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Probing murine methyltransferase Dnmt3a interactions with benzo[a]pyrene-modified DNA by fluorescence methods

Antonio S. Minero¹, Olga V. Lukashevich¹, Natalia A. Cherepanova¹, Alexander Kolbanovskiy², Nicholas E. Geacintov², and Elizaveta S. Gromova¹

¹Department of Chemistry, Moscow State University, Moscow, Russia

²Department of Chemistry, New York University, New York, USA

Summary

The impact of bulky carcinogen-DNA adducts positioned at or near recognition sites (CpG) of eukaryotic DNA methyltransferases on their catalytic activities is poorly understood. In this work we have employed site-specifically modified 30-mer oligodeoxyribonucleotides containing stereoisomeric benzo[a]pyrene diol epoxide (B[a]PDE)-derived guanine (B[a]PDE-*N*²-dG) or adenine (B[a]PDE-*N*⁶-dA) adducts of different conformations as substrates of catalytic domain of murine Dnmt3a (Dnmt3a-CD). The fluorescence of these lesions was used to examine interactions between Dnmt3a-CD and DNA. In B[a]PDE-DNA•Dnmt3a-CD complexes the intensity of fluorescence of the covalently bound B[a]PDE residues is enhanced relative to the protein-free value when the B[a]PDE is positioned in the minor groove ((+)- and (–)-*trans*-B[a]PDE-*N*²-dG adducts in the CpG site) and when it is intercalated on the 5'-side of the CpG site ((+)-*trans*-B[a]PDE-*N*⁶-dA adduct). The fluorescence of B[a]PDE-modified DNA•Dnmt3a-CD complexes exhibits only small changes when the B[a]PDE is intercalated with base displacement in (+)- and (–)-*cis*- B[a]PDE-*N*²-dG adducts and without base displacement in the (–)-*trans*- B[a]PDE-*N*⁶-dA adduct. The initial rates of methylation were significantly reduced by the minor groove *trans*-B[a]PDE-*N*²-dG adducts regardless of their position in the substrate and by the intercalated *cis*-B[a]PDE-*N*²-dG adducts within the CpG site. The observed changes in fluorescence and methylation rates are consistent with the flipping of the target cytosine and a catalytic loop motion within the DNA•Dnmt3a-CD complexes. In the presence of the regulatory factor Dnmt3L, an enhancement of the methylation rates and fluorescence were observed which is consistent with a Dnmt3L-mediated displacement of the catalytic loop towards the CpG site.

Keywords

Benzo[a]pyrene-DNA; minor groove; intercalation; fluorescence; methylation

Corresponding author: Elizaveta S. Gromova, Department of Chemistry, Moscow State University, Moscow, 119992, Russia, Fax: +7-495-939-3181, Tel: +7-495-939-3144, gromova@genebee.msu.ru.

Enzymes: Dnmt3a, M.SssI, M.HhaI (C5 MTases, EC 2.1.1.37)

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Introduction

Benzo[*a*]pyrene (B[*a*]P), one of the most widely studied environmental polycyclic aromatic hydrocarbons (PAH), is metabolized *in vivo* to genotoxic 7,8-dihydrodiol-9,10-epoxides (B[*a*]PDE) with an *anti*-orientation of the 7-hydroxyl and 9,10 epoxide groups. The (+)-7*R*, 8*S*-dihydrodiol-9*S*,10*R* and (–)-7*S*,8*R*-dihydrodiol-9*R*,10*S* epoxides react with DNA via a nucleophilic attack of the *N*²- or *N*⁶-exocyclic amino groups of dG and dA, respectively, at the C10 position of the B[*a*]PDE molecule (Fig. 1), thus giving rise to stable covalent adducts. If not repaired, these DNA lesions can cause mutations that have been associated with the initiation of cancer in animal model systems [1, 2]. In spite of their bulk, certain PAH-DNA lesions are refractory to DNA repair, while others are easily excised by the mammalian nucleotide excision repair apparatus [3].

In mammals, DNA methylation plays important roles in cell differentiation, gene regulation, genomic imprinting, X chromosome inactivation, and disease related processes [4]. The B[*a*]PDE-*N*²-dG lesions may be formed at the guanine residue in CpG sequence contexts that are recognition sites of mammalian DNA methyltransferases (MTases) [5]. Such damage in the cellular DNA may disturb the functioning of MTases and change the genomic methylation pattern. This suggestion was supported by strong decreases in methylation efficiencies of the prokaryotic MTases M.EcoRII, M.HhaI and M.SssI in the case of 18-mer DNA duplexes containing (+)- and (–)-*trans*-B[*a*]PDE-*N*²-dG and (+)-*cis*-B[*a*]PDE-*N*²-dG adducts [6-8]. These studies have shown that (1) the B[*a*]PDE lesions affect primarily the methylation reaction by decreasing the efficiency of the catalytic step rather than the binding of the enzymes to the carcinogen-DNA adducts, and that (2) the methylation efficiency is strongly dependent on adduct conformation and its position within the DNA substrate. For prokaryotic M.SssI, the most significant effect on the methylation efficiency was observed when DNA containing (+)- and (–)-*trans*-B[*a*]PDE-*N*²-dG adducts, that are known to be positioned in the minor groove [9], were positioned on the 5'-side of the CpG recognition site of the enzyme [7]. However, a number of important questions remain that have not been resolved. It is not known how these and other related bulky B[*a*]P-derived DNA lesions located both within and outside of the CpG site affect DNA methylation catalyzed by eukaryotic MTases.

The mammalian MTase Dnmt3a and its Dnmt3-like co-protein (Dnmt3L) participate in establishing DNA methylation patterns during embryogenesis [10, 11]. The C-terminal domains of Dnmt3a and Dnmt3L form a tetrameric complex with two active sites [12]. Molecular modeling of DNA in a complex with a Dnmt3a dimer indicated that the two active sites are separated by about one DNA helical turn. Dnmt3L induces conformational changes in the catalytic loop of Dnmt3a that promotes its binding to DNA and stimulates DNA methylation by Dnmt3a [11, 12]. Dnmt3a binds to DNA by a cooperative multimeric mechanism [12] as well as forming of nucleoprotein filaments [13]. The binding parameters and rates of methylation of 18-mer B[*a*]PDE-modified DNA duplexes containing (+)- and (–)-*trans*-B[*a*]PDE-*N*²-dG adducts were explored employing the catalytic domain of the eukaryotic MTase Dnmt3a [14]. A slight enhancement of binding and 1.7-6.3-fold decreases in the methylation reaction rates for different site-specifically modified B[*a*]PDE-DNA duplexes was revealed. Here, the impact of the covalently bound bulky B[*a*]PDE residues on

the functioning of the catalytic domain of murine Dnmt3a (Dnmt3a-CD) was studied. The fluorescence of B[a]PDE- residues were used as a probe to monitor changes in conformation and/or local environment of these bulky lesions upon formation of DNA•Dnmt3a-CD complexes. The changes in fluorescence intensities were correlated with studies of methylation rates. A series of unique 30-mer B[a]PDE-DNA adduct-containing oligonucleotide duplexes were synthesized that contained conformationally defined single stereoisomeric B[a]PDE- N^2 -dG or B[a]PDE- N^6 -dA lesions positioned within the CpG site or in the flanking nucleotide sequences. The guanine lesions are known to be positioned within the minor groove in the case of the *trans*-B[a]PDE- N^2 -dG adducts [15, 16], or assume base-displaced intercalated conformations in the case of the stereoisomeric *cis*-B[a]PDE- N^2 -dG adducts [17, 18], while the B[a]PDE- N^6 -dA adducts are intercalated from the major groove without base displacement [9, 19, 20]. We made use of the fact that B[a]PDE residues of B[a]P-derived adducts in double- and single-stranded DNA can be used as fluorescent probes of their microenvironment for studying their interactions with different proteins [8, 21-24].

