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Mechanisms of action of NIP_{71} on N-myristoyltransferase activity

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Abstract

N-Myristoyl-CoA:protein N-myristoyltransferase (NMT) is the enzyme that catalyses the transfer of myristate from myristoyl-CoA to the N-terminal glycine of protein substrates. NMT was highly purified from bovine brain by procedures involving sequential column chromatography on DEAE-Sepharose CL-6B, phosphocellulose, hydroxylapatite, and mono S and mono Q f.p.l.c.. The highly purified NMT (termed NMT·II) possessed high specific activity with peptide substrates derived from the N-terminal sequences of the cAMP-dependent protein kinase and $pp60^{src}$ (29,800 and 47,600 pmol N-myristoylpeptide formed/min/mg, respectively), intermediate activity with a peptide based on the N-terminal sequence of a viral structural protein (μl) (M2; 17,300 pmol N-myristoylpeptide formed/min/mg) and very low activity with a peptide derived from the N-terminal sequence of myristoylated alanine-rich C-kinase substrate (MARCKS; 1500 pmol myristoylpeptide formed/min/mg). An NMT protein inhibitor (NIP_{71}) isolated from the particulate fraction of bovine brain (King MJ and Sharma RK: Biochem J 291: 635–639, 1993) potently inhibited highly purified NMT activity (IC_{50} 23.7 nM). A minor NMT activity (NMT·PU; 30% total NMT activity), which failed to bind to phosphocellulose, was insensitive to NIP_{71} inhibition. Inhibition of NMT was observed to be via mixed inhibition with respect to both the myristoyl-CoA and peptide substrates with NIP_{71} having an apparent higher affinity for NMT than the NMT·myristoyl·CoA complex. Inhibition by NIP_{71} at subsaturating concentrations of myristoyl-CoA and peptide resulted in a sigmoidal pattern of inhibition indicating that bovine brain possesses a potent and delicate on/off switch to control NMT activity. (Mol Cell Biochem **141:** 79–86, 1994)

Key words: N-myristoyltransferase, NIP_{71}, mixed inhibition, lipid

Abbreviations: NMT – N-myristoyl-CoA:protein N-myristoyltransferase; NMT·I – mono Q N-myristoyl-CoA:protein N-myristoyltransferase peak I; NMT·II, mono Q N-myristoyl-CoA:protein N-myristoyltransferase peak II; NMT·III, mono Q N-myristoyl-CoA:protein N-myristoyltransferase peak III; NIP_{71} – 71 kDa heat-stable N-myristoyltransferase inhibitor protein

N-myristoyltransferase (NMT; EC 2.3.1.97) catalyses the N-terminal myristoylation of a selection of cellular, viral and onco-proteins. Many of the known myristoylated proteins appear to be important in cellular regulation, signal transduction and virion assembly [1–5]. NMT appears to be ubiquitous in eukaryotes, having been observed in yeast [6] plants [7, 8] invertebrate [8] and vertebrate tissues [9–13]. While it is clear that myristoylation and consequently NMT is important in the functioning of the cell, since a lack of NMT is lethal to eukaryotic cells [14], the physiological function and regulation of NMT is at present poorly understood. The enzyme exhibits remarkable substrate specificity, having an absolute requirement for an N-terminal glycine residue as well as being unable to efficiently utilise any fatty acid other than myristate. The characterisation of NMT activity *in vivo* has indicated that myristoylation is a cotranslational event since protein inhibitors such as cycloheximide inhibited myristoylation as well as protein synthesis [15]. As such it has been thought of as a once only event, no cycles of acylation/deacylation/reacylation being observed, with the myristoyl-glycine bond having a half-life identical to that of the polypeptide chain. However, it is clear that simply possess-

