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Enhanced activity of human *N*-myristoyltransferase by dimethyl sulfoxide and related solvents in the presence of serine/threonine-containing peptide substrates

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Abstract

Human *N*-myristoyltransferase (hNMT) activity was found to be stimulated several-fold by DMSO and its analogues in the presence of serine-containing peptide substrates. DMSO caused a concentration-dependent 10-fold stimulation of hNMT activity in the presence of a $pp60^{src}$-derived peptide substrate (Gly-Ser-Ser-Lys-Ser-Lys-Pro-Lys-Arg). However, the stimulation of hNMT activity was not observed by DMSO when a cyclic AMP (cAMP)-dependent protein kinase-derived Ser-free peptide substrate (Gly-Asn-Ala-Ala-Ala-Ala-Lys-Lys-Arg-Arg) was used. These findings suggested that the effect of DMSO is on the substrate rather than on the enzyme. When a MARCKS (myristoylated alanine-rich C-kinase substrate)-derived peptide substrate (Gly-Ala-Gln-Phe-Ser-Lys-Thr-Ala-Arg-Arg) and the M2 gene segment of the reovirus type 3 peptide substrate (Gly-Asn-Ala-Ser-Ser-Ile-Lys-Lys-Lys) were used, hNMT activity was increased by approximately 8.5- and 7-fold, respectively. Dimethyl sulfone (20%) increased hNMT activity between 2.5- and 3.5-fold in the presence of $pp60^{src}$, MARCKS, and M2 gene segment peptides. Dimethyl formamide (20%) increased the hNMT activity by 8.5-, 8.5-, 5.5- and 3.5-fold when $pp60^{src}$, MARCKS, M2, and cAMP-dependent protein kinase-derived peptide substrates were used, respectively. Acetone (20%) also increased the hNMT activity by 20-fold in the presence of the $pp60^{src}$ peptide substrate. Dimethyl ammonium chloride (20%) caused about 6.5- and 2.5-fold increases in the hNMT activity in the presence of the $pp60^{src}$ and cAMP-dependent protein kinase-derived peptide substrates, respectively. Infrared spectroscopy showed a decreased intensity in the band at 3500–3600 cm^{-1} when the infrared spectrum of the $pp60^{src}$-derived peptide was determined in the presence of DMSO. These results suggest the involvement of hydrogen bonding between the heteroatoms of the organic molecules and the hydrogen atoms of the free hydroxyl groups of the serine/threonine-containing peptide substrates. Such interactions appear to enhance the activity of hNMT towards its serine-containing substrates.

Keywords: *N*-Myristoyltransferase; Regulation; Peptide substrates; Dimethyl sulfoxide

1. Introduction

NMT is an essential eukaryotic enzyme that catalyzes the co-translational transfer of myristic acid (a C:14 saturated fatty acid) from myristoyl-CoA onto the amino terminal of the glycine residue of a number of viral, cellular, and oncoproteins (see Refs. [1–6] for reviews). The known myristoylated proteins include the catalytic subunit of cAMP-dependent protein kinase, various tyrosine kinases ($pp60^{src}$, $pp60^{yes}$, $pp56^{lck}$, $pp59^{fyn/syn}$, and cAbl), the β-subunit of calmodulin-dependent protein phosphatase (calcineurin), the myristoylated alanine-rich C-kinase substrate (MARCKS), the α-subunit of several G proteins, and several ARF proteins involved in ADP ribosylation [1–6]. Several viruses possess myristoylated coat proteins, and the myristoylation has been demonstrated to be necessary for their normal replication [7–9].

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Abbreviations: NMT, *N*-myristoyl-CoA:protein *N*-myristoyltransferase; hNMT, human NMT; cAMP, cyclic AMP; DMSN, dimethyl sulfone; DMS, dimethyl sulfide; DMF, dimethyl formamide; DMA, dimethyl ammonium chloride; ARF, ADP ribosylation factor.

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NMTs have been purified and characterized in terms of their molecular properties [10–16]. We demonstrated for the first time that NMT was more active in colonic epithelial neoplasms than in the corresponding normal appearing colonic tissue; furthermore, an increase in NMT activity appeared at an early stage in colonic carcinogenesis [17]. Recently, over-expression of the NMT protein in colorectal adenocarcinomas and gallbladder carcinomas has been demonstrated [18,19]. Fungal, viral, and tumor growth can be inhibited by perturbation of myristoylation, and thus NMT has been identified as a potential target for the development of chemotherapeutic agents [7,20–25].