Results

Methylation of B[a]PDE-DNA by Dnmt3a-CD

The DNA repair and DNA replication/mutational pathways associated with PAH chemical carcinogenesis have been widely studied [25]. However, less attention has been paid to epigenetic mechanisms such as alterations in methylation patterns and little is known about the effects of DNA lesions on the rates of eukaryotic DNA methylation and the response of MTases to bulky DNA adducts with different conformational features. In order to answer these questions we have investigated the impact of site-specific, conformationally defined stereoisomeric B[a]PDE- N^2 -dG and B[a]PDE- N^6 -dA adducts on methylation catalyzed by the eukaryotic Dnmt3a-CD and prokaryotic M.SssI MTases.

Reaction of the diol epoxide B[a]PDE with the exocyclic amino groups of guanine and adenine in DNA occurs by *cis*- and *trans*- opening of the epoxide ring that generates a mixture of stereoisomeric B[a]PDE-DNA adducts with different configurations relative to the C10 position of B[a]PDE. Our studies focused on the structurally well characterized stereoisomeric (+)- and (-)-*trans*-B[a]PDE- N^2 -dG, (+)- and (-)-*cis*-B[a]PDE- N^2 -dG adducts, and the (+)- and (-)-*trans*-B[a]PDE- N^6 -dA adducts (Fig. 1). The aromatic ring systems of the (+)- and (-)-*trans*-B[a]PDE- N^2 -dG adducts (motif A) reside in the minor groove of the double helix, pointing toward the 5'- or 3'-directions of the modified strands, respectively [16, 26, 27]. The B[a]PDE aromatic residues assume intercalated conformations with displacements of the modified guanines and partner cytosines in the case of the (+)-*cis*- and (-)-*cis*-B[a]PDE- N^2 -dG adducts (motif B) [17, 18]. The modified guanine is displaced into the minor groove in the former case and the major groove in the latter, while the partner C residue is displaced into the major groove in both cases. In the (-)-*trans*-B[a]PDE- N^6 -dA (motif D) and (+)-*trans*-B[a]PDE- N^6 -dA (motif E) adducts the aromatic residues are intercalated on the 5'- (motif D) and 3'- (motif E) side of the modified A•T base pair without base displacement [28, 29]. Another important difference is that the (+)-*trans*-B[a]PDE- N^6 -dA adduct is significantly more distorting than the (-)-*trans* stereoisomeric adduct [19, 30].

The oligodeoxynucleotide sequences containing the stereoisomeric B[a]PDE-dG/dA adducts are shown in Table 1. Each of the modified 30-mer DNA duplexes contained the lesions either within the CpG site or adjacent to it (Table 2). The 5-methylcytosine was positioned within the unmodified strand instead of the target cytosine, thus yielding hemimethylated DNA. The initial methylation rates, v_1 , were determined under conditions of excess of substrate over Dnmt3a-CD (Table 2). The time dependences of these reactions were linear until 20 min in all cases (data not shown). The v_1 value for **ACGC/TGMG** was 1.7-fold higher than that for **GCGC/CGMG** (Table 2) in accordance with the finding that the **ACGC/TGCG** sequence was the preferred substrate [31].

In the first series of experiments, the position of the minor groove (+)-*trans*-B[a]PDE- N^2 -dG adduct in the DNA duplexes was varied. The efficiency of methylation (v_1^{rel}) decreased 12- and 9-fold when the damaged base was positioned within the CpG or adjacent to it in the **GCG⁺C/CGMG** and the **G⁺CGC/CGMG** duplexes, respectively (Table 2). However, when the damaged base containing stereochemically unresolved B[a]PDE- N^2 -dG adducts was positioned seven base pairs away from the CpG site, as in the case of the **GCGC---G*/CGCG---C** duplex (Table 2), the v_1^{rel} value was not affected.

The impact of minor groove (+)- and (-)-*trans*-B[a]PDE- N^2 -dG versus base-displaced intercalative (+)- and (-)-*cis*-B[a]PDE- N^2 -dG adducts positioned within the CpG site on DNA methylation was examined (Table 2). The v_1^{rel} value was diminished 9-12-fold when the (-)-*trans*-B[a]PDE- N^2 -dG adduct was positioned within CpG in the **GCG⁺C/CGMG** and **ACG⁺C/CGMG** duplexes relative to the unmodified sequences. The v_1^{rel} values in the case of the **GCG⁺C/CGMG**, **ACG⁺C/TGMG** and **ACG⁻C/TGMG** duplexes were reduced by 20-, 8- and 7-fold relative to their unmodified counterparts. Therefore, positioning these guanine lesions within the CpG site diminishes methylation rates regardless of the adduct conformations.

In the next series of experiments, the guanine adjacent to the CpG site was replaced either by the minor groove (+)-*trans*-B[a]PDE- N^2 -dG or the base-displaced intercalated (-)-*cis*-B[a]PDE- N^2 -dG adduct, or the intercalated (+)- and (-)-*trans*-B[a]PDE- N^6 -dA lesions (Table 2). Overall, the impact of the (+)-*trans*-B[a]PDE- N^2 -dG adduct was stronger than the effects of the two intercalated (+)- and (-)-*trans*-B[a]PDE- N^6 -dA adducts. The v_1^{rel} value for the (-)-*cis*-B[a]PDE- N^2 -dG adduct was not affected. Significant differences were observed in the case of the two stereoisomeric (+)- and (-)-*trans*-B[a]PDE- N^6 -dA adducts. The v_1^{rel} value for the **A⁺CGC/TGMG** sequence (5'-side intercalation) was 1.3 times greater, and the v_1^{rel} value for the **A⁺CGC/TGMG** sequence (3'-side intercalation) was smaller by a factor of 3.7 relative to the unmodified sequence.

In addition, the binding of Dnmt3a-CD to B[a]PDE-modified DNA was monitored using a fluorescence polarization technique with FAM-labeled unmodified strands in the presence of the co-factor analogue AdoHcy which mimics the experimental conditions of the methylation assays and facilitates the formation of a specific complex between C5-MTases and DNA [32, 33]. Typical binding curves are shown in Fig. S1. The curves can be interpreted in terms of Scheme 1 (Materials and Methods) yielding two binding constants, K_{d1} and K_{d2} , for binding of the two Dnmt3a-CD homodimers to B[a]PDE-DNA duplexes

(Table S1). For substrates containing *trans*-B[a]PDE- N^2 -dG adducts, the binding affinities ($K_{d1}K_{d2}$) did not differ significantly from those of the undamaged GCGC/CGMGf duplex. However, in the case of both *cis*-B[a]PDE- N^2 -dG adducts, the binding affinities were smaller than in the case of the undamaged GCGC/CGMGf duplex. Hence, the binding affinities of B[a]PDE-modified DNA sequences to Dnmt3a-CD depend on adduct stereochemistry. The $K_{d1}K_{d2}$ values were approximately the same for GCG^{c+}C/CGMG and G^c-CGC/CGMG duplexes. However, the methylation rates were strongly dependent on the positions of the B[a]PDE lesions (Table 2). Thus, in the case of *cis*-adducts, differences in binding are not directly correlated with the methylation rates. Overall, the observed decreases in methylation rates of all B[a]PDE-modified DNA duplexes did not result from their altered Dnmt3a-CD binding affinities. Furthermore, the $P_{max} - P_0$ values, proportional to the volume of the bound protein, was 0.28-0.31 for all Dnmt3a-CD complexes (Fig. S1); the analogous value was 0.08 for M.SssI complexes with GCGC/CGMGf. Since the molar masses of Dnmt3a-CD and M.SssI are 36 and 44 kDa, respectively, the $P_{max} - P_0$ value is consistent with the binding of about four Dnmt3a-CD monomers per 30-mer B[a]PDE-modified duplex containing one CpG site. Therefore, the observed changes in catalytic activities are not due to an effect of the B[a]PDE residues on the oligomerization of Dnmt3a-CD.

