Effects of L-Histidine and Its Structural Analogues on Human *N*-Myristoyltransferase Activity and Importance of EEVEH Amino Acid Sequence for Enzyme Activity†

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Received April 20, 1998; Revised Manuscript Received July 14, 1998

ABSTRACT: Myristoyl-CoA:protein *N*-myristoyltransferase (NMT) is an essential eukaryotic enzyme that catalyzes the cotranslational transfer of myristate to the NH_2-terminal glycine residue of a number of important proteins of diverse function. Human NMT (hNMT) activity was found to be activated by L-histidine in a concentration-dependent manner. In contrast, two structural analogues of L-histidine, L-histidinol and histamine, inhibited hNMT activity in a noncompetitive manner with half-maximal inhibitions of 18 and 1.5 mM, respectively. The inhibition of hNMT activity by L-histidinol was reversed by a 2-fold molar excess of L-histidine, suggesting that L-histidine and L-histidinol were competing for a common site on NMT. Kinetic data indicated that whereas L-histidine enhanced the V_{max}, both L-histidinol and histamine decreased the V_{max}; none of these compounds altered the K_m. Our studies suggest that L-histidine and its analogues may be interacting with His-293, involved in myristoyl-CoA transfer, rather than His-218, and implicated in the transfer of myristoyl-CoA to the peptide substrates. Site-directed mutagenesis of His-293, Val-291, and Glu-290 resulted in proteins with no measurable NMT activity. The most conserved region in the catalytic domain EEVEH (289−293) is critical for the myristoyl-CoA transfer in the NMT-catalyzed reactions. This region will be useful for the design of regulators of NMT function.

Myristoyl-CoA:protein *N*-myristoyltransferase (NMT)[1] catalyzes the cotranslational transfer of myristate from myristoyl CoA to the amino-terminal glycine residue of a number of cellular, viral, and oncoproteins (*1*−*4*). In addition, many viruses have been shown to have myristoylated coat proteins, and myristoylation of these proteins has been demonstrated to be important for their replication (*5*). Heteroatom-substituted analogues of the myristoyl group and inhibitors of NMT have been reported to inhibit the replication of a number of viruses, including HIV (*6*). Fungi also myristoylate a number of proteins which are important to the viability of these organisms (*7*). The incorporation of myristoyl group analogues into the fungal proteins was reported to result in cell death (*7*). We were the first to report that animal and human colon cancer tissues had elevated levels of NMT activity compared to their normal tissue counterparts, suggesting a role for NMT in tumor progression (*8*). More recently, we demonstrated overexpression of NMT protein in colorectal adenocarcinomas (*9*). An endogenous inhibitor of NMT purified from bovine brain (*10*) inhibited NMT activity in the rat colonic tumors in vitro (*8*). The availability of specific inhibitors of this enzyme will facilitate further study of its role in the development of cancer.

The fact that viral (*6*), fungal (*7*), and tumor growth (*8*) can be inhibited by perturbation of myristoylation suggest that the enzyme responsible for this protein modification could provide a suitable target for therapeutic agents (*11*, *12*). Synthetic inhibitors of NMT activity have been developed on the basis of analogues of the substrates and products of NMT (acyl-CoA), peptide, and the acyl peptide (*4*, *6*).

Human NMT (hNMT) exhibits a sequential ordered bi−bi reaction mechanism (*13*, *14*). For *Saccharomyces cerevisiae* NMT (Nmt1p), an intermediate of fatty acyl coenzyme A associated with the enzyme can be detected by isoelectric focusing (change in pI from 8.15 for apoenzyme to 6.7 for bound form) or fluorography of SDS−PAGE gels (binding of radiolabled myristoyl-CoA) (*15*). Chymotryptic digestion of the fatty acyl-CoA:Nmt1p complex produces fragments that retain labeled myristic acid but that are hydrolyzable by hydroxylamine (*15*). These results suggest that, in *S. cerevisiae* Nmt1p, a covalent intermediate forms that involves a serine or cysteine residue located between Asn-42 and Thr-220 (hNMT region Asp-41 to Trp-217) (*16*).

† This work was partially supported by the Heart and Stroke Foundation of Saskatchewan, Canada (RKS), and by the National Cancer Institute of Canada, from funds collected by the Canadian Cancer Society (RCW).

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[1] Abbreviations: NMT, *N*-myristoyl-CoA:protein N-myristoyltransferase; hNMT, human NMT; sNMT, spleen NMT; IPTG, isopropyl β-D-thiogalactopyranoside; SDM; site-directed mutagenesis; DEPC, diethylpyrocarbonate.

10.1021/bi980891b CCC: $15.00
Published on Web 10/02/1998

Table 1: Deletion Mutagenesis of HNMT[a]

deletion[b]	primer	expressed protein (between amino acids)	kDa	NMT activity (nmol/min/mg) Histidinol −	NMT activity (nmol/min/mg) Histidinol +
WT	S 5′ CCC AAG CTT ATG AAC TCT TTG CCA A 5′ CCG GAA TTC GTG GTG GGG TTC CAT TTC	1−416	50	10	5.5
N	S 5′ CAG CAG AGA GGA TCC AGG A 5′ CCG GAA TTC GTG GTG GGG TTC CAT TTC	9−416	49	10	5.5
N[b]	S 5′ CGC GGA TCC GTG GTG GTC AGG GAC CTG CCA A A 5′ CCG GAA TTC GTG GTG GGG TTC CAT TTC	21−416	47	33	20
N	S 5 CGC GGA TCC ATA ACC CCC ATG GCC CCG TG A 5′ CCG GAA TTC GTG GTG GGG TTC CAT TTC	52−416	43	7.8	3.2

[a] Deletion mutants were generated and proteins were expressed as described in the Experimental Procedures. S, sense primer; A, antisense primer, WT, wild-type. Transferase assay was carried out with WT (1.0 μg/assay), N^6 (1.2 mg/assay), N^{21} (1.2 μg/assay), and N^{51} (1.0 μg/assay) in the absence and presence of L-histidinol (20 mM) using cAMP-dependent protein kinase derived peptide substrate (1.0 mM). Reaction was initiated by the addition of 50 mM [1-^{14}C]myristoyl-CoA. N-Deletion of 8, 20, and 51 amino acids from the N-terminal end of hNMT. [b] This N-terminal deletion mutant was previously shown to exhibit 3-fold higher activity than WT NMT (*19*).

