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Dimethyl sulfoxide stimulates the catalytic activity of de novo DNA methyltransferase 3a (Dnmt3a) in vitro

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Abstract

Mammalian DNA methyltransferase Dnmt3a is required for de novo methylation of CpG dinucleotides in genomic DNA. While DNA methyltransferase inhibitors have been extensively utilized both in vitro and in vivo, no stimulator of catalytic activity has been identified thus far. Here we show that the methyltransfer activity of Dnmt3a is stimulated by the addition of dimethyl sulfoxide (DMSO) to the reaction solution in vitro. Enzymatic analysis of initial reaction velocity suggests that the DMSO stimulation effect depends on the interaction between DMSO and the reaction substrates (DNA and AdoMet), but not the enzyme itself.

Keywords: DNA methyltransferase; Dnmt3a; Enzymology; Kinetics; Catalytic stimulator; Dimethyl sulfoxide

1. Introduction

In higher eukaryotes, the epigenetic methylation of DNA participates in embryogenesis, homeostasis, and carcinogenesis via transcriptional regulation in chromatin [1–3]. DNA methyltransferases (DNMTs) are the principal enzymes responsible for

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cytosine methylation of DNA [4,5]. Although previous in vivo studies suggested that mammalian de novo DNA methyltransferase 3a (Dnmt3a) is required for embryonic development [5,6], it remains unclear how Dnmt3a is involved in establishing DNA methylation patterns. Unlike the mammalian maintenance DNA methyltransferase DNMT1, Dnmt3a has been less well characterized since the enzymatic activity of Dnmt3a is significantly lower than that of DNMT1.

DNA methylation (and DNA methylation-related proteins) has been shown to be relevant to several genetic diseases in humans, including Rett and ICF syndromes, as well as cancer [7–10]. Thus, seeking novel reagents capable of either positively or negatively controlling the catalytic activity of a DNMT is a major research focus in both basic and industrial research. Generally, considering the interaction between an enzyme and its substrates, molecules that are structurally similar to the substrate/product are likely to be promising candidates as catalytic inhibitors [11]. In contrast, novel stimulators may be difficult to identify unless binding motifs in the primary structure or X-ray crystallography data are available. To our knowledge, there are no reports describing chemicals that stimulate the catalytic activity of DNA methyltransferases other than their natural substrates (DNA and AdoMet). In this report, we demonstrate that dimethyl sulfoxide (DMSO) stimulates Dnmt3a activity up to 4-fold due to its interaction with the reaction substrates (DNA oligonucleotides and AdoMet). This novel approach to positively affect DNA methyltransferase activity in vitro may be useful to examine the biochemical properties of mammalian DNMTs that have relatively lower catalytic activity, such as the Dnmt3s.

2. Materials and methods

2.1. Materials

S-adenosyl-L-[methyl-^{3}H]methionine (^{3}H-AdoMet) was purchased from Amersham Biosciences. Streptoavidin-coated magnetic beads were purchased from Dynal. General chemicals including AdoMet derivatives were purchased from Sigma, Life Technologies, Novagen, and Roche. DNA oligonucleotides were synthesized by MWG Biotech and New England Biolabs (Table 1). Preparation of double-stranded DNA oligonucleotides was described previously [12]. Bacterial methylases *Sss*I and *Hha*I were purchased from New England Biolabs.

2.2. Preparation of recombinant proteins (Dnmt3a and DNMT1)

The infection and expression of His-tagged recombinant Dnmt3a and DNMT1 utilizing a baculovirus system in Sf9 cells was described previously [12]. Purification of recombinant protein was modified as follows. Nickel-agarose resin (Novagen) (200 μl as bed volume) was washed twice with distilled water (400 μl), twice with 50 mM $NiSO_4$ (400 μl), and once with Ni-binding buffer (20 mM Tris–HCl, pH 8.0, 10% glycerol, 500 mM NaCl, and 5 mM imidazole). His-tagged protein was eluted