Dnmt3L produced 3-5-fold enhancements in the initial rates (v_2) of methylation by Dnmt3a-CD (Table 2). It is noteworthy that this effect did not depend on the conformations of the B[a]PDE-DNA adducts.

The prokaryotic MTase M.SssI has a recognition site that is identical to that of Dnmt3a [34]. The impact of (+)- and (-)-*trans*-B[a]PDE- N^2 -dG adducts and (+)-*cis*-B[a]PDE- N^2 -dG adduct on methylation of 18-mer DNA duplexes by M.SssI was studied earlier [7, 8]. However, in the present work, we focused on the impact of two types of intercalative adducts that were not examined before. Specifically, we compared the effects of *cis*-B[a]PDE- N^2 -dG base-displaced intercalated adducts, and the *trans*-B[a]PDE- N^6 -dA adducts that adopt “classical” intercalation conformations. The initial rates of methylation in the case of GCG^{c+}C/CGMG, G^c-CGC/CGMG, A^{t+}-CGC/TGMG, A^t-CGC/TGMG and the corresponding undamaged substrates are summarized in Table 3. The presence of the (-)-*cis*-base-displaced intercalative B[a]PDE- N^2 -dG lesion on the 5'-side adjacent to the CpG site virtually abolished the methylation reaction; however, the presence of the stereoisomeric, also base-displaced intercalated (+)-*cis*-B[a]PDE- N^2 -dG positioned within the CpG site, practically did not alter the methylation rate; these effects are opposite to those observed in the case of Dnmt3a-CD. The effects of (+)- and (-)-*trans*-B[a]PDE- N^6 -dA adducts on methylation rates were much less pronounced. The observed differences in behavior of M.SssI and Dnmt3a-CD are consistent with the previous conclusion concerning the sensitivity of M.SssI to base modifications 5'-adjacent to a CpG site [7, 8], and the sensitivity of Dnmt3a primarily associated with the modifications positioned within a CpG site [14].

Fluorescence characteristics of B[a]P residues in modified DNA-Dnmt3a-CD complexes

The fluorescence of the pyrene-like B[a]PDE residues are sensitive to π -stacking interactions with neighboring bases [35] and to the polarity of the microenvironment [22, 36, 37]. It is well established that the fluorescence of B[a]PDE-DNA adducts exhibits broad pyrene-like vibronic emission bands with maxima at ~ 380 -385 and 400-405 nm [8, 21, 22, 38]. The fluorescence of B[a]PDE- N^2 -dG adducts in DNA is strongly quenched by a solvent polarity-dependent electron transfer mechanism [36, 39-42]. Therefore, this fluorescence can be used as a probe for monitoring conformational changes that occur when these B[a]PDE-modified DNA sequences bind to proteins as shown earlier [8, 21-24].

In this work, we monitored, as a function of protein concentration, the fluorescence intensities of the stereoisomeric B[a]PDE- N^2 -dG and B[a]PDE- N^6 -dA lesions positioned at different sites in the vicinity of the methylation target cytosines in CpG recognition sites. Our objectives were to determine how the structurally distinct stereoisomeric lesions [28] and their locations relative to the target cytosine residues, affect methylation efficiencies. While the ultimate conformations of these lesions are not known once the DNA•MTase•AdoHcy complexes are formed, we demonstrate here that the initial conformations of the lesions and their positions relative to the methylation target in naked DNA affect (1) the fluorescence intensities upon DNA•MTase complex formation, and (2) the methyltransferase activities.

B[a]PDE-DNA—When excited at 350 nm, the emission spectrum of the B[a]PDE-DNA sequences display two fluorescence emission bands located near 385 and 405 nm (Fig. 2). The short wavelength maximum is different for minor groove and intercalative conformations of the B[a]PDE residues with 382 and 389 nm fluorescence emission maxima observable in the case of duplexes $\text{ACG}^{\text{t}}\text{C/TGMG}$ (motif A) and $\text{A}^{\text{t}}\text{CGC/TGMG}$ (motif D), respectively (data not shown); the differences in the emission spectra indicate differences in conformations between these guanine and adenine adducts as described earlier [28, 37, 43]. The fluorescence of the minor groove *trans*-B[a]PDE- N^2 -dG adducts (motif A) was strongly quenched relatively to the B[a]P-tetraol fluorescence, and when the bulky polycyclic aromatic residue was intercalated into the DNA helix with base displacement (motif B), or (+)-*trans*-B[a]PDE- N^6 -dA adducts that is 3'-intercalated and that distorts and destabilizes the duplex (motif E) (Table S2, Fig. 3). By contrast, the duplex with the (-)-*trans*-B[a]PDE- N^6 -dA 5'-intercalated adduct without base displacement, $\text{A}^{\text{t}}\text{CGC/TGMG}$ (motif D), was characterized by a relatively high fluorescence intensity which was close to that of the B[a]P-tetraol fluorescence intensity manifested in DNA-free aqueous solutions [36] (Fig. 3). In the case of the (+)- or (-)-*trans*-B[a]PDE- N^2 -dG-containing duplexes, the low fluorescence intensity is attributed to an electron-transfer mechanism [39, 41, 42] and to the high accessibility of the B[a]P residue to the polar microenvironment (water molecules) [22, 36, 37]. The quenching of the fluorescence of intercalative B[a]P residues in $\text{ACG}^{\text{c}}\text{C/TGMG}$, $\text{GCG}^{\text{c}}\text{C/CGMG}$, $\text{G}^{\text{c}}\text{CGC/CGMG}$ and $\text{A}^{\text{t}}\text{CGC/TGMG}$ duplexes is mostly due to the quenching effect of the neighbouring bases [35, 40, 44, 45]. Also, the fluorescence observed in the case of the $\text{A}^{\text{t}}\text{CGC/TGMG}$ sequence is quenched because the aromatic B[a]PDE residue is accessible to the polar microenvironment [22, 36, 37]. The

high fluorescence of the duplex $A^t\text{-CGC/TGMG}$ may be attributed to the low effectiveness of electron transfer [46] and the non-aqueous microenvironment of the B[a]PDE residue.

B[a]PDE-DNA in complexes with Dnmt3a-CD and Dnmt3a-CD/Dnmt3L—We determined fluorescence spectra of complexes of B[a]PDE-modified DNA duplexes with Dnmt3a-CD and the co-factor analog, AdoHcy as exemplified for the duplex $GCG^{t+}C/CGMG$ (Fig. 2). To ensure the saturation of protein binding sites in the fluorescence titration experiments, excess B[a]PDE-DNA concentrations of 200 nM that exceeded the K_d of B[a]PDE-DNA•Dnmt3a-CD•AdoHcy complexes (10-70 nM, Table S1) were employed. No further changes in fluorescence intensities were observed at still higher concentrations (500 nM) of $G^{t+}CGC/CGMG$ or $GCG^{t+}C/CGMG$ duplexes. At each protein concentration, a 5 min incubation time sufficed to reach a stable fluorescence intensity. The fluorescence values at 384 nm (I^{384}) were normalized to the fluorescence of B[a]PDE-DNA in the absence of protein (I_r^{384}) and plotted as a function of Dnmt3a concentration (Fig. 2, insert). The fluorescence values corresponding to the plateau region shown in the insert in Fig. 2 are reported in Table S2. A four-fold excess of Dnmt3a-CD protein over the B[a]PDE-DNA concentration was required to reach this saturation level which corresponds to the binding of one protein tetramer per DNA molecule.