Comparisons of four yeast, human, and bovine NMT sequences reveal conserved cysteine residues at amino acids 169 and 214 in hNMT and bovine spleen NMT (sNMT) (172 and 217 in Nmt1p) (*17*−*19*). In addition, 4 histidine residues are conserved [His-131, His-171, His-217, and His-293 in hNMT and sNMT; His-134, His-174, His-221, and His-296 in Nmt1p (*17*−*19*)]. It has been shown that fatty acid transfer by hNMT is dependent upon conserved cysteine and histidine residues (*18*).

Histidine residues appear to play a critical role in NMT catalysis (*20*, *21*). Both hNMT and sNMT are inhibited by diethylpyrocarbonate (DEPC); this DEPC-mediated inactivation is blocked by coadministration of histidine. We have also reported previously the activation of sNMT by imidazole buffer (*22*). L-Histidinol, a structural analogue of the essential amino acid L-histidine in which the α-carboxylic group has been reduced to a primary alcohol (*23*, *24*) is a potent, reversible inhibitor of both steps of the reaction catalyzed by histidyl-tRNA synthetase (*23*, *24*) which inhibits protein synthesis at the initiation step (*24*, *25*). Liver perfusion studies by Kimball et al. (*26*) demonstrated that L-histidinol inhibits protein synthesis through its action on a specific phosphatase which, in turn, governs the activity of initiation factors. Myristoylation is a cotranslational process, and it has been shown recently that NMT is associated with the ribosomal subcellular fraction (*27*). In this paper, the response of hNMT to histidine and its imidazole-containing analogues L-histidinol and histamine were investigated. Site-directed mutagenesis and 5′ deletion mutagenesis were also employed not only to map out the essential regions involved in the NMT activity, but also to address the possible mechanism of NMT inhibition by histidine analogues.

EXPERIMENTAL PROCEDURES

Materials. [1-^{14}C]Myristoyl-CoA (54.7 mCi/mmol) was obtained from Amersham International, and [9,10-^{3}H] myristic acid (39.3 Ci/mmol) was purchased from DuPont NEN. pTrcHisC vector was obtained from Invitrogen. Site-directed mutagenesis kit was obtained from Stratagene Inc., La Jolla, CA. Agarose Gel DNA extraction kit was purchased from Boehringer Mannheim, Canada. *Pseudomonas* acyl CoA synthetase, coenzyme A, benzamidine, phenylmethanesulfonyl fluoride, leupeptin, imidazole, L-histidine, D-histidine, L-histidinol, and histamine were obtained from Sigma. General laboratory chemicals were of analytical grade. The following peptide was synthesized from Alberta Peptide Institute, Edmonton, Alberta, Canada: Gly-Asn-Ala-Ala-Ala-Ala-Lys-Lys-Arg-Arg (based on the NH_2 terminal sequence of type II catalytic subunit of cAMP-dependent protein kinase).

Construction of Escherichia coli Expression Vector. The hNMT cDNA (*28*), which contains the entire coding sequence except the first 8 amino acids at the N-terminal end, was isolated from the pBluescript SK (+) vector by digesting the plasmid with *Bam*HI and *Eco*RI and separating the fragment on 0.8% agarose gel. The cDNA insert was purified using Agarose Gel DNA Extraction Kit (Boerhinger Mannheim, Canada). The fragment was ligated into pTrcHisC which had been digested with *Bam*HI and *Eco*RI. The derivative recombinant plasmid was designated as pTrcHisC.hNMT.

Expression and Purification of Recombinant hNMT. E. coli DH5a with the recombinant pTrcHisC.hNMT was grown to stationary phase at 37 °C in LB medium containing 50 mg/L ampicillin and 1 mM final concentration of isopropyl β-D-thiogalactopyranoside (IPTG). The bacteria were harvested by centrifugation at 10000*g* for 20 min. The bacteria were suspended in buffer A (50 mM Tris-HCl, pH 8.0 containing, 2 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, 0.5 μg/mL leupeptin, and 0.1% Triton X-100). The bacterial suspension was sonicated for 10 s on ice. The lysate was cleared by centrifugation at 15000*g* for 20 min, and the pellet was discarded. The protein was purified according to the method described earlier (*29*).

5′ Deletion Mutagenesis. Deletions were generated at the 5′-end of hNMT cDNA using internal primers employing a PCR-based approach. Recognition sequences for *Bam*HI at 5′- and *Eco*RI at 3′-ends were added to the PCR primers. PCR amplification was carried out in 30 μL volume using 2 ng of template DNA, 1 μM each of the primers (Table 1), 200 μM dNTPs, 6.6% formamide, and 1 unit of Pfu DNA polymerase. The conditions were as follows: following denaturation at 96 °C for 2 min and 25 cycles of amplification at 94 °C for 30 s, 55 °C for 40 s, and 72 °C for 2 min, final extension was done at 72 °C for 15 min. The PCR products were purified with a PCR cleanup kit (Wizard, Promega) and further sequenced to check any errors occur-