The specific molecular mechanisms for the regulation of NMT activity are poorly understood. A 71-kDa *N*-myristoyltransferase inhibitor protein (NIP_{71}) was purified to near homogeneity from bovine brain [26]. A 45-kDa *N*-myristoyltransferase activator protein (NAF_{45}) also has been discovered [27]. The physiological roles of NIP_{71} and NAF_{45} are, at present, unknown; however, they may serve to regulate the myristoylation reaction. In an earlier study, we had observed that hNMT activity was stimulated by L-histidine, whereas its two analogues, L-histidinol and L-histamine, inhibited hNMT activity [25]. Furthermore, this inhibition of hNMT activity by L-histidinol and L-histamine was reversed by an excessive concentration of L-histidine, suggesting that L-histidine and L-histidinol were competing for a common site on hNMT.

Work in our laboratory is aimed at understanding the molecular mechanisms involved in the inhibition and activation of NMT activity, and discovering novel potent inhibitors and activators. Our recent focus is on the development of potential inhibitors and activators of NMT. Such compounds may aid in the understanding of the regulation of this mammalian enzyme. During the search for modulators of NMT, we found that certain compounds under investigation were poorly soluble in aqueous buffers and required DMSO as a solvent for the preparation of stock solutions. DMSO is a convenient solvent used frequently for the study of lipophilic compounds. In our preliminary work, we observed that hNMT activity was stimulated several-fold by DMSO alone. Therefore, these results motivated us to further investigate the role of DMSO in the myristoylation reaction. In this paper, we report the effects of DMSO and its analogues on hNMT activity *in vitro*. Except for DMS, these DMSO analogues increased hNMT activity for serine-containing peptide substrates.

2. Materials and methods

2.1. Chemicals and reagents

DMSO, acetone, and DMF were analytical grade reagents obtained from BDH; DMSN, DMS, and DMA were purchased from the Aldrich Chemical Co. The chemical structures of these compounds are given in Fig. 1.

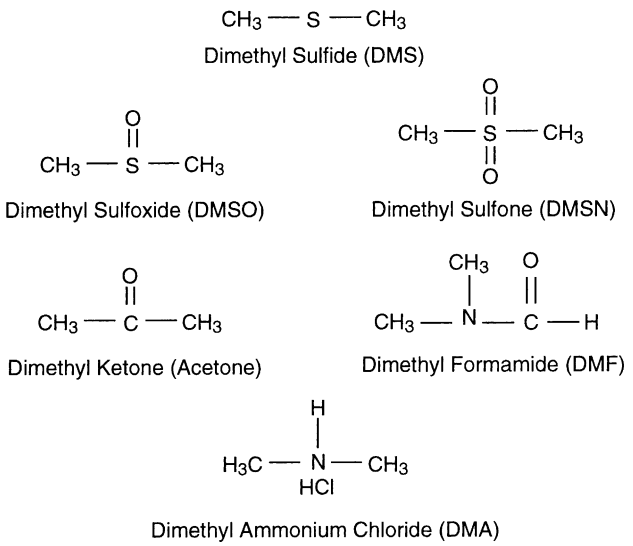

Fig. 1. Structures of DMSO and its analogues.

[9, 10-^{3}H]Myristic acid (39.3 Ci/mmol) was purchased from DuPont NEN. pTrcHis-C vector was obtained from Invitrogen. *Pseudomonas* acyl-CoA synthetase, CoA, benzamidine, phenylmethylsulfonyl fluoride, and leupeptin were obtained from the Sigma Chemical Co. General laboratory chemicals were of analytical grade. The peptides Gly-Asn-Ala-Ala-Ala-Ala-Lys-Lys-Arg-Arg (based on the NH_2-terminal sequence of the type II catalytic subunit of cAMP-dependent protein kinase) and Gly-Ser-Ser-Lys-Ser-Lys-Pro-Lys-Arg (based on the NH_2-terminal sequence of $pp60^{src}$) were obtained from Research Genetics. The peptide Gly-Asn-Ala-Ser-Ser-Ile-Lys-Lys-Lys (the NH_2-terminal sequence of the M2 gene segment of reovirus type 3) was from the Alberta Peptide Institute. The following peptide was synthesized by the solid phase manual protocol developed by Merrifield [28]: Gly-Ala-Gln-Phe-Ser-Lys-Thr-Ala-Arg-Arg (NH_2-terminal sequence of MARCKS). This peptide was purified further by CM-cellulose column chromatography and G-25 Sephadex gel filtration.