The fluorescence of B[a]PDE-DNA•Dnmt3a-CD•AdoHcy complexes relative to the fluorescence of free B[a]PDE-modified DNA sequences was greater except in the case of the $A^t\text{-CGC/TGMG}$ •Dnmt3a-CD•AdoHcy complex (Table S2). At the same time, no significant shifts of the emission bands were observed during the entire titration range which suggest that the B[a]PDE-DNA adduct conformation may be similar in free B[a]PDE-DNA as well as in the B[a]PDE-DNA•Dnmt3a-CD•AdoHcy complexes (Figure 2) since DNA strand separation is known to lead to significant changes in the spectroscopic properties of B[a]PDE-DNA adducts [37, 43, 47].

The effect of (+)-*trans*-B[a]PDE- N^2 -dG adducts in DNA substrates (motif A) on the fluorescence of the B[a]PDE-DNA•Dnmt3a-CD•AdoHcy complex was studied. The largest (3-fold) enhancement of the fluorescence of the B[a]P residue was observed when the B[a]PDE- N^2 -dG lesion was within the CpG site (Table S2 and Fig. 4A). The enhancement was diminished when the adduct was adjacent to the CpG site in $G^{t+}CGC/CGMG$ or moved away from it in $GCGC---G^*/CGCG---C$ which is predominantly (+)-*trans*-B[a]PDE- N^2 -dG, but with minor proportions of other stereoisomeric adducts (Table S2 and Fig. 4A). Unlike $GCG^{t+}C/CGMG$ (motif A), the (+)-*cis* adduct (motif B) positioned at the same site in the same duplex sequence, $GCG^{c+}C/CGMG$, exhibited an enhancement in the fluorescence intensity by a factor of 1.2 only in a complex with Dnmt3a-CD (Table S2 and Fig. 4B). Thus, site-specifically modified minor groove B[a]PDE-residues with (+)-*trans*-B[a]PDE- N^2 -dG adduct exhibits a greater fluorescence enhancement than the stereoisomeric base-displaced intercalative (+)-*cis*-B[a]PDE- N^2 -dG adduct and demonstrate that fluorescence changes do depend on the stereochemical properties of the lesions in naked DNA. These effects are most likely due to a greater hydrophobic microenvironment associated with specific B[a]PDE-DNA•Dnmt3a-CD•AdoHcy complex conformations experienced by B[a]PDE residues located in the minor groove as compared to an intercalated conformation.

This hypothesis is supported by the changes in the fluorescence intensities of B[a]PDE-DNA upon complex formation with Dnmt3a-CD/Dnmt3L (Table S2 and Fig. 5). Such complexes with $\text{GCG}^{\text{t}+}\text{C}/\text{CGMG}$ and $\text{ACG}^{\text{t}+}\text{C}/\text{TGMG}$ duplexes (minor groove adducts, motif A) showed a 4- to 5-fold larger fluorescence enhancement in comparison with the same complexes in the absence of Dnmt3L. The impact of Dnmt3L on the fluorescence of the B[a]PDE-DNA•Dnmt3a-CD/Dnmt3L•AdoHcy complexes with other stereoisomeric lesions was very small or entirely absent. Therefore, Dnmt3L resulted in the greatest enhancements in fluorescence intensities when the B[a]PDE residues were located in the minor groove and oriented towards the 5'-direction relative to the modified guanine residue.

The effects of protein complex formation on the fluorescence of various intercalative B[a]PDE-DNA adducts were also compared (Table S2 and Fig. 4C). The damaged nucleotide was positioned adjacent to the CpG site. The 1.7-fold fluorescence enhancement in the presence of Dnmt3a-CD was observed in the case of the $\text{G}^{\text{c}}\text{CGC}/\text{CGMG}$ duplex (motif B). The 3-fold enhancement of the fluorescence of B[a]PDE residues was observed in the case of $\text{A}^{\text{t}+}\text{CGC}/\text{TGMG}$ when A•T base pair flanking the target dC on its 3'-side is distorted by (+)-*trans*-B[a]PDE- N^6 -dA adduct (motif E); however, an opposite effect, the quenching of the fluorescence by a factor of 1.4 was observed in the case of the non-distorted (-)-*trans*-B[a]PDE- N^6 -dA adduct (motif D). Thus, the positions of intercalated (-)-*trans*-B[a]PDE- N^6 -dA, (+)-*trans*-B[a]PDE- N^6 -dA or (-)-*cis*-B[a]PDE- N^2 -dG adducts in DNA duplexes in complexes with Dnmt3a can lead either to an enhancement or a quenching of the fluorescence.

Overall, the binding of Dnmt3a-CD to B[a]PDE-DNA resulted in significant fluorescence enhancements when the B[a]PDE residue was located within the CpG site in the minor groove of DNA or intercalated on the 5'-side of the CpG site (intercalative motif E).

B[a]PDE-DNA duplexes in complexes with the MTases M.SssI or M.HhaI—In order to compare the functions of Dnmt3a with prokaryotic enzymes, the fluorescent properties of complexes of M.HhaI and M.SssI with the 30-mer DNA duplexes $\text{G}^{\text{t}+}\text{CGC}/\text{CGMG}$, $\text{GCG}^{\text{t}+}\text{C}/\text{CGMG}$, $\text{GCG}^{\text{t}+}\text{C}/\text{CGMG}$ (motif A), $\text{A}^{\text{t}+}\text{CGC}/\text{TGMG}$ (motif E) and $\text{A}^{\text{t}+}\text{CGC}/\text{TGMG}$ (motif D) were investigated (Table S3). The DNA substrates contained overlapping recognition sites of M.HhaI (5'-GCGC), M.SssI (5'-CG) and Dnmt3a (5'-CG). It was shown that the MTase binding affinities for 18-mer canonical DNA and 18-mer (+)- and (-)-*trans*-B[a]PDE-DNA decrease in the order M.HhaI > M.SssI > Dnmt3a-CD, with K_d values in the range 10 pM – 70 nM [7, 8, 14]. The enhancement of fluorescence intensities (I^{384}) by a factor 45-50 relative to protein-free B[a]PDE-DNA duplexes was observed in the case of $\text{GCG}^{\text{t}+}\text{C}/\text{CGMG}$ •M.SssI•AdoHcy, $\text{G}^{\text{t}+}\text{CGC}/\text{CGMG}$ •M.HhaI•AdoHcy, and $\text{GCG}^{\text{t}+}\text{C}/\text{CGMG}$ •M.HhaI•AdoHcy complexes with (+)-*trans*-B[a]PDE- N^2 -dG adducts positioned within the MTase recognition sites (Fig. 6). These enhancements were ~15-fold greater than in the case of $\text{GCG}^{\text{t}+}\text{C}/\text{CGMG}$ •Dnmt3a-CD•AdoHcy complexes. Enhancement of I^{384} by factors of 7 and 4 was observed in the case of $\text{GCG}^{\text{t}+}\text{C}/\text{CGMG}$ •M.SssI•AdoHcy and $\text{GCG}^{\text{t}+}\text{C}/\text{CGMG}$ •M.HhaI•AdoHcy complexes, respectively (Table S3). A 3-fold enhancement of the fluorescence of B[a]PDE-DNA duplexes in $\text{A}^{\text{t}+}\text{CGC}/\text{TGMG}$ •M.SssI•AdoHcy complexes was observed which was similar to the fluorescence

enhancement observed in the $A^{T^+}\text{CGC/TGMG}\cdot\text{Dnmt3a}\cdot\text{AdoHcy}$ complex. No changes in the fluorescence of $A^{T^+}\text{CGC/TGMG}\cdot\text{M.SssI}\cdot\text{AdoHcy}$ complexes was observed. Thus, in the case of prokaryotic MTases, dramatic enhancements of the fluorescence occurs when the B[a]PDE-modified guanine residue was placed within the recognition site and the B[a]PDE residue was positioned in the minor groove of DNA pointing towards the 5'-direction of the modified strand.