Table 2: Site-Directed Mutagenesis of EEVEH Region of HNMT[a]

desired mutation	mutant oligonucleotide sequence[b]	NMT activity (%)
wild-type	CAG CAG AGA GGA TCC AGG CCG GAA TTC GTG GTG GGG TTC CAT TTC	100
H293N	CAG GAG GAG GTG GAG AAC TGG TTC TAC CCC GGG GGT AGA ACC AGT TCT CCA CCT CCT CCT	0.0
H293G	CAG GAG GAG GTG GAG GGC TGG TTC TAC CCC GGG GGT AGA ACC AGC CCT CCA CCT CCT CCT	0.0
E292G	GCC AGG AGG AGG TGG GGC ACT GGT TCT ACC GGG TAG AAC CAG TGC CCC ACC TCC TCC TGC	77.6
E292H	GCC AGG AGG AGG TGC ACC ACT GGT TCT ACC C GGG TAG AAC CAG TGG TGC ACC TCC TCC TGG	76.0
V291G	GTG ATG AGC CAG GAG GAG GGG GAG CAC TGG GTA GAA CCA GTG CTC CCC CTC CTC CTG GGT CAT GAC	0.0
E289G	GTC ATG AGC CAG GGG GAG GTG GAG CAC TGG TTC TAC GTA GAA CCA GTG CTC CAC CTC CCC CTG GCT CAT GAC	37.8
E290G	GTA GAA CCA GTG CTC CAC CCC CTC CTG GCT CAT GAC GTC ATG AGC CAG GAG GAG GGG GAG CAC TGG TTC TAC	0.0
E289,290G	GTC ATG AGC CAG GGG GGG GTG GAG CAC TGG TTC TAC GTA GAA CCA GTG CTC CAC CCC CCC CTG GCT CAT GAC	0.0
E289,292G	GTC ATG AGC CAG GGG GAG GTG GGG CAC TGG TTC TAC GTA GAA CCA GTG CCC CAC CTC CCC CTG GCT CAT GAC	23.0
E290,292G	GTC ATG AGC CAG GAG GGG GTG GGG CAC TGG TTC TAC GTA GAA CCA GTG CCC CAC CCC CTC CTG GCT CAT GAC	7.0
E289,290,292G	GTC ATG AGC CAG GGG GGG GTG GGG CAC TGG TTC TAC GTA GAA CCA GTG CCC CAC CCC CCC CTG GCT CAT GAC	0.0

[a] Myristoyltransferase assay was carried out with wild-type and mutant NMTs in the presence of cAMP-dependent protein kinase derived peptide substrate (1.0 mM). The reaction was initiated by the addition of 50 μM [1-^{14}C]myristoyl-CoA and incubated at 30 °C for 30 min as described in the Experimental Procedures. [b] Top and bottom primers, sense and antisense. Data from eight observations.

ring during PCR amplification. The PCR products were then digested with *Bam*HI and *Eco*RI and subjected to agarose gel electrophoresis. The purified fragments were ligated into *Bam*HI/*Eco*RI-digested pTrcHisC vector. After identifying the recombinants, the cultures were grown in LB medium and induced by IPTG (1mM) and the extracts were assayed for NMT activity.

Site-Directed Mutagenesis (SDM). Site-directed mutagenesis was carried out by using quickchange site-directed mutagenesis kit (Stratagene Inc., La Jolla, CA). The reaction mixture contained SDM buffer (200 mM Tris-HCl, pH 8.8, 100 mM KCl, 100 mM $(NH_4)SO_4$, 20 mM $MgSO_4$, 1% Triton X-100, and 1 mg/mL nuclease-free bovine serum albumin), 1 mM deoxynucleotide mix (dATP, dCTP, dTTP, and dGTP), 50 ng of pTrcHisC.hNMT (5.6 kb), 125 ng of sense and antisense primer with mutation(s) (Table 2) in a total volume of 50 μL followed by the addition of 2.5 units of pfu DNA polymerase using PTC100 programmable thermal controller (MJ Research Inc, Watertown, Mass MA). The extension parameters of SDM were as follows: following denaturation at 95 °C for 30 s, 16 cycles at 95 °C for 30 s, 55 °C for 1 min, and at 68 °C for 12 min (2 min/kb of plasmid length). Following temperature cycling, we placed the reaction on ice for 2 min to cool, then added 10 units of DPN I restriction enzyme, mixed, and incubated the reaction at 37 °C for 60 min. Transformation was carried out using 1 μL of the DPN I treated reaction to Epicurian Coli XL1-blue supercompetent cells and the reaction was plated on LB/Amp (100 μg/mL) plates. The cDNAs of all mutants were sequenced from end-to-end after PCR; the only mutations observed were those intentionally introduced to create each desired mutation. The clones were induced with IPTG (1 mM) and the extracts were assayed for NMT activity.

Assay of N-Myristoyltransferase. Myristoyltransferase activity was measured as described earlier (*30*, *31*). For the standard enzyme assay, the reaction mixture contained either 50 μM [1-^{14}C] myristoyl-CoA or 1 μM [^{3}H]myristoyl-CoA, 50 mM Tris-HCl, pH 7.4, 0.5 mM EGTA, 1% Triton X-100, cAMP-dependent protein kinase derived peptide, and NMT in a total volume of 25 μL. The transferase reaction was initiated by the addition of radiolabeled myristoyl-CoA and was incubated at 30 °C for 10−30 min. The reaction was terminated by spotting aliquots of incubation mixture onto P81 phosphocellulose paper disks and drying them under a stream of warm air. The P81 phosphocellulose paper disks were washed in three 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 nmol of myristoyl peptide formed/min.

Effect of L*-Histidine,* L*-Histidinol, and Histamine on hNMT Activity.* The NMT activity was carried in the absence and presence of either L-histidine (0−40 mM), D-histidine (0−40 mM), L-histidinol (0−34 mM), or histamine (0−4 mM) using cAMP-dependent protein kinase derived peptide (1.0 mM) as substrate. Kinetic analysis was also carried in the absence and presence of histidine and their structural analogues.