Table 1
Synthetic DNA oligonucleotides utilized in this study

Name appearing in text and figures	Sequence[a]
Unmethylated ssDNA	5′-B-GATCCGACGACGACGCGCGCGCGACGACGAGATC
Fully methylated ssDNA	5′-B-GATCMGAMGAMGAMGMGMGMGMGAMGAMGAGATC
Unmethylated dsDNA	5′-B-GATCCGACGACGACGCGCGCGCGACGACGAGATC CTAGGCTGCTGCTGCGCGCGCGCTGCTGCTCTAG
Hemimethylated dsDNA	5′-B-GATCCGACGACGACGCGCGCGCGACGACGAGATC CTAGGMTGMTGMTGMGMGMGMGMTGMTGMTCTAG
Fully methylated dsDNA	5′-B-GATCMGAMGAMGAMGMGMGMGMGAMGAMGAGATC CTAGGMTGMTGMTGMGMGMGMGMTGMTGMTCTAG

[a] M, 5-methyl-2′-deoxycytosine; B, biotin.

three times (total 210 μl) with 70 μl of Ni-elution buffer (20 mM Tris–HCl, pH 8.0, 10% glycerol, 10 mM NaCl, and 1 M imidazole) and pooled.

2.3. DNMT-magnetic beads assay

A protocol for the DMB (DNMT-magnetic beads) assay has been described previously [12]. A standard reaction contains 300 nM Dnmt3a, 125 nM DNA oligonucleotides (2.5 μM in the concentration of CpG dinucleotides when unmethylated dsDNA was utilized as the substrate), and 900 nM tritium-labeled AdoMet (Amersham Biosciences, 1 mCi/ml) in reaction buffer (50 mM Tris–HCl, pH 8.0, 5 mM EDTA, 10% glycerol, 10 mM 2-mercaptoethanol, and 0.5 mM PMSF). A typical reaction is incubated for 30 min at 37 °C. Changes in the conditions for a particular experiment are described in the figure legends. Mean values of independent duplicate or triplicate experiments are presented in figures and tables. Error bars represent the standard deviations from the mean.

3. Results and discussion

3.1. Catalytic activity of recombinant Dnmt3a

Recombinant Dnmt3a expressed from a baculovirus system was purified and utilized for all studies. We first determined the reaction plots of the methyltransfer activity of recombinant Dnmt3a with regard to time- and enzyme–dose dependency (Fig. 1), while substrate–dose dependency for DNA and AdoMet on Dnmt3a activity has been reported previously [12]. The reaction was found to be linear up to 1.2 μM Dnmt3a (Fig. 1A) and for the first 150 min (Fig. 1B). All experiments described in this report were performed under conditions yielding tritium incorporation within the linear range.

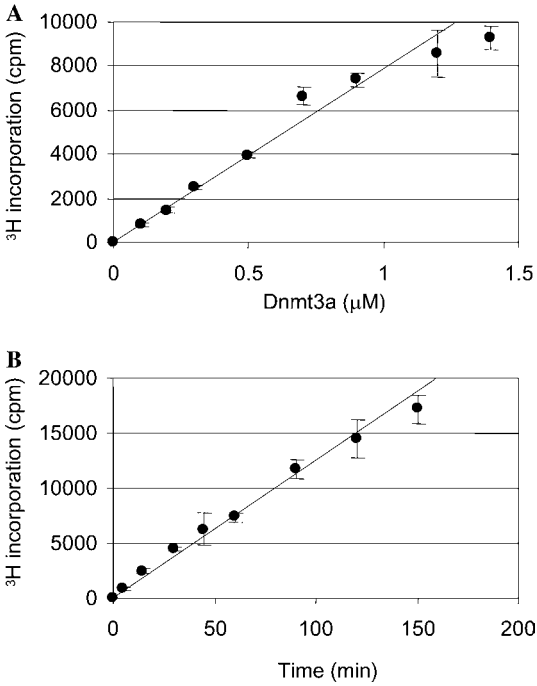

Fig. 1. Reaction progress plots for the methylation activity of mammalian de novo DNA methyltransferase Dnmt3a. (A) Linearity of the methylation reaction as a function of enzyme concentration. Each reaction contains recombinant Dnmt3a at 0, 100, 200, 300, 500, 700, 900 nM, 1.2, 1.4 μM in the standard reaction conditions. (B) Time course of the methylation reaction catalyzed by Dnmt3a. Duplicate reactions (400 μl) containing 300 nM Dnmt3a, 125 nM unmethylated dsDNA (2.5 μM in CpG concentration), and 900 nM AdoMet were incubated at 37 °C.