Discussion

The impact of site-specific, conformationally defined stereoisomeric B[a]PDE- N^2 -dG and B[a]PDE- N^6 -dA adducts on DNA methylation catalyzed by Dnmt3a-CD and the changes in fluorescence that occur upon complex formation, were examined.

Structural and biochemical studies of prokaryotic MTase M.HhaI revealed that, upon binding to DNA, the catalytic loop of the enzyme undergoes a full conformational change that is required for catalysis, and the target cytosine is flipped out from the double helix in the DNA•M.HhaI•AdoHcy complex. This extrusion of the target cytosine residue occurs simultaneously with a movement of the catalytic loop [48-50]. It is usually assumed that flipping of the target cytosine occurs in DNA complexes of all C5-cytosine MTases. However, to our knowledge, the conformational transitions within DNA•Dnmt3a complexes have not yet been experimentally characterized.

Methylation of the 30-mer B[a]PDE-DNA duplexes by Dnmt3a-CD is dependent, in a pronounced manner, on the position of the adducts and their conformational properties (Table 2). Minor groove adducts (motif A), both in the CpG sites or 5'-adjacent to these sites, decrease methylation rates by factors of 6-20. Assuming that the DNA conformations are not markedly different in the protein complexes than in naked DNA (see below), these results suggest that the minor groove location of the bulky B[a]PDE residues affects (1) the flipping of the target cytosines which in the case of M.HhaI occurs via the minor groove [48, 49], and (2) the movement of the Dnmt3a-CD catalytic loop which stabilizes the flipped-out cytosine. Intercalative motif B guanine adducts positioned within the CpG sites (e.g., in the $\text{GCG}^{C^+}\text{C/CGMG}$, $\text{ACG}^{C^+}\text{C/TGMG}$ and $\text{ACG}^{C^+}\text{C/TGMG}$ duplexes) cause decreases in v_1 by factors of 7-21; by contrast, lesions positioned outside of the CpG site (e.g., $\text{G}^{C^+}\text{CGC/CGMG}$) do not exhibit any significant impact on methylation rates (Table 2 and Fig. 4B and C). In the first case, the modified guanine and the partner target cytosine are displaced from their normal positions [28] which may interfere with the formation of important intermolecular contacts with Dnmt3a-CD that are necessary for the formation of reaction-competent DNA•Dnmt3a-CD•AdoMet complexes.

As expected [37, 43], the fluorescence of the B[a]PDE-DNA duplexes was largely dependent on the modified base (G or A) and the conformational properties of B[a]PDE- N^2 -dG adducts (Fig. 2). Furthermore, structurally diverse stereoisomeric B[a]PDE-DNA lesions exhibit different fluorescence intensity changes upon formation of complexes with Dnmt3a-CD (Table S2). Although the adduct conformations could, in principle, be different in the DNA-protein complexes than in naked DNA, we note that the fluorescence spectral shifts upon formation of B[a]PDE-modified DNA•MTase complexes are negligible (Fig. 2). These observations suggest that there are no observable, gross conformational changes due to DNA

strand separation since the spectroscopic characteristics of B[a]PDE-DNA adducts are distinguishable in single- and double-stranded [37, 43, 47]. The observed changes in fluorescence intensities without changes in emission wavelengths (Fig. 2) are therefore most likely due to changes in the local polarities of the microenvironments of the adducts [36, 43, 51]. Based on these considerations, our hypothesis is that the overall conformational features of the B[a]PDE-DNA lesions play a role in modulating the methyltransferase activities in a manner that depends on the positions and stereochemical features of these lesions. The changes in fluorescence intensities (Table S2) provide information about the electrostatic polarity of the microenvironment of these lesions in Dnmt3a-CD complexes.

The fluorescence of the B[a]PDE residues is enhanced in complexes of Dnmt3a-CD in the case of the minor groove adducts in G^{t+}CGC/CGMG and GCG^{t+}C/CGMG duplexes (Fig. 4A), presumably because of the enhanced hydrophobicity of the microenvironment possibly mediated by interactions with the catalytic loop. This effect suggests that the Dnmt3a-CD hydrophobic groups approach the CpG site from the minor groove in which the covalently bound B[a]PDE residues are located. We speculate that such groups are part of the catalytic loop of Dnmt3a which may move towards the minor groove of DNA upon complex formation (see also below). Unlike minor groove adducts, in the case of the site-specific intercalative base-displaced adduct GCG^{c+}C/CGMG (motif B), no change in fluorescence of the covalently bound B[a]PDE residues was observed (Fig. 4B), possibly because this B[a]PDE residue is buried between adjacent base pairs [17] and is therefore insensitive to the proximity of the hydrophobic groups of the Dnmt3a-CD.

It is important to note that the Dnmt3a-CD methylation activity is sensitive to some DNA adduct structural features that are positioned outside of the CpG site in naked DNA as exemplified by the impact of the (+)-*trans*-B[a]PDE-N⁶-dA (motif E) and (-)-*trans*-B[a]PDE-N⁶-dA (motif D) adducts on the methylation rates and the relative fluorescence intensities of the corresponding B[a]PDE-DNA•Dnmt3a-CD•AdoHcy complexes (Fig. 4C). The properties of B[a]PDE-DNA•Dnmt3a-CD•AdoHcy complexes with (+)- and (-)-*trans*-B[a]PDE-N⁶-dA adducts were different which is consistent with their intercalated conformations on the 3'- and 5'-sides of the modified adenine residues, respectively [28, 37]. In the case of the (+)-*trans*-B[a]PDE-N⁶-dA adducts, the B[a]PDE residue is intercalated on the 3'-side of the B[a]PDE-modified A•T base pair and is thus located in the immediate vicinity of the target cytosine. We suggest that the observed strong enhancement of the fluorescence of the A^{t+}CGC/TGMG duplex upon binding of Dnmt3a-CD may be caused both by an increase in the local hydrophobicity in the immediate microenvironment of the B[a]PDE residue, as well as a reduction of the well-established fluorescence quenching effect of neighboring cytosine residues [40] because it is flipped out from the double helix (Fig. 7A). The 3.6-fold decrease of the Dnmt3a-CD-catalyzed methylation rate is probably due to the distortion of the CpG site caused by the intercalation of the B[a]PDE residue. By contrast, in the case of the A^{t+}CGC/TGMG•Dnmt3a-CD•AdoHcy complex with the B[a]PDE residue intercalated on the 5'-side of the modified A•T base pair, the B[a]PDE residue is more distant from the target cytosine and thus the methylation rate is practically unchanged and only a small fluorescence quenching effect was observed (Fig. 4C). Such

effects are consistent with a proper base flipping which promotes the enhanced exposure of the B[a]PDE residue to the polar microenvironment (Fig. 7B).