Other Methods. Protein concentration was determined by the method of Bradford (*32*) using bovine serum albumin as a standard.

RESULTS

NMT activity was found to be activated by L-histidine in a concentration-dependent manner (Figure 1). That similar results were obtained with D-histidine (data not shown) suggested that the NMT activation by either D- or L-histidine is due to the imidazole moiety. Indeed, we have demonstrated the activation of sNMT by imidazole buffer (*22*).

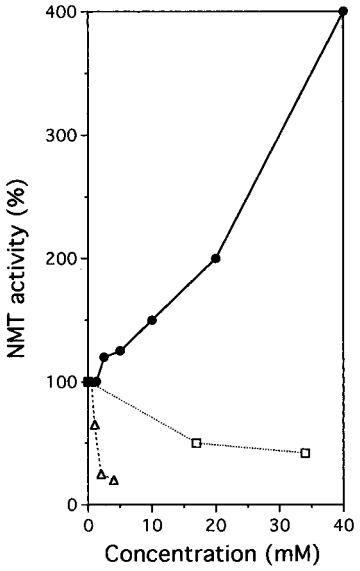

FIGURE 1: Effect of L-histidine, L-histidinol, and histamine on hNMT. Human NMT (1.7 μg/assay) was incubated with either L-histidine [0−40 mM (●)] or L-histidinol [0−34 mM (□)] or histamine [0−4 mM (△)] in the presence of cAMP-dependent protein kinase derived substrate (1.0 mM) at 30 °C for 10 min. The reaction was initiated with 50 μM [1-^{14}C]myristoyl-CoA as described in the Experimental Procedures. Results are expressed as percent of control (NMT activity in the absence of additives). Assays were done in duplicate.

Table 3: Kinetic Properties of NMT in the Presence of L-Histidine and Their Structural Analogs[a]

additives	K_m (μM)	V_{max} (nmol/min/mg)
Nil	200	36.0
L-histidine	195	60.0
L-histidinol[b]	192	18.0
Histamine[b]	205	18.0

[a] Human NMT (1.73 μg/assay) was incubated with cAMP-dependent protein kinase derived peptide substrate (0−1.0 mM) in the absence and presence of either L-histidine (20 mM) or L-histidinol (20 mM) or histamine (1 mM). The reactions were initiated by the addition of 1.0 μM [^{3}H]myristoyl-CoA and incubated at 30 °C for 10 min as described in the Experimental Procedures. [b] Close to half-maximal inhibition of L-histidinol (20 mM) and histamine (1 mM) were used for kinetic analysis.

However, both D- and L-histidine are much more effective activators of NMT than imidazole, suggesting that both the imidazole and either the α-amino or α-carboxyl moieties were needed for activation. That histidine-*O*-methyl ester inhibited NMT activity (data not shown) implicated the α-carboxyl group of histidine. That several other amino acids (L-tryptophan, L-isoleucine, and glycine) failed to influence NMT activity (data not shown) suggested that "full" NMT activation depended upon both an imidazole moiety and a nearby carboxyl-residue. This notion was supported by the finding that NMT activity was also inhibited, in a concentration-dependent manner, by L-histidinol and histamine with a half-maximal inhibition of 18 and 1.5 mM, respectively (Figure 1). Kinetic analysis was carried out in the absence and presence of L-histidine, L-histidinol and histamine using peptide derived from the N-terminal sequence of cAMP-dependent protein kinase (Table 3). These results indicate that L-histidinol and histamine appear to be noncompetitive inhibitors, because inhibition of the NMT by these compounds does not change

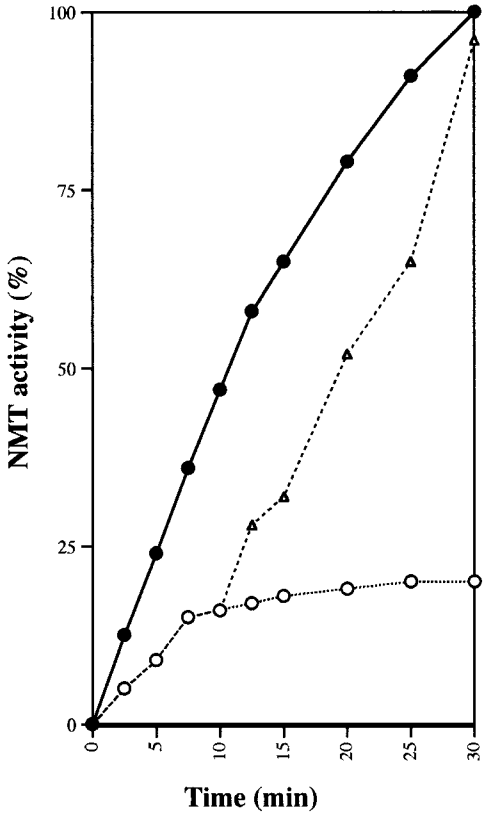

FIGURE 2: Reversal of the L-histidinol inhibition of NMT by L-histidine. Human NMT (1.7 μg/assay) was incubated in the presence of either 40 mM L-histidine (●) 34 mM L-histidinol (○) using cAMP-dependent protein kinase derived peptide substrate (1.0 mM) at 30 °C for 30 min. The reaction was initiated with 50 μM [1-^{14}C]myristoyl-CoA as described in the Experimental Procedures. After 10 min L-histidine was added to one tube to a final concentration of 80 mM (△) and the progress of the reaction was followed. Results were expressed as percent of activation (NMT activity in the presence of L-histidine). Assays were done in duplicate.

the Michaelis constant, whereas V_{max} is changed. The inhibition of NMT by L-histidinol can be reversed by excess concentration of L-histidine (Figure 2), suggesting that NMT is not covalently modified by the histidine analogues. Similar results were also obtained with histamine (data not shown). The results further suggest that L-histidine, L-histidinol, and histamine could be competing for a common site on NMT.