3.2. Inhibitory effect of substrate/product analogs on Dnmt3a activity

DNA methyltransferases utilize two substrates, DNA and *S*-adenosyl-L-methionine (AdoMet), to produce two products, methylated DNA and *S*-adenosyl-L-homocysteine (AdoHcy) (Fig. 2A), suggesting that analogs of these molecules may inhibit Dnmt3a. We demonstrate that our assay system is sufficiently reproducible to detect subtle differences among similar molecules (Fig. 2B) as inhibitors. Sinefungin, the only naturally occurring AdoHcy analog [13,14], inhibited Dnmt3a as efficiently as AdoHcy, indicating that the structural differences between AdoHcy and sinefungin in the central portion of the side chain are not important for binding to Dnmt3a. In contrast, 5′-isobutylthio-5′-*S*-deoxyadenosine (SIBA) [15], which possesses a structure around the sulfur atom that is highly similar to that of AdoHcy, was less effective as an inhibitor than sinefungin. This result suggests that the terminal structure of the side chain is more important than a sulfur atom for recognition by Dnmt3a. This notion was supported by utilizing 5′-deoxy-5′-(methylthio)-adenosine (MTA) as an inhibitor, which was the least

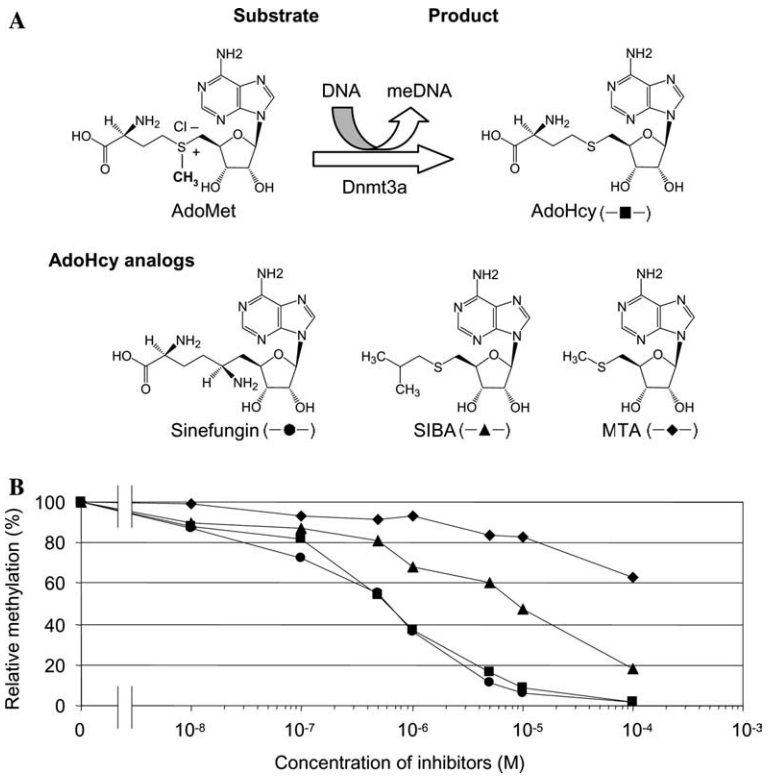

Fig. 2. The chemical structure of the AdoHcy analog side-chain is responsible for the differential inhibitory effects on Dnmt3a activity. (A) The substrate, *S*-adenosyl-L-methionine (AdoMet), is converted to the product, *S*-adenosyl-L-homocysteine (AdoHcy), by the Dnmt3a-mediated methylation reaction on DNA. The methyl group that is transferred from AdoHcy to cytosine is shown in bold. Stereochemical structures of the AdoHcy analogs including sinefungin, 5′-isobutylthio-5′-*S*-deoxyadenosine (SIBA), and 5′-deoxy-5′-(methylthio)-adenosine (MTA) are shown. (B) Differences in the effects of the reaction product (AdoHcy) and its analogs on Dnmt3a activity. Inhibitors employed are as follows; AdoHcy (squares), sinefungin (circles), SIBA (triangles), and MTA (diamonds). The methylation activity is presented as a relative value (%) to the activity without added inhibitor (100%). An approximate half-maximal inhibition (IC50) for each inhibitor is as follows; AdoHcy (600 nM), sinefungin (600 nM), SIBA (8 μM), and MTA (>1 mM).

effective inhibitor and has the most divergence at the terminal end of the side chain relative to AdoHcy.