2.2. Expression and purification of recombinant hNMT

Escherichia coli DH5α with recombinant pTrcHisC.hNMT was grown to stationary phase at 37° in Luria broth medium containing 50 mg/L of ampicillin and 1 mM isopropyl-β-D-thiogalactopyranoside for 2 hr. The cells were harvested by centrifugation at 10,000 *g* for 30 min at 4° and suspended in 50 mM Tris–HCl (pH 8.0) containing 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.5 μg/mL of leupeptin, and 0.1% Triton X-100. The suspension was sonicated for 10 sec on ice. The lysate was cleared by centrifugation at 15,000 *g* for 20 min, and the supernatant was used for the purification of the expressed protein. The recombinant hNMT was purified as described elsewhere [25,29].

2.3. Assay of N-myristoyltransferase

N-Myristoyltransferase activity was measured according to procedures described earlier [30,31]. The reaction mixture contained 40 mM Tris–HCl (pH 7.4), 0.5 mM EGTA, 1% Triton X-100, 1 μM [^{3}H]myristoyl-CoA, 500 μM peptide, and hNMT in a total volume of 25 μL. The myristoylation reaction was initiated by the addition of radiolabeled myristoyl-CoA and was incubated at 30° for 30 min. The reaction was terminated by spotting aliquots of the incubation mixture onto P81 phosphocellulose paper disks and drying them under a stream of warm air. The P81 phosphocellulose paper disks were then washed in two changes of 40 mM Tris–HCl (pH 7.3) for 90 min. The radioactivity was quantified in 7.5 mL of Beckman Ready Safe Liquid Scintillation mixture, in a Beckman Liquid Scintillation Counter. One unit of NMT activity was expressed as 1 pmol of myristoyl peptide formed/min.

2.4. Synthesis of [^{3}H]myristoyl-CoA

[^{3}H]Myristoyl-CoA was synthesized enzymatically as described earlier [30], except where modified as mentioned below. The reaction mixture contained 40 mM Tris–HCl (pH 7.4), 10 mM $MgCl_2$, 0.1 mM EGTA, 5 mM ATP, 1 mM LiCoA, 1 μM [^{3}H]myristic acid (7.5 μCi), and 0.3 unit/mL of *Pseudomonas* acyl-CoA synthetase in a total volume of 200 μL. The reaction was carried out for 30 min at 30°. The conversion to [^{3}H]myristoyl-CoA usually was greater than 95%. The enzyme assay was carried out as described above.

2.5. Infrared spectroscopy

The infrared spectra were recorded on a Bio-Rad (FTS-40 Mid IR) Fourier-transform spectrometer either neat or as potassium bromide discs.

2.6. Other methods

The various percentages of liquid and solid compounds used in the NMT assays were dissolved in distilled water (v/v) and (w/v), respectively.

3. Results and discussion

In the present study, hNMT activity was found to be stimulated approximately 10-fold by 30% DMSO using a pp60^{src}-derived peptide substrate (Fig. 2). The kinetic properties of hNMT were also examined in the presence and absence of 30% DMSO, using the pp60^{src}-derived peptide as substrate (data not shown). These results suggest that hNMT activity is stimulated approximately 12-fold in the presence of DMSO. On the other hand, DMSO caused no stimulation of hNMT activity in the presence of a cAMP-dependent protein kinase-derived peptide substrate, suggesting that DMSO did not have an effect on the solubility of myristoyl-CoA (Fig. 2). To determine whether the stimulation of hNMT activity was due to a decrease in product inhibition, a time course of hNMT activity was carried out with and without 30% DMSO (data not shown). The fold stimulation of hNMT activity by 30% DMSO as a