To further characterize the inhibitory properties of histidinol on NMT activity, 5′ deletions were generated, as described in Table 1. Previous studies on NMT indicated that deletion of 51 amino acids from the amino-terminal end had no effect on the enzyme activity, whereas C-terminal deletions resulted in the complete loss of NMT activity (*19*). L-Histidinol was also found to inhibit the NMT activity of N-terminal deletion mutants similar to wild-type NMT, suggesting that the N-terminal end (51 aa) is not essential for the inhibition.

As reported by others (*20*, *33*), the reagent DEPC totally inactivated NMT (Table 4). The presence of 20 mM histidine or imidazole during DEPC treatment preserved 83 and 50% of the enzyme activity, respectively, but had no protective capacity if added at the end of treatment period (Table 4). In contrast, DEPC-treatment performed in the presence of either L-histidinol or histamine (20 mM) gave

Table 4: Effect of L-Histidine and Its Structural Analogs on NMT Activity in the Presence of Diethylpyrocarbonate (DEPC)[a]

treatment	concn (mM)	NMT activity (%)
none		100
L-histidine	20	204
imidazole	20	112
L-histidinol	20	25
histamine	20	10
DEPC	10	1.5
during DEPC treatment		
L-histidine	20	83
imidazole	20	50
L-histidinol	20	8.2
histamine	20	11
histidine and its structural analogs after DEPC treatment	20	1.5

[a] Human NMT (4.0 μg/assay) was incubated with various treatments in the presence of cAMP-dependent protein kinase derived peptide substrate (1.0 mM) for 10 min at 30 °C as described in the Experimental Procedures. Reaction was initiated by the addition of 50 μM [1-^{14}C]myristoyl-CoA. The results are expressed as percent of control (NMT activity in the absence of treatment). All assays were done in duplicate.

almost no protection from DEPC (Table 4). These results suggest that functional groups in association with the imidazole of histidine and/or its derivatives may participate by either protection or inhibition of NMT activity, alone or in combination of DEPC.

That substantial levels of L-histidine and structural analogues (20−40 mM) were employed suggested that their effects could be mediated by alterations in pH, perhaps by influencing the capture/retention of the acyl-peptide on the P81 phosphocellulose papers. Indeed, 50 mM Tris-HCl at pH 7.4 is at the margin of its buffering capacity, and at 30 °C, this capacity will be further compromised (pH will be lower). However, incubation of 50 mM Tris-HCl, pH 7.4, at 30 °C in the presence and absence of L-histidine or its structural analogues did not shift the pH, as determined by the CIBA Corning 634 Ca^{2+}/pH analyzer (data not shown). These findings argue that neither L-histidine nor its structural analogues were exhibiting their effects on pH of the assay medium.

There are several possible mechanisms to explain NMT inhibition by L-histidinol: (1) thioesterase activity, (2) proteolytic degradation, (3) demyristoylation of the myristoylated-peptide, or (4) direct interaction of L-histidinol with NMT. To test whether the L-histidinol possesses thioesterase activity, [^{14}C]myristoyl-CoA was incubated in the presence and absence of L-histidinol. The mixture was extracted four times with 2 vol of heptane, and radioactivity in the aqueous and pooled organic phases was determined: 98% of the radioactivity remained in the aqueous phase (Table 5). Control experiments with [^{3}H]myristic acid resulted in 98% of the radioactivity being extracted into the organic heptane phase. These results demonstrate that the L-histidinol does not possess any "endogenous" thioesterase activity.

To investigate if L-histidinol possesses any protease activity, two approaches were used. In the first of these, a 10 min preincubation of L-histidinol with either peptide substrate or NMT resulted in no increase in inhibitory activity (Table 6). Second, L-histidinol added 10 min after the initiation of the transferase reaction gave the same degree of inhibition as was observed for L-histidinol added before

Table 5: Effect of L-Histidinol on Myristoyl-CoA Concentration[a]

addition	L-histidinol	proportion of radioactivity in phase: aqueous	proportion of radioactivity in phase: organic
myristoyl-CoA	−	0.98	0.02
	+	0.985	0.015
myristic acid	−	0.02	0.98
	+	0.02	0.98

[a] The [^{14}C] myristoyl-CoA (50 μM) was incubated in the presence and absence of L-histidinol (40 mM) for 30 min at 30 °C and then extracted four times with 2 vol of heptane and the radioactivity in the aqueous and organic phases was determined by liquid scintillation counting according to the method of Hosaka et al. (*53*). Control experiments were performed with [^{3}H]myristic acid. The extractions were 98% efficient. Results are depicted as the proportion of radioactivity (cpm) recovered in each phase.

Table 6: Effect of Preincubation of L-Histidinol on Peptide and NMT

treatment	NMT activity (%)	
control NMT	100	
	preincubation with L-histidinol	
treatment	−	+
peptide (cAMP-dependent protein kinase)	29.3	29
NMT	32.6	32

[a] The cAMP-dependent protein kinase derived peptide (1.0 mM), and NMT (8 μg) were incubated with 40 mM L-histidinol for 10 min at 30 °C. Respective controls were added with 40 mM L-histidinol before the start of NMT assay. The reaction was initiated by the addition of 50 μM [1-^{14}C]myristoyl-CoA and carried out the NMT assay as described in the Experimental Procedures. The results are expressed as percent of control (NMT activity in the absence of L-histidinol). All assays were done in duplicate.

the initiation of the transferase reaction (Figure 3). This experiment also argues against the L-histidinol exerting its effect by a demyristoylation of the radiolabeled myristoyl-peptide. If the L-histidinol possessed any demyristoylase activity, a time-dependent loss of radioactivity recovered in the assay would be observed in Figure 3. We conclude therefore that the L-histidinol may act directly on NMT.