3.3. Antithetical effects of magnesium ions on Dnmt3a activity

DNA methyltransferase activity of Dnmt3a was inhibited in the presence of >100 mM NaCl or KCl (Fig. 3A), which is consistent with a previous report [16]. In contrast, a divalent cation such as Mg^{2+} showed not only severe inhibition compared to monovalent cations, but also stimulation of up to 50% (1.5-fold) at $MgCl_2$ concentrations between 1 and 10 mM (Fig. 3A). It should be noted that

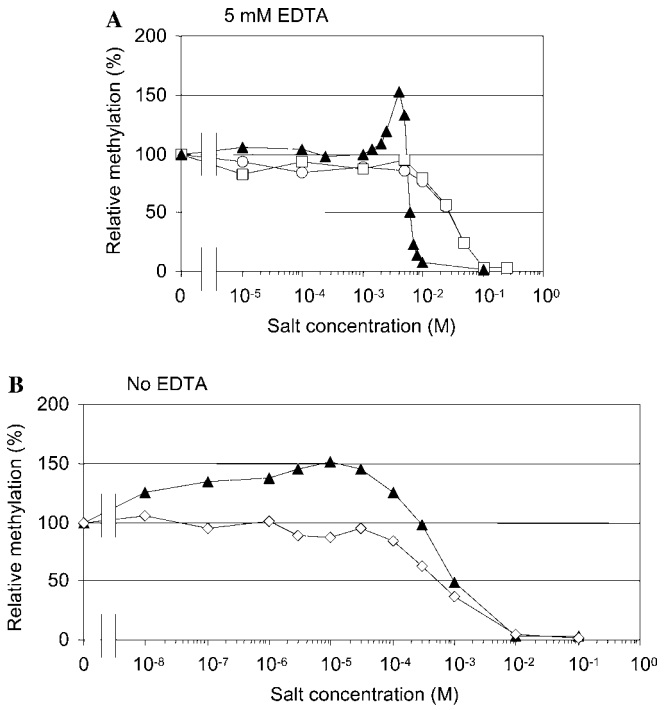

Fig. 3. Magnesium ions have both stimulatory and inhibitory effects on the methylation reaction catalyzed by Dnmt3a. Salt sensitivity of Dnmt3a in the presence (A) or absence (B) of 5 mM EDTA. Chloride salts utilized are as follows; NaCl (open circles), KCl (open squares), $MgCl_2$ (closed triangles), and $CaCl_2$ (open diamonds). The methylation activity is presented as a relative value (%) to the activity without salt addition (100%).

our standard reaction conditions contained 5 mM EDTA, a potent chelating reagent for divalent metal cations. In the absence of EDTA, the stimulatory curve of Mg^{2+} broadened and its peak shifted to a lower concentration (10 μM) (Fig. 3B). Other divalent cations, such as Ca^{2+} (Fig. 3B) and Cu^{2+} (not shown), did not stimulate Dnmt3a. Each metal ion interacts with DNA at particular binding sites, which leads to specific conformational changes in the DNA [17,18]. Thus, it is assumed that Dnmt3a may recognize the specific structure of a DNA–Mg^{2+} complex as a favorable substrate for its methylation at lower Mg^{2+} concentrations, and as an unfavorable one at higher Mg^{2+} concentrations. Currently, there is no "standard" condition in regard to salt concentrations in a mammalian DNA methyltransferase reaction in vitro. Our results suggest that monovalent salts (NaCl or KCl) are not required for Dnmt3a under the experimental condition in vitro. Although a slight stimulation of Dnmt3a activity was observed in the presence of a broad range of $MgCl_2$ concentration, it is difficult to control the precise dose of such a residual concentration of $MgCl_2$. Thus, a fixed concentration of EDTA should be added in a reaction buffer to eliminate the antithetical effects of divalent cations and to maintain the reproducibility of the enzymatic analysis.

3.4. Stimulatory effect of dimethyl sulfoxide on Dnmt3a activity

We were particularly interested in searching for factors or conditions that could stimulate the activity of Dnmt3a in vitro. Thus, we conducted a systematic screening of a broad range of reagents including nucleotide derivatives, cofactors of other enzymes, inorganic salts, and small and macromolecular organic compounds. We found that a typical polar solvent, dimethyl sulfoxide (DMSO), could dramatically stimulate DNA methyltransferase activity (Fig. 4). Catalytic activity of Dnmt3a, as well as DNMT1, was stimulated by the addition of DMSO to 35% (Fig. 4A). In contrast, activities of the bacterial cytosine methylases *Sss*I and *Hha*I were not enhanced by DMSO addition, indicating that DMSO stimulation of enzymatic activity is not a property common to all DNA methyltransferases. Generally, the catalytic domains of DNA methyltransferases are highly conserved from bacteria to humans. However, there are minor differences even in the conserved methyl transfer motifs between the mammalian and bacterial enzymes. For instance, motif IX is EXXRXXGFP in