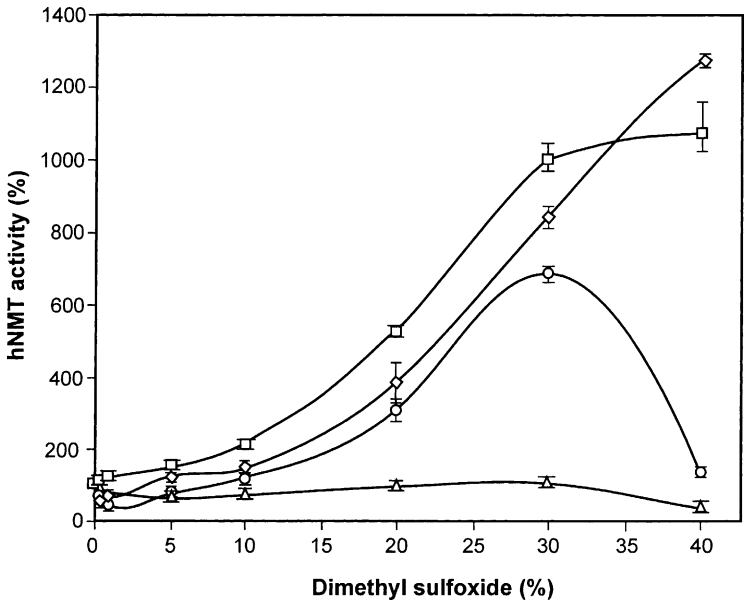

Fig. 2. Effect of DMSO on hNMT. hNMT activity was determined as described in Section 2. Purified hNMT (1.7 μg/assay) was assayed with a pp60^{src}-derived peptide (□); a MARCKS-derived peptide (◇); the M2 gene segment of the reovirus type 3 peptide (○); and a cAMP-dependent protein kinase peptide (△) in the presence of various percentages of DMSO. Control experiments were performed in the absence of DMSO, and the hNMT activity was considered as 100%. The control values, in pmol/mL, observed for the cAMP-dependent protein kinase-derived peptide, the pp60^{src}-derived peptide, the MARCKS-derived peptide, and the M2 gene segment of the reovirus type 3-derived peptide were: 0.271 ± 0.05, 0.121 ± 0.02, 0.164 ± 0.04, and 0.178 ± 0.03, respectively. Each experiment was performed in quadruplicate, and data are the means ± SD of four experiments.

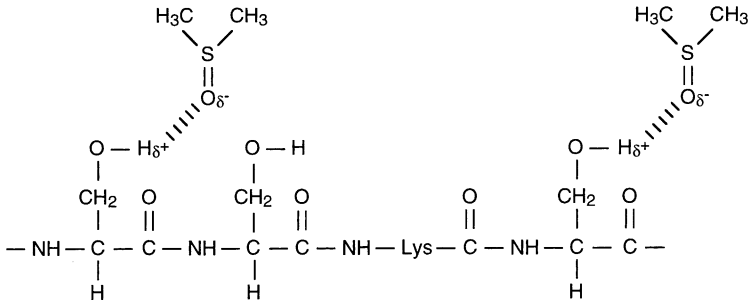

Fig. 3. Possible mechanism for the stimulation of hNMT activity by hydrogen bonding via the interaction of the hydrogen atoms of free hydroxyl groups of peptide substrates with the oxygen atom of DMSO. Peptide, pp60^{src}-derived peptide (Gly-Ser-Ser-Lys-Ser-Lys-Pro-Lys-Arg).

function of incubation time did not change, suggesting that the stimulation of hNMT was not due to the amount of product inhibition. In addition, the effect of DMSO was examined on the other serine-containing substrates. Fig. 2 shows that 30% DMSO also caused a similar stimulation of hNMT activity when a MARCKS-derived peptide and the M2 gene segment of the reovirus type 3 peptide substrates were used. However, hNMT activity was inhibited by a higher percentage of DMSO when the M2 gene segment of the reovirus type 3 peptide substrate was used (Fig. 2). These results suggested further that hNMT has a higher activity toward serine-containing peptide substrates. Therefore, we hypothesized that the stimulation of NMT activity in the presence of serine-containing peptide substrates could be due to hydrogen bonding involving the free hydrogen atoms of the hydroxyl groups of serine moieties with the oxygen atom of DMSO. The data in Fig. 2 imply that there is an increased interaction of DMSO with the substrate as the concentration of DMSO is elevated. Thus, a hydrogen bond could be formed between the oxygen atom of DMSO and the proton of the hydroxy group of a serine residue. A second molecule of DMSO could interact in a similar fashion, as illustrated in Fig. 3. As the concentration of DMSO is increased further, the third hydroxyl group of the pp60^{src} peptide substrate could interact by hydrogen bond formation.