NMT has a common binding site for both peptide and fatty acyl-CoA substrates (*15*, *33*). The acyl-peptide generated in the enzyme assay is a competitive inhibitor for fatty acyl-CoA and a noncompetitive inhibitor for peptide (*33*), suggesting that the acyl-peptide can bind to the enzyme at its fatty acyl-CoA binding site but not at its peptide-binding site (*15*, *33*). Previously, it has been shown that mutation of His-293 interfered with the formation of acyl-enzyme intermediate while mutation of His-218 attenuated the generation of the myristoylated product (*18*). We wished to determine whether the inhibition of NMT activity by L-histidinol and histamine are interfering at His-293 or His-218.

For the purpose of assay convenience, we intentionally modified cAMP-dependent protein kinase peptide at its C-terminal end by introducing an Arg^{+} (positive charge) to facilitate binding to the negatively charged P81 phosphocellulose paper (*30*, *31*). NMT can also bind to P81 phosphocellulose paper through its lysine and arginine residues. If L-histidinol and histamine are interfering with His-218, i.e., at the level of peptide transfer, one would

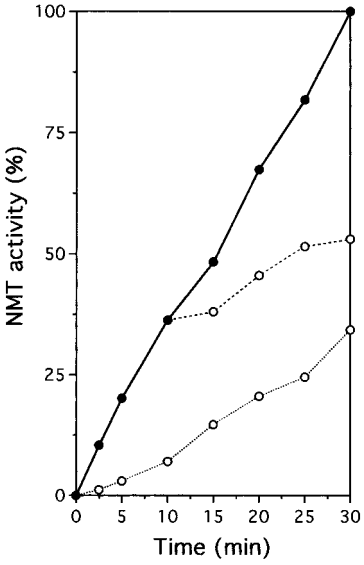

FIGURE 3: Time-dependent inhibition of NMT by L-histidinol. Human NMT (8 μg/assay) was incubated in the presence of cAMP-dependent protein kinase derived peptide substrate (1.0 mM) in a total volume of 150 μL. The reaction was initiated by the addition of 50 μM [1-^{14}C]myristoyl-CoA. At time 0 or 10 min (○) L-histidinol (40 mM) was added to the incubations; control incubations were performed by the addition of buffer (●). Samples (15 μL) of the reaction mixture were assayed at the indicated times as described in the Experimental Procedures.

expect retention of radiolabel on NMT as an acyl-enzyme intermediate. Such intermediates have been reported previously in human and yeast NMTs (*15*, *18*). Thus, the assay method cannot distinguish between radiolabel retained by the NMT (as an acyl-enzyme intermediate) or that bound to the peptide product. It is known, however, that thioester and reactive oxyester bonds are susceptible to hydroxylamine-mediated hydrolysis at pH 7.0 (*15*). The latter finding offers an opportunity to distinguish between isotope retained as an acyl-enzyme intermediate or as the stable, peptide-linked end product. Further, in all assays, the enzyme concentrations were in the micromolar range vs nanomolar concentrations of the acyl-peptide formed. L-Histidine at 20 mM gives 2-fold stimulation and 40 mM gives 4-fold stimulation (Figure 1). To determine whether this activity is due to the formation of acyl-enzyme intermediate (myristate trapped) or the acyl-peptide product, hydroxylamine treatments were performed. Thus, NMT assays were carried out in the presence of histidine and its structural analogues. After 10 min, aliquots were spotted on P81 phosphocellulose paper and the paper disks were washed for 2 h with either 1.0 M Tris-HCl, pH 7.5, or 1.0 M hydroxylamine, pH 7.0. The results indicated that the NMT activity in control is similar to the hydroxylamine treatment (Table 7), suggesting that L-histidinol analogues are interfering with the His-293 in the acyl-enzyme intermediate formation. If these compounds were acting at His-218, allowing the formation of an acyl-enzyme intermediate but not allowing the subsequent transfer to the peptide substrate, one would expect loss of radioactivity with hydroxylamine.

Four histidine residues are highly conserved in human, yeast, bovine, nematode, and drosophila NMTs (*17*−*19*, *34*−*37*). Our studies, along with the existing knowledge, implicate the crucial role of histidine residues in the enzyme activity (*18*, *20*, *21*, *33*). Carboxylic groups from spatially

Table 7: Effect of L-Histidine and Its Structural Analogs on NMT Activity in the Presence of Hydroxylamine[a]

treatment	1.0 M Tris-HCl, pH 7.5	1.0 M hydroxylamine, pH 7.0
none	100	100
L-histidine	203	200
imidazole	102	95
L-histidinol	25	26
histamine	10	11

[a] Human NMT (4.0 μg/assay) was incubated with various treatments with L-histidine and its structural analogues (20 mM) in the presence of cAMP-dependent protein kinase derived peptide substrate (1.0 mM). The reaction was initiated by the addition of 50 μM [1-^{14}C]myristoyl-CoA for 10 min at 30 °C. After 10, min reaction was spotted on to P81 phosphocellulose paper disks, and subjected to washing either in the presence of 1.0 M Tris-HCl, pH 7.5, or 1.0 M hydroxylamine, pH 7.0, for 2 h. The P81 phosphocellulose paper disks were again washed in three changes of 40 mM Tris-HCl, pH 7.3 for 90 min as described in the Experimental Procedures. The results are expressed as percent of control (NMT activity in the absence of treatment). All assays were done in duplicate.

proximate aspartic and glutamic acid residues may also cooperate with the histidines to optimize acid−base properties (*18*, *38*). In hNMT, proximate amino acids near His-131 (LPQWH^{131}CGVR), His-171 (FLCVH^{171}KKLR), His-218 (CRYWH^{218}RSLN), and His-293 (EEVEH^{293}WFYP) suggested that His-293 may require involvement with acidic (glutamic acid) residues in the enzyme catalysis.