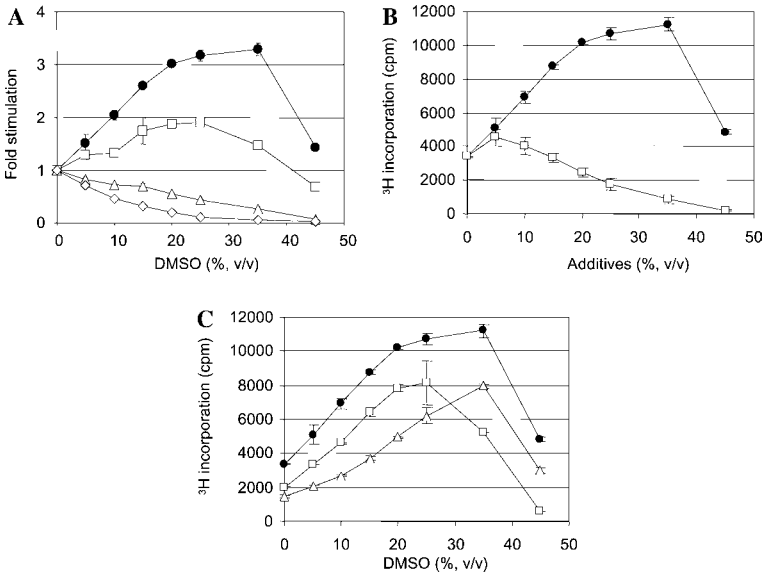

Fig. 4. Dimethyl sulfoxide (DMSO) stimulates the enzymatic activity of Dnmt3a. (A) Effects of DMSO on typical cytosine methyltransferase activities. The methyl transfer reaction of Dnmt3a (closed circles), DNMT1 (open squares), bacterial methylase *Sss*I (open triangles), and *Hha*I (open diamonds) was examined in the presence of various concentrations of DMSO. Reaction conditions for each enzyme are as follows; Dnmt3a (300 nM enzyme, 125 nM unmethylated dsDNA, 900 nM AdoMet), DNMT1 (10 nM enzyme, 125 nM hemimethylated dsDNA, 300 nM AdoMet), *Sss*I (0.005 U/μl enzyme, 125 nM unmethylated dsDNA, 900 nM AdoMet), and *Hha*I (0.125 U/μl enzyme, 125 nM unmethylated dsDNA, 900 nM AdoMet). The methylation activity is presented as a relative value to the activity without DMSO. (B) Effects of organic solvents on Dnmt3a activity. Two additives, DMSO (closed circles) and DMF (open squares), were examined. (C) Stimulatory effect of DMSO with different DNA substrates. DNA substrates utilized are as follows; unmethylated dsDNA (closed circles), hemimethylated dsDNA (open triangles), and single-stranded DNA (open squares).

mammalian DNA methyltransferases, but EXXLXXGFD in *Sss*I and EXXRXXGYP in *Hha*I. These differences may be responsible for the differential stimulatory effect of DMSO. Other organic solvents such as dimethyl formamide (DMF) (Fig. 4B), ethanol, dimethylsulfone, and 2-propanol (data not shown) did not show significant stimulation of Dnmt3a activity, suggesting that alterations in solubility of substrates are not the principal reason for DMSO stimulation of Dnmt3a activity. DMSO is frequently utilized as a denaturant in PCR and DNA sequencing reactions. Our results, however, demonstrated that Dnmt3a activity was stimulated by DMSO even when ssDNA was utilized as substrate (Fig. 4C), suggesting that DMSO's ability to enhance denaturation of the double-stranded DNA is not responsible for the stimulation effect.