To substantiate further the involvement of hydrogen bonding between DMSO and the pp60^{src}-derived peptide substrate, infrared spectra were recorded for the peptide in the absence and presence of DMSO. The pp60^{src}-derived peptide substrate showed a strong broad band at 3500–3600 cm^{-1} in its infrared spectrum. The broad band was diminished at 3500–3600 cm^{-1} when the pp60^{src}-derived peptide substrate was combined with DMSO (Fig. 4). These results suggest that the free hydroxyl groups of serine moieties are involved in hydrogen bonding. Therefore, hydrogen bonding between the DMSO solvent and the pp60^{src}-derived peptide (as well as other serine-containing peptides) may promote accessibility of these substrates to hNMT by unfolding mechanisms. It is well known that organic solvents influence protein conformations via alterations in electrostatic interactions through changes in solvent dielectric constants (compared to the aqueous milieu) as well as by their effects on the counterion atmosphere and on the binding of solvents or other solutes to the peptides [32]. Formation of the hydrogen-bonded complexes by DMSO involving hydrogen bond donor organic compounds such as phenols and other alcohols through their hydroxyl groups has been well established [33,34].

DMSO has been shown to stimulate or inhibit various enzymes *in vitro* and *in vivo* [35,36]. For example, a 2-fold stimulation of trypsin activity was observed with 20% DMSO [37]. Activities of pancreatic deoxyribonuclease and microbial deoxyribonuclease were also enhanced with a maximum rate occurring at 20% DMSO [38].

Furthermore, DMSO is widely used in pharmaceutical applications, enzyme-catalyzed reactions, veterinary medicine, dermatology, experimental immunology, and microbiology [35,36,39,40]. The observation in this study that stimulation of hNMT activity by DMSO takes place in the presence of serine-containing peptide substrates therefore has significant implications in biological systems in general and in medicine in particular.

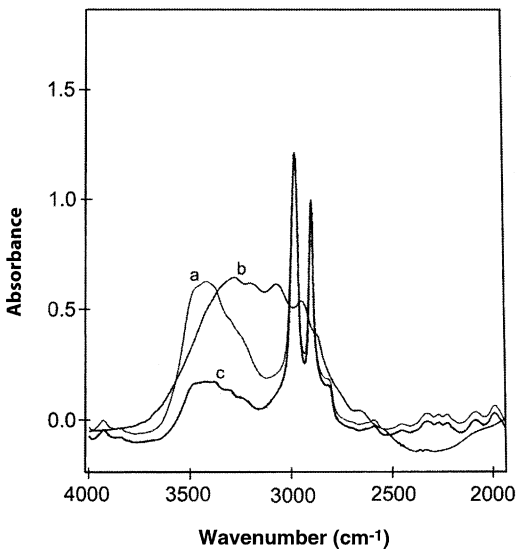

Fig. 4. Infrared spectrum of (a) dry DMSO, (b) pp60^{src}-derived peptide, and (c) a mixture of dry DMSO and the pp60^{src}-derived peptide in a 5:1 molar ratio.