To determine the functional significance of the acidic residues adjacent to His-293 (EEVEH) in enzyme catalysis, site-directed mutagenesis was carried out. Table 2 shows the impact of substitution of residues preceded by His-293 on NMT activity. The results indicated that substitution of His-293 with either Asn or Gly resulted in the loss of NMT activity. In the absence of His-293, exogenous histidine cannot facilitate the transfer of myristoyl-CoA (data not shown). Substitution of Val-291 with Gly also resulted in the loss of NMT activity. Substitution of Glu-292 with either Gly or His resulted in 20% decrease in NMT activity, whereas substitution of Glu-290 with Gly resulted in complete loss of NMT activity. Unless the other two Glu residues (289, 292) have an effect on NMT activity, the complete loss of activity with the Glu 290 mutant is expected. Substitution of combinations of Glu-289 and -290, Glu-289 and -290, and Glu-290 and -292 with Gly resulted in the loss of 100, 77, and 93%, respectively. Substitution of three Glu (289, 290, and 292) with Gly resulted in the complete abolishment of NMT activity, suggesting that Glu-290 is essential for enzyme catalysis.

DISCUSSION

Human NMT activity was found to be activated by L-histidine in a concentration dependent manner. Two structural analogues of L-histidine, L-histidinol and histamine, were found to inhibit hNMT activity in a noncompetitive manner, with half-maximal inhibitions of 18 and 1.5 mM, respectively. The inhibition of hNMT activity by the analogues was reversed by increasing the concentration of L-histidine, suggesting that histidine, histidinol, and histamine were competing for a common site on NMT. Kinetic data indicated that, whereas L-histidine enhanced the V_{max}, both

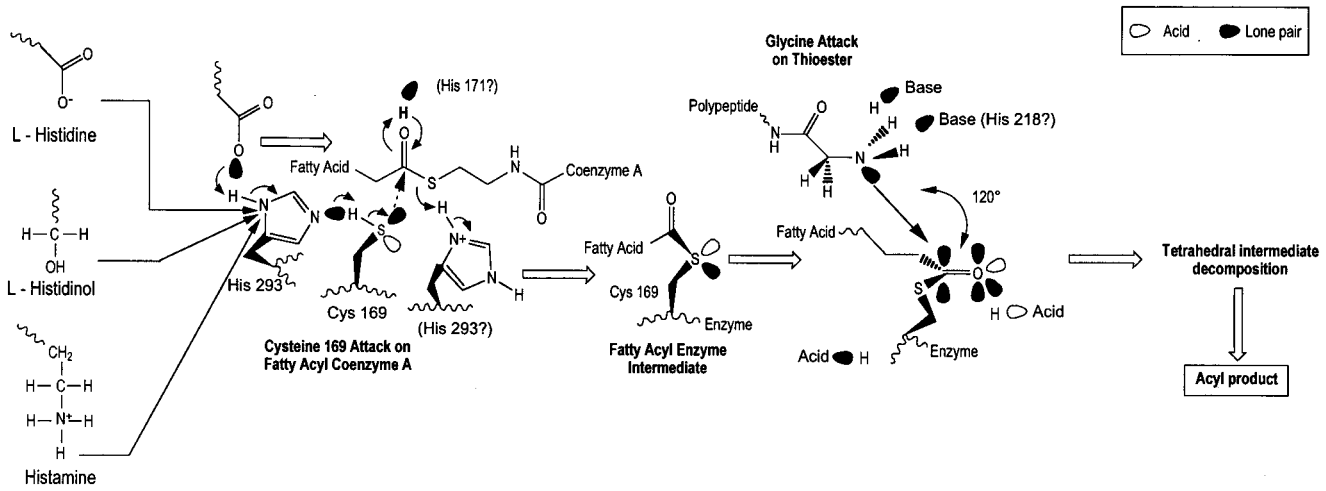

FIGURE 4: Possible mechanism of inhibition of NMT activity. The model was adapted from Peseckis and Resh (*18*).

L-histidinol and histamine decreased the V_{max}; none of these compounds altered the K_m. Results of our study indicated that L-histidine and their analogues may be competing for the His-293 in the myristoyl-CoA transfer rather than His-218 in the transfer of myristoyl-CoA to the peptide substrates. A working model for the active site of hNMT has been suggested (*18*). In this model, Cys-169 serves as the fatty acid attachment site for the covalent myristoyl enzyme intermediate (*18*). It was also suggested that His-171 acted as a general acid−base and His-293 functioned as a specific acid−base during the cysteine thiol nucleophilic on the carbonyl of the myristoyl-CoA (ref *18*; Figure 4). A tetrahedral intermediate of myristate, CoA, and Cys-169 would then decompose to acylated cysteine and CoA (ref *18*; Figure 4). The possibility that His-218 could then act as an acid or base to catalyze the transfer of the acyl-group from the acyl-enzyme intermediate to a polypeptide substrate was also suggested (*18*).