3.5. The DMSO effect depends on the interaction between DMSO and both substrates of Dnmt3a

Enzyme (Dnmt3a), DNA, and AdoMet are the three essential factors to reconstitute the methyltransfer reaction in vitro. To identify which factor(s) interacts with DMSO to stimulate the Dnmt3a catalytic reaction, we employed conditions in which one of three factors is decreased to examine whether the DMSO concentration yielding maximal activity would be affected (Table 2 and Fig. 5). When the amount of DNA was decreased, the peak of the stimulation curve shifted to the left (from 35 to 25% DMSO concentration), indicating that the stimulatory effect of DMSO is dose dependent with respect to DNA (Fig. 5B, center panel). In contrast, when the enzyme concentration was decreased, tritium incorporation still increased continuously between 0 and 35% DMSO, suggesting that DMSO was not acting on Dnmt3a itself (Fig. 5B, left panel). Decreasing the concentration of AdoMet also yielded a peak shift on the stimulation plot (Fig. 5B, right panel). Therefore, interactions between DMSO and DNA, as well as DMSO and AdoMet, participate in the DMSO-mediated stimulation of Dnmt3a activity. These results suggest that the stimulatory effect by DMSO depends on the specific interaction between DMSO and substrates (DNA and AdoMet), and not between DMSO and enzyme. As DMSO is a highly polar solvent that is similar to water, DMSO molecules may be capable of substituting for water molecules around DNA and AdoMet substrates, resulting in alterations in

Table 2
Summary of peak shift assay

Symbol	Experimental condition				Result		
	Factor decreased	Concentration (μM) of			Enzymatic activity (cpm) at		DMSO concentration (%) yielding maximal activity
		Dnmt3a	CpG	Adomet	0% DMSO	Peak	
–●–	N/A	0.3	2.5	0.9	3377 ± 11	11221 ± 388	35
–○–	Enzyme	0.1	2.5	0.9	1042 ± 54	4518 ± 394	35
–□–	DNA	0.3	0.15	0.9	1053 ± 13	3469 ± 53	25
–△–	Adomet	0.3	2.5	0.09	1068 ± 87	2662 ± 53	20

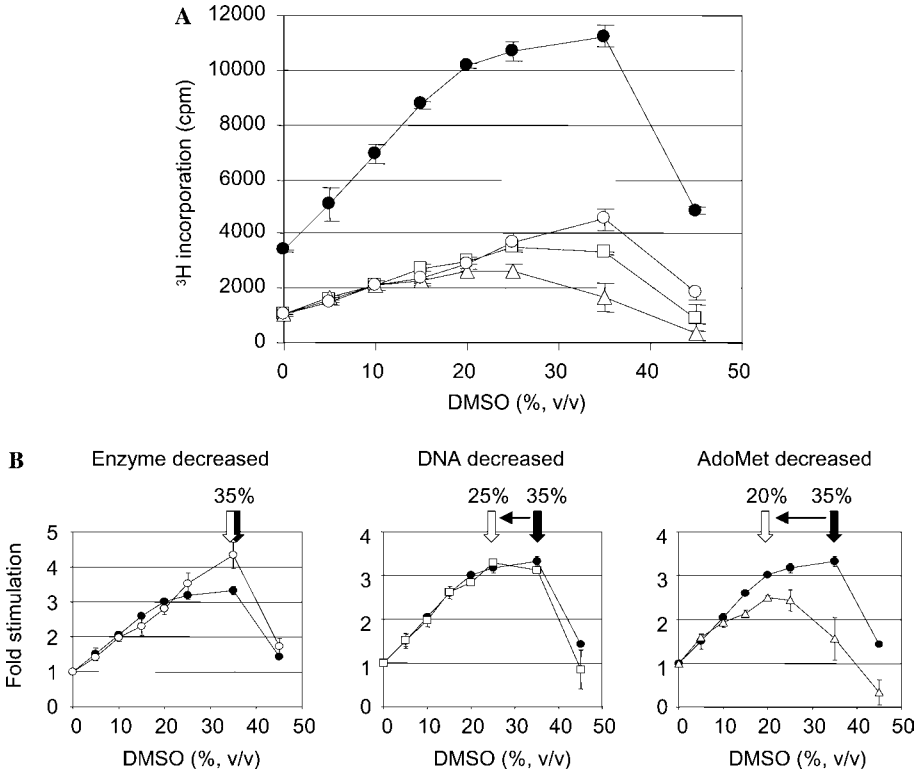

Fig. 5. DMSO stimulates Dnmt3a activity via interaction with both substrates. Several reaction conditions (presented in Table 2) were employed to identify the factor(s) affected by DMSO, which participates in the Dnmt3a stimulation. Reaction conditions were chosen such that enzymatic activity in the absence of DMSO yielded approximately 1000 cpm of tritium incorporation as follows; standard condition (0.3 μM Dnmt3a, 2.5 μM CpG, 0.9 μM AdoMet) (closed circles), enzyme decreased (0.1 μM Dnmt3a, 2.5 μM CpG, 0.9 μM AdoMet) (open circles), DNA decreased (0.3 μM Dnmt3a, 0.15 μM CpG, 0.9 μM AdoMet) (open squares), AdoMet decreased (0.3 μM Dnmt3a, 2.5 μM CpG, 0.09 μM AdoMet) (open triangles). Unmethylated dsDNA was utilized as the DNA substrate. Direct plot (A) and its relative comparison under each condition (B) are shown.