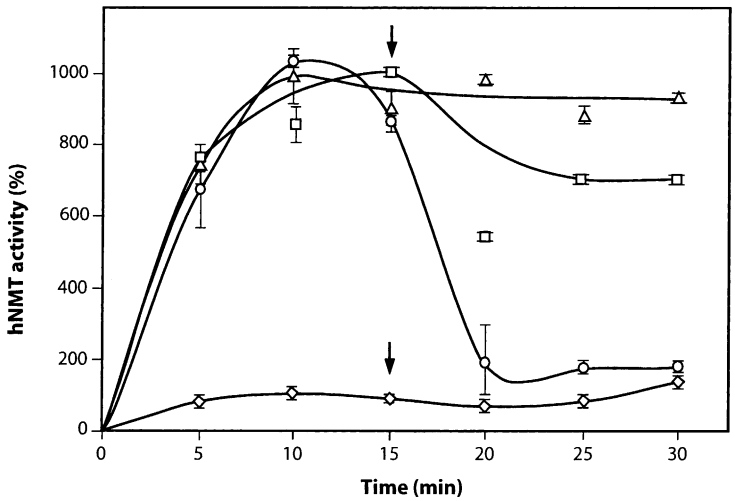

Fig. 5. Reversibility of DMSO stimulation of hNMT. The reaction mixture contained 20.4 μg hNMT in a total volume of 303 μL in the standard assays described in Section 2. The reactions were performed in the presence of 30% DMSO (○, □, △) or without DMSO (◇). Aliquots were taken at various time intervals. After 15 min, the reaction mixture, devoid of DMSO, was added (point of addition indicated by arrow) to one sample containing 30% DMSO to reduce the final DMSO concentration to 10% (○); reaction mixture with 30% DMSO was added to a sample containing 30% DMSO (□); and no reaction mixture was added to a sample containing 30% DMSO (△). All the reaction ingredients were maintained at their initial concentrations, and the progress of the reactions was followed. The control values for 100% activity, in pmol/mL, for pp60^{src}-derived peptide substrate at 5, 10, 15, 20, 25, and 30 min were: 0.088 ± 0.02, 0.127 ± 0.02, 0.112 ± 0.02, 0.117 ± 0.04, 0.09 ± 0.006, and 0.158 ± 0.02, respectively. Each experiment was performed in quadruplicate, and the data are the means ± SD of four experiments.

To test whether the stimulation of hNMT by DMSO, through hydrogen bonding with a serine-containing peptide, can be reversed by lowering the percentage of DMSO, a myristoylation reaction was carried out in the presence of 30% DMSO using the pp60^{src}-derived peptide substrate. After 15 min, the percentage of DMSO was brought from 30 to 10% by the addition of the reaction mixture devoid of DMSO. Fig. 5 shows that the stimulation of hNMT could be reversed completely by lowering the DMSO content in the reaction mixture. However, a slight decrease in the stimulation was observed when the reaction mixture with 30% DMSO was added after 15 min to the reaction initially containing 30% DMSO (Fig. 5). This slight decrease in the stimulation may be due either to the change of reaction temperature or to dilution. These results further suggest that the stimulation of hNMT activity by the pp60^{src}-derived peptide substrate is through hydrogen-bond formation.

We hypothesized that if there were an additional oxygen atom bound to the sulfur atom of DMSO, then the NMT activity should increase through increased hydrogen bonding. We therefore examined the effect of DMSN (Table 1) on hNMT activity. DMSN (20%) increased the hNMT activity by approximately 2.5-fold in the presence of the pp60^{src} peptide substrate, whereas no effect was observed in the presence of the cAMP-dependent protein kinase substrate (Table 1). Surprisingly, this DMSN-mediated increase in hNMT activity was less than that induced by DMSO (Fig. 2). The decreased stimulation of hNMT activity by DMSN could be due to a steric hindrance, caused by the second oxygen atom of DMSN, on hydrogen

Table 1
Effect of various analogues of DMSO on hNMT activity

Compound (20%)	Stimulation (-fold)			
	cAMP-dependent protein kinase peptide	pp60^{src} peptide	MARCKS peptide	M2 gene segment of reovirus type 3 peptide
None	1.0	1.0	1.0	1.0
DMSN	1.0 ± 0.2	2.5 ± 0.1	3.5 ± 0.7	3.0 ± 0.8
DMF	3.5 ± 0.1	8.5 ± 2.3	8.5 ± 1.9	5.5 ± 0.06
Acetone	2.5 ± 0.9	20 ± 0.16	NC[a]	NC
DMA	2.5 ± 0.06	6.5 ± 0.79	NC	NC

NMT activity was assayed as described in Section 2. Since 40 mM Tris–HCl buffer (pH 7.4) was used in the standard hNMT assay, the pH of the reaction mixture did not change with the addition of DMSO and its analogues. Purified hNMT (1.7 μg/assay) was incubated with 20% analogues of DMSO in a final volume of 25 μL. Control experiments were performed in the absence of DMSO analogues, and the hNMT activity was considered as 100%. The control values in pmol/mL observed: for cAMP-dependent protein kinase-derived peptide, 0.271 ± 0.05; for pp60^{src}-derived peptide, 0.121 ± 0.02; for MARCKS-derived peptide, 0.164 ± 0.04; and for M2 gene segment of reovirus type 3-derived peptide, 0.178 ± 0.03. Each experiment was performed in quadruplicate, and data are the means ± SD of four experiments.