Interestingly, another frequently occurring DNA lesion, 8-oxoguanine, which is associated with oxidative stress, causes a significant decrease in the v_1^{rel} values when the damage is positioned within the CpG site [52]. Together with the significant reduction in methylation rates observed in the case of B[a]PDE-DNA duplexes containing any of the stereoisomeric adducts within the CpG site (Fig. 4B), these findings indicate that the intact structure of the CpG site is an important condition for the proper functioning of Dnmt3a-CD. DNA adducts positioned outside of the CpG sites affect methylation to a lesser extent (Fig. 4C). In contrast, B[a]PDE-modified nucleotides that flank the CpG sequences on their 5'-side is crucial for the prokaryotic CpG-recognizing MTase M.SssI. In this case, methylation was either abolished in 18-mer duplexes containing B[a]PDE residues in the minor groove (motif A) [7], or strongly decreased in the case of 30-mer duplexes with the base-displaced intercalative (-)-*cis*-B[a]PDE- N^2 -dG adduct (motif B) (Table 3). The B[a]PDE- N^6 -dA adducts in the same position have less impact on the methylation rates of M.SssI.

The enzymatically inactive Dnmt3a regulatory factor, Dnmt3L, causes an increase in the DNA methylation rate because it stabilizes the conformation of the flexible catalytic loop of Dnmt3a [12]. When Dnmt3a-CD interacts with B[a]PDE-DNA duplexes in the presence of Dnmt3L, an enhancement in the methylation rate was also observed with all of the conformationally different adducts (Table 2). A model of DNA•Dnmt3a-CD/Dnmt3L complexes was proposed that predicts the binding of two monomers of Dnmt3a-CD and two monomers of Dnmt3L forming a tetramer with one Dnmt3a-Dnmt3a interface and two Dnmt3a-Dnmt3L interfaces [12]. The complex has two DNA-binding surfaces, i.e. one for each Dnmt3a-CD molecule. Therefore, in view of an absence of direct interactions of Dnmt3L with DNA in the MTase-substrate complex, the stimulatory effect of Dnmt3L should not depend on the conformation of the B[a]PDE adducts, as was observed by us experimentally. The fluorescence of B[a]PDE-DNA•Dnmt3a-CD/Dnmt3L•AdoHcy complexes was only slightly enhanced except in the case of the $\text{GCG}^{\text{t}+}\text{C/CGMG}$ and $\text{ACG}^{\text{t}+}\text{C/TGMG}$ duplexes that exhibited strong enhancements in the fluorescence (Fig. 5). This latter effect may be attributed to the increase of the accessibility of the B[a]PDE residue in $\text{GCG}^{\text{t}+}\text{C/CGMG}$ and $\text{ACG}^{\text{t}+}\text{C/TGMG}$ duplexes to the non-aqueous microenvironment of Dnmt3a. Particularly noteworthy is the larger fluorescence enhancement for $\text{ACG}^{\text{t}+}\text{C/TGMG}$ •Dnmt3a-CD/Dnmt3L complex in comparison with the $\text{ACG}^{\text{t}+}\text{C/TGMG}$ •Dnmt3a-CD/Dnmt3L complex (Fig.5). This may be explained by the observation that the B[a]PDE aromatic ring system in the (+)-*trans*-B[a]PDE- N^2 -dG adduct is more exposed to the solvent than in the (-)-*trans*-B[a]PDE- N^2 -dG adduct [53]. A strong fluorescence enhancement was observed in the case of $\text{GCG}^{\text{t}+}\text{C/CGMG}$ •M.HhaI•AdoHcy complexes (Fig. 6). Based on structural and biochemical studies of these DNA•M.HhaI complexes [48, 49], this fluorescence enhancement is consistent with the B[a]PDE residue being located in a non-aqueous microenvironment created by the M.HhaI catalytic loop interacting with the DNA. It is reasonable to assume that a similar environment is formed as a result of conformational transitions that occur during the methylation reaction catalyzed by Dnmt3a-CD.

The fluorescence enhancement observed in the complexes of minor groove B[a]PDE-DNA with prokaryotic MTase M.HhaI or M.SssI was considerably higher than in the case of complexes with Dnmt3a-CD (Fig. 6). This observation suggests that the catalytic loop fits more tightly to the DNA in the case of prokaryotic enzymes than in the case of Dnmt3a-CD. As discussed above, Dnmt3L promotes a conformational tightening of the Dnmt3a-CD catalytic loop. This is consistent with the additional enhancement of the fluorescence intensity (Fig. 6) and the inferred increase in the local hydrophobicity when Dnmt3L binds to such B[a]PDE-DNA-Dnmt3a complexes, and a model of a DNA•Dnmt3a/Dnmt3L complex in which the active-site loop of Dnmt3a is superimposable on the closed conformation of the catalytic loop of M.HhaI [54]. Such prominent fluorescence enhancements are not observable in the case of A⁺CGC/CGMG in complexes with M.SssI, Dnmt3a-CD, and Dnmt3a-CD/Dnmt3L (Tables S2 and S3) because intercalated B[a]PDE residues are not susceptible to conformational changes of the catalytic loop.

In conclusion, B[a]PDE-N²-dG and B[a]PDE-N⁶-dA adducts can alter DNA methylation rates when positioned within CpG or adjacent, flanking sites. Such effects might contribute to epigenetic changes in gene expression and, potentially, the development of cancer in humans, thus contributing to the genotoxic effects associated with DNA adduct-induced mutagenic mechanisms [55-57]. The impact of the B[a]PDE-N²-dG and N⁶-dA lesions on methylation rates and changes in fluorescence of the covalently bound B[a]PDE residues upon complex formation with Dnmt3a and Dnmt3a/Dnmt3L indicate that conformational transitions occur both within the DNA and the proteins. Our results suggest that the movement of the Dnmt3a-CD catalytic loop is disturbed by the bulky B[a]PDE residues when they are positioned in the DNA minor groove, thus leading to decreases in the catalytic methylation rates. The catalytic loop creates a local hydrophobic environment that leads to the observed B[a]PDE-DNA adduct stereochemistry-dependent fluorescence enhancement of the B[a]PDE residues. Finally, The fluorescence characteristics of some of the different stereoisomeric B[a]P-DNA•Dnmt3a-CD•AdoHcy complexes are consistent with the target cytosines flipping out of the double-helix.

Materials and Methods

Chemicals—AdoHcy was purchased from Sigma (St. Louis, MO). [CH₃-³H]AdoMet (15 Ci/mmol, 66.7 μM) was obtained from Amersham Biosciences (Little Chalfont, U.K.). The following buffers (A–E) were used: A, 50 mM Tris-HCl, pH 7.6, 10 mM MgCl₂, 5 mM dithiothreitol, 0.1 mM spermidine, 0.1 mM EDTA, 1mM ATP; B, 40 mM Tris-HCl, pH 7.8, 10 mM MgCl₂, 10 mM dithiothreitol, 0.5 mM ATP; C, 20 mM HEPES-NaOH, pH 7.5, 100 mM KCl, 1 mM EDTA, 10 mM mercaptoethanol; D, 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM EDTA, 10 mM mercaptoethanol; E, 10 mM Tris-HCl, pH 7.9, 50 mM NaCl, 2 mM mercaptoethanol.

Oligonucleotides—The oligonucleotide sequences employed are shown in Table 1. The 30-mer oligonucleotides, unmodified flanked oligonucleotides and templates for ligation were received from Syntol (Moscow, Russia) and IDT (Coralville, IA). The FAM label was

introduced at the 5'-end of the oligodeoxynucleotide by means of an aminoalkyl linker containing six methylene groups.