Our results indicate that L-histidine increased the NMT activity, whereas L-histidinol and histamine inhibited NMT. One interpretation of our data is that the carboxylic group of L-histidine might donate its lone pair of electrons (carbonyl oxygen) to the nitrogen group of the imidazole of His-293 to enhance the thiol nucleophilicity for the formation of acylated cysteine. In the case of L-histidinol, the primary alcohol (occupying the same position as the carboxyl-group in histidine) is very weak acid; not only weaker than −COOH, but even more than H_2O. This situation would diminish thiol nucleophilicity. Similar reasoning is applicable to the effects of histamine. This agent lacks an α-carboxyl-residue; its basic amino group would likely more strongly diminish thiol nucleophilicity than L-histidinol, again via effects mediated through the imidazole group of nitrogen (His-293), resulting in even more effective inhibition of myristoyl-CoA transfer. Indeed, our results indicated that histamine inhibits NMT at a 10-fold lower concentration (IC_{50}, 1.5 mM) than L-histidinol (IC_{50}, 18 mM). Histidine-*O*-methyl ester was also found to inhibit NMT activity (data not shown). Collectively, these observations suggest not only the importance of both an imidazole and an α-carboxyl group for the activation by L- and D-histidine, but also that varying the functional group linked to α-carbon adjacent to the imidazole moiety in the histidine analogues has a profound effect on the NMT reaction mechanism. Neither L-histidinol nor histamine were able to protect NMT from the DEPC-mediated inactivation of histidine residues (Table 4), even though L-histidine offered substantial protection, again implicating a critical role for the α-carboxyl group. We propose that the divergent effects of histidine and its structural analogues on NMT activity reported herein reflect the nature of functional groups linked to the α-carbon group of imidazole derivatives, which in turn, alter the electronic behavior of the His-293 moiety in the NMT enzymatic reaction.

Table 8: Conserved EEVEH Region in Various Orthologous NMTs[a]

species	289	290	291	292	293
H−NMT-1	E	E	V	E	H
H−NMT-2	E	E	V	A	H
B−SPL	E	E	V	E	H
B−CAR	E	E	V	E	H
C. elegans	E	E	L	A	H
C. albicans	E	E	F	K	H
S. cerevisiae	E	E	F	E	H
C. neoformans	D	E	V	E	H
H. capsulatum	K	E	V	D	H
Drosophila	E	E	F	R	H

[a] The peptide sequence (EEVEH) of human (hNMT-1), *C. elegans*, *C. albicans*, *S. cerevisiae*, *C. neoformans*, and *H. capsulatum* NMTs were taken from Zhang et al. (*34*). Bovine spleen and cardiac muscle NMT peptide sequence were taken from Raju et al. (*19*, *36*). Drosophila NMT peptide sequence was taken from Ntwasa et al. (*35*). Human NMT-2 peptide sequence was taken from Giang and Cravatt (*37*).

Site-directed mutagenesis to His-293, Val-291, and Glu-290 resulted in the protein with no measurable NMT activity. The crystal structure of anti-fungal target *Candida albicans* NMT has a long, curved, uncharged groove and a deep pocket at the center (*39*). The pocket floor has been reported to be negatively charged due to the vicinity of the C-terminal carboxylate and nearby conserved glutamic acid residues, which separates the pocket from the cavity (*39*). Multiple alignment of −EEVEH− region in various orthologous NMTs indicated that E-290 and His-293 are highly conserved (Table 8). Glutamate-289 is also well conserved in human, bovine (spleen and cardiac muscle), *Caenorhabditis elegans*, *Drosophila*, and yeast (*Candida albicans and S. cerevisiae*). Valine-291 is highly conserved in mammalian NMTs (*18*, *19*, *34*, *36*, *37*). The functional implication of these residues

suggest that this region could be a target for development of potential regulators of NMT function.

Previously, it has been shown that imidazole-substituted dipeptide amides are selective inhibitors of *Cadiada albicans* NMT and that these agents have antifungal activity (*40*). L-Histidinol has been shown to modulate cancer drug toxicity (*41*), both in vitro (optimum concentration 1 mM in conventional tissue culture media) and in DBA/2J mice bearing either L1210 leukemia (*42*, *43*), P815 mastocytoma (*44*), or P388 leukemia (*45*), as well as in C57/BL mice bearing disseminated B16f10 melanoma (*46*). L-Histidinol has also been shown to reverse several types of drug resistance (*41*), including a unique form of resistance observed in MDCK-T1, a tumorigenic epithelial cell line (*47*). Although the capacity of L-histidinol to enhance cancer drug toxicity in tumor cells and to circumvent drug resistance traits has been interpreted as dependent upon its protein synthesis inhibitory attribute (*48*), it was also demonstrated that the action of L-histidinol and its mimetics cannot be attributed solely to this property (*48*, *49*). L-Histidinol possesses antihistamine activity (*50*) and can be considered a substituted imidazole, in which case it could alter eicosanoid metabolism (*51*) or inhibit cytochrome P450s (*52*). Thus, it is unlikely that L-histidinol acts exclusively as a protein synthesis inhibitor; it may evoke a number of responses from cells, any, or all of which might be implicated in its capacity to modulate anticancer drug action (*41*). The data presented here suggest that various imidazoles may elicit effects upon myristoylation as well. It is also of interest that the drug-modulating action of L-histidinol on tumor cells is eliminated by excess L-histidine, both in tissue culture (*48*) and in tumor-bearing animals (unpublished observations, Warrington). Although the concentrations needed to elicit these effects of L-histidinol and L-histidine in vivo were considerably lower (10−100-fold) than are required in this study, it is conceivable that the myristoyltransferase enzyme, in its native state, is substantially more sensitive to "imidazole-mediated" alteration than it is in its purified state. Myristoylation has been identified as a potential target for the development of chemotheurapeutic agents (*11*). The results presented here suggest that histidine analogues and other imidazole derivatives may be useful as potential inhibitors of protein myristoylation. Studies to investigate this possibility and its relevance, if any, to the capacity of L-histidinol and its mimetics to modulate anticancer drug toxicity are underway.

ACKNOWLEDGMENT

R.V.S R. is a recipient of a Research Fellowship from the Health Services Utilization and Research Commission of Saskatchewan.

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BI980891B