[a] NC, not carried out.

bond formation. Additionally, it has been reported that DMSN forms weaker hydrogen bonds with alcohols compared to DMSO [41]. The decreased stimulation of hNMT activity by DMSN could, therefore, also be due to the formation of weak hydrogen bonds caused by the presence of DMSN. To test further the hypothesis that the oxygen atoms attached to the sulfur in DMSO and DMSN are involved in the stimulation of hNMT activity through hydrogen bonding, we examined the effect of various concentrations of DMS, which lacks oxygen atoms, on hNMT activity. DMS did not cause an increase in hNMT activity (data not shown), suggesting that the oxygen atom is required for the formation of hydrogen bonds and the subsequent alteration of the peptide substrate structure.

To further substantiate our hypothesis that hydrogen bonding is involved in making free hydroxyl groups containing peptides to stimulate NMT, we studied the myristoylation reaction in the presence of other analogues of DMSO (Table 1). Acetone (20%) caused an approximately 20- and 2.5-fold increase in the hNMT activity with the pp60^{src} peptide and the cAMP-dependent protein kinase-derived peptide substrate, respectively (Table 1). We speculate that the 2.5-fold increase in the hNMT activity by 20% acetone in the presence of the cAMP-dependent protein kinase-derived peptide substrate was due to the ability of acetone to form hydrogen bonds with the free hydrogen atoms of the amino group of peptide linkages in the peptide substrate. The 20-fold increase in the hNMT activity for the pp60^{src}-derived peptide substrate may be due to the collective effect of hydrogen bonding of the free hydrogen atoms of amino groups of peptide bonds and the hydroxyl groups of serine and threonine moieties.

DMF also has an oxygen atom attached to a carbon with an adjacent nitrogen atom. Therefore, it was anticipated that the delocalization of the lone pair of electrons on the nitrogen atom would increase the electron density on the oxygen atom, which could participate in hydrogen bonding. The experimental results showed that 20% DMF caused an increase in the hNMT activity by 3.5-, 8.5-, 8.5-, and 5.5-fold, respectively, when the cAMP-dependent protein kinase, pp60^{src}, MARCKS, and M2 gene segment of reovirus type 3 peptide substrates were used (Table 1). In this case, the hydrogen atoms of the free hydroxyl groups of the serine and threonine moieties may be involved in hydrogen bonding with the lone pair of electrons on the oxygen atom of the amide linkage in DMF.

To find out whether a nitrogen atom can also participate in hydrogen bond formation, we studied the effect of DMA (Table 1) on hNMT activity. The results showed that 20% DMA increased the hNMT activity approximately 6.5-fold for the pp60^{src}-derived peptide substrate (Table 1). This result further substantiates our hypothesis of the involvement of hydrogen bonding between the solvent and substrate to increase the activity of hNMT. There was approximately a 2.5-fold increase in activity in the presence of the cAMP-dependent protein kinase-derived peptide substrate.

The question arises as to why DMSO and not water molecules led to increases in NMT activity. It is possible that the hydrogen atoms of the serine and threonine hydroxyl groups form much stronger bonds with the oxygen atom of DMSO than water. In addition, the interaction of DMSO may permit perturbations of the substrate, which are reflected by increased NMT activity. Alternatively, DMSO may impede changes in the shape of the substrate caused by water molecules. It is conceivable that the changes (or lack thereof) induced in the shape of the substrate by DMSO and its analogues may facilitate the interactions with NMT.

In conclusion, the results obtained from these *in vitro* studies suggest that the hydrogen atoms of the free hydroxyl groups of serine/threonine moieties in peptide substrates may be involved in hydrogen bonding with the oxygen and nitrogen atoms of organic solvent molecules. This interaction of such peptides with organic solvents can mediate the stimulation of NMT activity. It will be important now to assess whether the stimulation of DMSO and its analogues occurs *in vivo*. Therefore, further studies are warranted. However, this study has revealed the important contributions of solvents to bioactivities and has served as a template for further investigations in this area.

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