The 30-mer B[a]PDE-oligonucleotides (Table 1) were generated by enzymatic ligation of B[a]PDE-modified 12-mers with two 9-mers, or one 18-mer hybridized with a fully complementary template strand. The site-specifically modified 12-mer oligonucleotides containing a single B[a]PDE-*N*²-dG or B[a]PDE-*N*⁶-dA residue were synthesized by reacting the oligonucleotides with racemic *-anti*-B[a]PDE as described in detail elsewhere [58]. These oligonucleotides were phosphorylated by incubation for 1 h at 37 °C in 20 µL of buffer A containing 10 units of T4-polynucleotide kinase and 1 nmole of each oligonucleotide. The other non-phosphorylated fragment and 2 nmoles of the template strand were then added. The solution was heated to 95° C and then slowly cooled to room temperature, and finally to 16° C. The ligation was performed with 5 unit of T4 DNA ligase in 60 µL of buffer B for 16 h. Subsequently, the sample was then heated at 90° C for 5 min, and the ligation products were purified using 12.5% denaturing polyacrylamide gel. The 30-mer oligonucleotides were excised from the gel by elution with 1M LiClO₄, and precipitation with acetone. The oligodeoxynucleotide concentrations were estimated spectrophotometrically using a Cary Eclipse (Varian) spectrophotometer as described [7].

Enzymes—T4 polynucleotide kinase (5000 U/mL) and T4 DNA ligase (5000 U/mL) were purchased from MBI Fermentas (Lithuania). M.HhaI (120 µM) was kindly provided by S. Klimašauskas (Vilnius, Lithuania).

The isolation of recombinant Dnmt3a-CD and M.SssI was described previously [14, 59]. The purification protocol for Dnmt3L was the same as described for Dnmt3a-CD. The Dnmt3a-CD was overexpressed as the N-terminally His₆-tagged protein with use of pET28a plasmid in BL21(DE3) cells. Dnmt3L was overexpressed as the N-terminally His₈-tagged protein with the use of pET41b in BL21(DE3) cells. The M.SssI was overexpressed as the N-terminally His₆-tagged protein by using the pCAL/nH plasmid in ER1821 cells. All proteins were purified by cobalt chelate chromatography.

Fluorescence measurements—Fluorescence spectra were recorded from 360 to 500 nm utilizing a Cary Eclipse (Varian) or FluoroMax-3 (Jobin Yvon) spectrofluorometers with slit widths of 5 nm for excitation and 5-10 nm for emission, λ_{ex} 350 nm.

The fluorescence spectra of protein-free B[a]PDE-modified DNA were recorded in C, D, and E buffer solutions and corrected for the corresponding buffer background.

Fluorescence titrations of B[a]PDE-modified DNA samples (200-500 nM, except in experiments with Dnmt3a-CD/Dnmt3L when 50 nM concentrations were used) were performed by adding of 1-2 µL aliquots of 10 µM Dnmt3a-CD, 5 µM M.HhaI, 5 µM M.SssI or 500 nM Dnmt3a-CD/Dnmt3L to the DNA solutions. The reaction mixtures were incubated for 5 min at 25 °C in the presence of 100 µM AdoHcy in buffers C, D, and E in the case of Dnmt3a-CD (or Dnmt3a-CD/ Dnmt3L), M.HhaI and M.SssI, respectively, and the fluorescence spectra were recorded in the range of 360–500 nm. The spectra of the enzymes incubated in buffers C, D, and E in the presence of 100 µM AdoHcy in the absence

of B[a]PDE-DNA were subtracted from those of the B[a]PDE-DNA•MTase•AdoHcy complexes. For each complex of B[a]PDE-DNA with the MTases, the fluorescence intensities at 384 nm were determined (I^{384}) and normalized to the corresponding B[a]PDE-DNA fluorescence intensities and the normalized values are denoted by I_f^{384} .

Fluorescence polarization measurements—were performed with a Cary Eclipse (Varian) spectrofluorometer with excitation at 495 nm and emission at 520 nm, and 10 nm slit widths at 25 °C in 120 μ L microcuvettes with 1 cm pathlength. Oligodeoxynucleotide duplexes (10 nM) containing the FAM label in the methylated DNA strand (CGMGf) were preincubated with 0.1 mM AdoHcy, and fluorescence polarization of free DNA (P_0) was measured. Dnmt3a-CD was added as 1-2 μ L aliquots of 3 μ M stock solution and the polarization value (P) was recorded 2 min after each addition. Measurements were conducted up to a final concentration of Dnmt3a-CD of 300-400 nM; at these values, the fluorescence polarization value achieves a plateau ($P_{\max}$). Dependences of P on the total concentration of Dnmt3a-CD were obtained and titration curves were analyzed in terms of Scheme 1 [14]:

where E_2 represents the homodimeric Dnmt3a-CD, S is FAM-labeled DNA, E_2S and E_4S are the enzyme-substrate complexes. The K_{d1} and K_{d2} values were obtained as described [14].

Methylation Assays—DNA methylation by Dnmt3a-CD was monitored by measuring the amount of tritium incorporated into the oligodeoxynucleotide target duplexes at 37°C [14]. The reaction mixtures contained 1.5 μ M oligodeoxynucleotide duplex, 2 μ M [CH_3 - ^3H] AdoMet and either 0.88 μ M Dnmt3a-CD in buffer C or 0.1 μ M of M.SssI in buffer E. The reaction was initiated by the addition of the MTase. After 5–20 min of incubation, 9.7 μ L aliquots of the reaction mixtures were pipetted onto DE-81 paper disks (Whatman, Brentford, U.K.). The filters were washed six times with 50 mM KH_2PO_4 , dried and placed into 3 ml of Zhs-106 scintillation fluid (Reakhim, Russia). The filter-bound radioactivity was measured using a Tracor Analytic Delta 300 scintillation counter (ThermoQuest/CE Instruments, Piscataway, USA) and the amount of methylated DNA was determined according to a previously described protocol [14]. The reaction mixtures containing 1.5 μ M oligodeoxynucleotide duplex, 2 μ M [CH_3 - ^3H] AdoMet, 0.3 μ M Dnmt3a-CD and 0.3 μ M Dnmt3L in buffer C were prepared. The Dnmt3a-CD and Dnmt3L solutions were preincubated for 30 min at 37°C and then the reaction was started by the addition of the enzyme to these solutions. The progress of the methylation reaction was monitored as described above.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AdoHcy	S-adenosyl-L-homocysteine
AdoMet	S-adenosyl-L-methionine
B[<i>a</i>]P	benzo[<i>a</i>]pyrene
anti-B[<i>a</i>]PDE	r7,t8-dihydroxy-t9,10-epoxy-7,8,9,10-tetrahydrobenzo[<i>a</i>]pyrene
Dnmt3a	DNA methyltransferase Dnmt3a
Dnmt3a-CD	catalytic domain of Dnmt3a
M	5-methylcytosine
MTase	DNA-(cytosine C5)-methyltransferase
PAH	polyaromatic hydrocarbons

Fig. 1.
Structures of B[a]PDE-DNA adducts.

Fig. 2.

Fluorescence spectra of free $GCG^{t+}C/CGMG$ and $GCG^{t+}C/CGMG$ duplexes in the presence of AdoHcy (500 nM duplex) and increasing concentrations of Dnmt3a-CD. Insert: fluorescence yields of $GCG^{t+}C/CGMG$ duplexes as a function of Dnmt3a-CD concentration.

Fig. 3.

Fluorescence intensities of B[a]PDE-DNA duplexes measured at 384 nm. The error bars represent standard deviations derived from at least three independent experiments.

Fig. 4.

Dependence of the relative fluorescence intensities at 384 nm (I_r^{384}) of B[a]PDE-DNA•Dnmt3a-CD•AdoHcy complexes (crosshatched) and relative initial rates of methylation (v_1^{rel}) of B[a]PDE-DNA by Dnmt3a-CD (white) on the position of the damaged nucleotide (A). Effects of adduct conformation in the CpG site (B), or adjacent to the CpG site (C). The error bars represent standard deviations derived from at least three independent experiments. Fluorescence of $\underline{ACG}^*C/TGMG$ •Dnmt3a-CD•AdoHcy complex was not determined.

