously studied DNA duplexes containing another type of damaged guanine, 7,8-dihydro-8-oxoguanine [30]. In the case of hemimethylated substrates, formation of nonproductive complexes, in contrast to that of productive ones (Fig. 4a), seems to be due to Dnmt3a-CD binding to DNA at the methylated strand side (Fig. 4b). Dnmt3a-CD is able to bind with 30-mer substrate containing fully methylated CpG region [30]. Another kind of nonproductive Dnmt3a-CD binding to DNA is also possible when both of the enzyme dimers bind nonspecifically, i.e. none of the dimers interact with a CpG region (Fig. 4c). This supposition is confirmed by Dnmt3a-CD complex formation with 30-mer DNA duplex not containing recognition site (data not provided). The ratio of possible complex types depends on affinity between the enzyme and DNA site binding the first and, too, is likely to depend on binding rate, as formed complex between the enzyme and DNA dissociate very slowly [41] and Dnmt3a-CD does not seem to redistribute during the methylation reaction.

For G**C̲G**C/C**XM**G duplex, when a damaged guanine residue is located in already methylated strand opposite to the target cytosine, *R* value is similar to that of G**C̲G**C/C**GM**G, while v_o value rises (Table 2). For G**C̲X**C/C**GM**G duplex, whose affinity to the enzyme and v_o value were observed to decrease, *R* value was also observed to decrease. For (X**C̲G**C/C**GM**G) duplex *R* value increased, which corresponded to v_o value increase. Except for G**C̲G**C/C**XM**G duplex, whose k_{st} value increased, k_{st} values of all other duplexes did not change. Variations in v_o values observed when O^{6}meG was in the methylated strand (G**C̲X**C/C**GM**G and X**C̲G**C/C**GM**G) seemed to be due to variations in the ratio of productive and nonproductive enzyme–substrate complexes, while when O^{6}meG was in the strand opposite to the target cytosine k_{st} increased (acceleration of the methylation reaction itself).

It is important to investigate molecular bases for the impact of O^{6}meG on functioning of Dnmt3a-CD and M.SssI. The presence of O^{6}meG opposite to cytosine in DNA double helix breaks its structure quite strongly: between the bases in C·O^{6}meG pair, only two hydrogen bonds are formed (Fig. 6), cytosine and O^{6}meG residues drift into the minor and major groove, respectively, and the structure of the sugar-phosphate backbone changes [42, 43]. The methyl group in O6 position is exhibited to the major groove. When bound with DNA, M.SssI makes contact with the N7 nitrogen atom of the guanine residue adjoined to the target cytosine and with internucleotide phosphates in the recognition site and near to it [44, 45]. From data of computer modeling of the complex between M.SssI, DNA, and AdoHcy, it follows that M.SssI contacts with the O6 oxygen atom of the guanine residue located opposite to the target cytosine [46]. Thus, the impact of O^{6}meG on DNA methylation by MTase can be related with breakdown of M.SssI contacts with damaged DNA caused by local deformations of double helix structure as well as by steric barriers caused by the methyl group at O6 position. Relying on the fact that M.SssI and Dnmt3a-CD possess identical sequence specificity as well as on high homology of primary structure of C5 MTases and Dnmt3a-CD [20, 39], it can be supposed that deterioration of G**C̲X**C/C**GM**G methylation by Dnmt3a-CD is due to breakdown of the enzyme contact with the N7 nitrogen atom of the guanine residue located near the target cytosine.

Comparing damaging impact on Dnmt3a-CD and M.SssI functioning, M.SssI can be said to be more sensitive to the presence of O^{6}meG in DNA as well as to its location relative to the recognition site and methylated

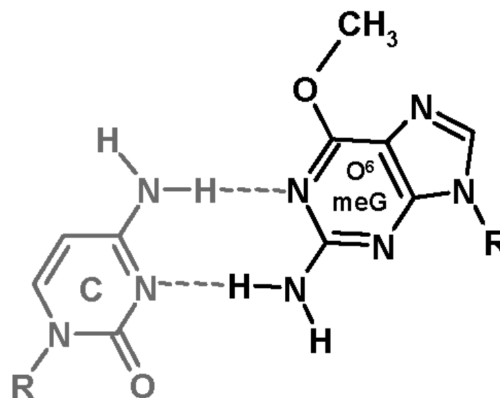

Fig. 6. Structure of C·O^{6}meG nucleotide pair (from [42, 43]).

cytosine. This fact suggests that the Dnmt3a-CD active center possesses better ability to adjust to damaged DNA than the procaryotic M.SssI. In the case of O^6meG, such adjustment can be realized by steric tension reduction in the Dnmt3a-CD active center as a consequence of methyl group displacement relative to the N1–C6 bond of the damaged guanine residue. The possibility of such alterations in orientation of the O^6meG methyl group was shown for human RNA polymerase II [13]. Thus, introduction of an O^6meG residue, depending on its location, into DNA leads to small decrease or increase in Dnmt3a-CD DNA methylation. We cannot accept as fact that *in vivo* in the presence of Dnmt3L regulatory factor, which stabilizes Dnmt3a-CD catalytic loop [36, 42], the impact of O^6meG on functioning of full-size Dnmt3a can be greater. For instance, despite the fact that in several cases *in vitro*, O^6meG stimulates DNA methylation by Dnmt1 MTase [27], DNA hypomethylation was observed when human lymphoblasts were treated with methylating agent [26]. This can be connected with the presence of a regulatory mechanism of DNA methylation in cells, in which other DNA binding proteins are involved. Recently, it was established that, in cells, hemimethylated CpG sites are recognized by UHRF1 protein, which then attracts Dnmt1 to these sites [47]. Interacting with DNA, UHRF1 was shown to form numerous contacts with internucleotide phosphates both in the recognition site and near to it (from 5′- and 3′-side) and to the bases (also to O6 and N7 atoms of guanine opposite to the target cytosine) [48, 49]. Obviously, replacement of guanine in the CpG site or near to it with O^6meG can break down the interaction of UHRF1 with DNA and, thereby, prevent the interaction of Dnmt1 with the given site. DNA hypomethylation observed in work [26] seems to be also caused by this mechanism. The mechanism of attraction of Dnmt3a to particular DNA sites in a cell is not studied yet; therefore, in the case of Dnmt3a, disturbance of DNA methylation through interaction with mediator proteins is also possible and requires further investigations.

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