their hydrophobicity and affinity for the enzyme. Such a DMSO cluster around the substrates, or "micelle," may have an advantage in facilitating electron transfer and the formation of covalent bonds under the transition state at the active site of Dnmt3a.

4. Conclusion

Since Dnmt3a has significantly lower activity compared to DNMT1 [12,16,19–21], in vitro "performance enhancers" may be quite useful when experiments require the detection of a low activity DNMT. At this time, there are five DNMT family

members in mammalian cells, of which the catalytic activity of Dnmt2, Dnmt3b1, and Dnmt3L has not yet been well characterized in vitro [22,23]. Our results may provide clues that could be used to reveal hitherto unidentified activities of these enzymes, which play important roles in mammalian embryogenesis and development [5,24].

Acknowledgments

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References

[1] M. Ehrlich, J. Cell Biochem. 88 (2003) 899–910.
[2] K.D. Robertson, Oncogene 20 (2001) 3139–3155.
[3] T.M. Geiman, K.D. Robertson, J. Cell Biochem. 87 (2002) 117–125.
[4] E. Li, T.H. Bestor, R. Jaenisch, Cell 69 (1992) 915–926.
[5] M. Okano, D.W. Bell, D.A. Haber, E. Li, Cell 99 (1999) 247–257.
[6] D. Watanabe, I. Suetake, T. Tada, S. Tajima, Mech. Dev. 118 (2002) 187–190.
[7] H. Leonhardt and M.C. Cardoso, J Cell Biochem. Suppl. (2000) 78–83.
[8] K.D. Robertson, A.P. Wolffe, Nat. Rev. Genet. 1 (2000) 11–19.
[9] A. Kumar, J. Biosci. 25 (2000) 213–214.
[10] T.H. Bestor, Hum. Mol. Genet. 9 (2000) 2395–2402.
[11] I.H. Segel, Enzyme Kinetics, Wiley, New York, 1993.
[12] T. Yokochi, K.D. Robertson, J. Biol. Chem. 277 (2002) 11735–11745.
[13] R.L. Hamil, M.M. Hoehn, J. Antibiot. (Tokyo) 26 (1973) 463–465.
[14] R.S. Gordee, T.F. Butler, J. Antibiot. (Tokyo) 26 (1973) 466–470.
[15] M. Vedel, M. Robert-Gero, M. Legraverend, F. Lawrence, E. Lederer, Nucleic Acids Res. 5 (1978) 2979–2989.
[16] A. Aoki, I. Suetake, J. Miyagawa, T. Fujio, T. Chijiwa, H. Sasaki, S. Tajima, Nucleic Acids Res. 29 (2001) 3506–3512.
[17] R.V. Gessner, G.J. Quigley, A.H. Wang, G.A. van der Marel, J.H. van Boom, A. Rich, Biochemistry 24 (1985) 237–240.
[18] Y.G. Gao, M. Sriram, A.H. Wang, Nucleic Acids Res. 21 (1993) 4093–4101.
[19] M. Okano, S. Xie, E. Li, Nat. Genet. 19 (1998) 219–220.
[20] F. Lyko, B.H. Ramsahoye, H. Kashevsky, M. Tudor, M.A. Mastrangelo, T.L. Orr-Weaver, R. Jaenisch, Nat. Genet. 23 (1999) 363–366.
[21] H. Gowher, A. Jeltsch, J. Mol. Biol. 309 (2001) 1201–1208.
[22] K. Liu, Y.F. Wang, C. Cantemir, M.T. Muller, Mol. Cell. Biol. 23 (2003) 2709–2719.
[23] M. Okano, S. Xie, E. Li, Nucleic Acids Res. 26 (1998) 2536–2540.
[24] D. Bourc'his, G.L. Xu, C.S. Lin, B. Bollman, T.H. Bestor, Science 294 (2001) 2536–2539.