Fig. 5. Comparison of relative fluorescence intensities at 384 nm (I_r^{384}) of different B[a]PDE-DNA complexes with Dnmt3a-CD (crosshatched bars) and Dnmt3a-CD/Dnmt3L (white bars) in the presence of AdoHcy. The error bars represent standard deviations derived from at least three independent experiments.

Fig. 6.

Comparison of relative fluorescence intensities at 384 nm (I_r^{384}) of B[a]PDE-DNA duplexes in complexes with M.HhaI, M.SssI, Dnmt3a-CD or Dnmt3a-CD/Dnmt3L in the presence of AdoHcy; $G^{t+}CGC/CGMG$ duplexes (crosshatched bars) and $GCG^{t+}C/CGMG$ duplexes (white bars). The error bars represent standard deviations derived from at least three independent experiments.

Fig. 7.

Schematic representations of accessibility of B[a]PDE residues in B[a]PDE-DNA duplexes to the aqueous solvent in complexes with Dnmt3a-CD; $A^{\dagger}\text{-CGC/TGMG}$ duplex (A), and $A^{\dagger}\text{-CGC/TGMG}$ duplex (B). The grey sickles represent the Dnmt3a-CD catalytic loop. The multipointed stars indicate the most enhanced fluorescence yields associated either with the B[a]PDE-DNA•Dnmt3a-CD•AdoHcy complexes (A), or protein-free DNA duplexes in aqueous solutions (B).

Scheme 1.

Table 1

Definitions of oligodeoxynucleotide sequences. S1 = 5'-CTGAATACT-3', S2 = 5'-TAACCTGAT-3', S3 = 3'-GACTTATGA-5', S4 = 3'-ATTGGACTA-5'; G⁺ and G⁻, (+)- and (-)-*trans*-B[a]PDE-N²-dG; G⁺ and G⁻, (+)- and (-)-*cis*-B[a]PDE-N²-dG; A⁺ and A⁻, (+)- and (-)-*trans*-B[a]PDE-N⁶-dA; G*, predominantly (+)-*trans*-B[a]PDE-N²-dG, but with minor proportions of other stereoisomeric adducts [28], FAM (f), 6-carboxyfluorescein.

Oligonucleotide	Designation
5'-S1-ACTTGCGCTCTC-S2-3'	GCGC
5'-S1-ACTTG ⁺ CGCTCTC-S2-3'	G ⁺ CGC
5'-S1-ACTTGCG ⁺ CTCTC-S2-3'	GCG ⁺ C
5'-S1-ACTTGCG ⁻ CTCTC-S2-3'	GCG ⁻ C
5'-S1-ACTTG ⁻ CGCTCTC-S2-3'	G ⁻ CGC
5'-S1-ACTTGCG ⁺ CTCTC-S2-3'	GCG ⁺ C
3'-S3-TGAACGMGAGAG-S4-5'	CGMG
3'-S3-TGAACGMGAGAG-S4-FAM-5'	CGMGf
5'-S1-ACTTACGCTCTC-S2-3'	ACGC
5'-S1-ACTTACG ⁺ CTCTC-S2-3'	ACG ⁺ C
5'-S1-ACTTACG ⁻ CTCTC-S2-3'	ACG ⁻ C
5'-S1-ACTTACG ⁺ CTCTC-S2-3'	ACG ⁺ C
5'-S1-ACTTA ⁺ CGCTCTC-S2-3'	A ⁺ CGC
5'-S1-ACTTA ⁻ CGCTCTC-S2-3'	A ⁻ CGC
3'-S3-TGAATGMGAGAG-S4-5'	TGMG
5'-S1-ACTTGCGCTCTCTTGCCTTAT-3'	GCGC---G
5'-S1-ACTTGCGCTCTCTTG*CCTTAT-3'	GCGC---G*
3'-S3-TGAACGCGAGAGAACGGAATA-5'	CGCG---C

Table 2

Kinetic parameters of methylation reactions of B[a]PDE-DNA catalyzed by Dnmt3a-CD and Dnmt3a-CD/Dnmt3L. The B[a]PDE-DNA duplexes are defined in Table 1; the recognition site of Dnmt3a-CD is marked in bold, the target cytosine is underlined; v_1^{rel} represents the rates of methylation relative to the methylation rate of the corresponding unmodified GCGC/CGMG, ACGC/TGMG or GCGC---G/CGMG---C duplexes.

B[a]PDE-DNA	Schematic view	Brief description of adduct conformation	B[a]PDE-DNA + Dnmt3a-CD		B[a]PDE-DNA + Dnmt3a-CD/ Dnmt3L	
			v_1 , nM/min	v_1^{rel}	v_2 , nM/min	
<u>GCGC</u> CGMG			1.5 ± 0.2	1.00	6.0 ± 0.4	
ACGC TGMG			2.5 ± 0.1	1.00	7.8 ± 0.5	
GCGC---G CGCG---C			1.7 ± 0.3	1.00	5.3 ± 0.6	
G ⁺ <u>GCGC</u> CGMG		Minor groove location	0.16 ± 0.05	0.11	0.83 ± 0.11	
G <u>G</u> ⁺ C CGMG			0.12 ± 0.04	0.08		
ACG ⁺ C TGMG			0.13 ± 0.03	0.05	0.37 ± 0.12	

B[α]PDE-DNA	Schematic view	Brief description of adduct conformation	B[α]PDE-DNA + Dnmt3a-CD		B[α]PDE-DNA + Dnmt3a-CD/ Dnmt3L	
			ν_1 , nM/min	ν_1^{rel}	ν_2 , nM/min	
$\overline{\text{GCG}}^{\text{t}}\text{C}$ CGMG			0.17 ± 0.09	0.11		
$\overline{\text{ACG}}^{\text{t}}\text{C}$ TGMG			0.21 ± 0.05	0.08	0.68 ± 0.15	
$\overline{\text{GCGC}}^{\text{t}}\text{---G}^*$ $\overline{\text{CGCG}}^{\text{t}}\text{---C}$			1.6 ± 0.3	0.95	5.7 ± 0.5	
$\text{G}^{\text{s}}\text{---}\overline{\text{CGC}}$ CGMG		Base-displaced intercalation	1.5 ± 0.1	1.00	5.1 ± 0.4	
$\overline{\text{GCG}}^{\text{e}}\text{C}$ CGMG			0.07 ± 0.03	0.05		

B[α]PDE-DNA	Schematic view	Brief description of adduct conformation	B[α]PDE-DNA + Dnm3a-CD		B[α]PDE-DNA + Dnm3a-CD/ Dnm3L	
			ν_1 , nM/min	ν_1^{rel}	ν_2 , nM/min	
ACG ^c C TGMG			0.31 ± 0.07	0.12	0.85 ± 0.09	
ACG ^c C TGMG			0.34 ± 0.15	0.14	0.85 ± 0.15	
A ¹⁺ CGC TGMG		Classical intercalation without base displacement	0.69 ± 0.11	0.27	2.1 ± 0.4	
A ¹⁺ CGC TGMG			3.1 ± 0.1	1.25	12 ± 2	

Table 3

Catalytic properties of B[*a*]PDE-DNA in reactions with M.SssI. The B[*a*]PDE-DNA duplexes are defined in Table 2.

B[<i>a</i>]PDE-DNA	v_1 , nM/min
GCGC CGMG	8.8 ± 0.3
GCG ⁶⁺ C CGMG	7.9 ± 0.7
G ⁶⁺ CGC CGMG	0.27 ± 0.09
ACGC TGMG	6.8 ± 0.6
A ¹⁺ CGC TGMG	2.0 ± 0.3
A ¹⁺ CGC TGMG	4.7 ± 0.4