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ing an N-terminal glycine residue is not a signal for myristoylation, nor do all myristoylated proteins appear to be cotranslationally modified. A 68 kDa protein from *D. discoideum* [16] was demonstrated to be transiently myristoylated on the N-terminal glycine upon incubation with [^{3}H]myristate. Although the 68 kDa protein was stable, the half-life of the myristate moiety of the 68 kDa protein was only 15 min. Also, McIlhinney and McGlone [11] have provided evidence for a population of myristoylated alanine-rich C-kinase substrate (MARCKS) protein in rat brain which was unmyristoylated. Bacterial lipopolysaccharide has been shown to promote the myristoylation of several macrophage proteins, including a 68 kDa MARCKS protein [17]. We have recently purified and characterized a potent membrane-associated heat-stable inhibitor protein from bovine brain, called NIP_{71}, which could serve to regulate NMT [18].

The yeast enzyme has been purified, characterized [10, 19, 20]. It has been cloned and expressed in bacteria [21]. Comparison of yeast NMT and rat liver NMT have indicated overlapping yet distinct peptide substrate specificities. Recently, NMT has been purified from bovine brain cortex and a partial peptide sequence obtained [22] which shows some similarity to the known yeast sequence and, in the case of three of the isolated peptides, sequence homology with the recently cloned human NMT [23]. NMT has recently been purified form bovine spleen [24]. While the yeast enzyme is a simple monomer, higher eukaryotic NMT appears to be more complex [18]. Also, initial purification from bovine brain, using DEAE-Sepharose CL-6B column chromatography, has indicated that bovine brain possesses multiple forms of NMT [13]. However, whether these activities represent different isoforms from different regions of the brain, or a different make-up of multisubunit complexes is at present unclear. Recently multiple forms of NMT have also been observed from a murine leukaemia cell line L1210 [25]. This communication documents the characterisation with respect to enzyme activity and NIP_{71} interactions of one of the forms of NMT highly purified from bovine brain.

Experimental procedures

[1-^{14}C]Myristoyl-Coenzyme A (54.7 mCi/mmol) and [9,10-^{3}H]myristic acid (39.3 Ci/mmol) were purchased from Amersham International plc. Peptides used were based on the N-terminal sequences of the type II catalytic subunit of cAMP-dependent protein kinase (Gly-Asn-Ala-Ala-Ala-Ala-Lys-Lys-Arg*-Arg*), pp60^{src} (Gly-Ser-Ser-Lys-Ser-Lys-Pro-Lys-Arg*), MARCKS (Gly-Ala-Gln-Phe-Ser-Lys-Thr-Ala-Arg*-Arg*) and the protein product (μl) of the M2 gene sequence of reovirus type 3 Dearing (M2; Gly-Asn-Ala-Ser-Ser-Ile-Lys*-Lys*-Lys*) [26]. Where necessary (*) additional lysine (Lys) or arginine (Arg) residues were added to the C-terminal of the peptides to ensure binding to P81 phosphocellulose paper. Peptide substrates were synthesised as described by King and Sharma [12], except the M2 viral peptide which was purchased from Alberta Peptide Institute. All other chemicals and biochemicals were synthesised or obtained from sources described by King and Sharma [12].

NMT assay

NMT assays were performed as previously described [12], except where detailed below. Generally, 2–10 μl of eluate (as indicated) isolated from various column chromatography steps were incubated in the presence of peptide substrate (10–1000 μM) at 30°C for 4–30 min in a final volume of 25 μl. Reactions were initiated by the addition of 0.27 μM [^{3}H]myristoyl-CoA (74.25–87.25 dpm/fmol) or 0.1–50 μM [^{14}C]myristoyl-CoA (121.4 dpm/pmol), as indicated. During purification NMT activity was generally followed utilising the enzymatic synthesis of [^{3}H]myristoyl-CoA and using the peptide based on the N-terminal sequence of the catalytic subunit of cAMP-dependent protein kinase as substrate. Results are expressed either as pmol myristoylpeptide formed/min/mg or units/mg, where one unit of NMT catalysed the formation of 1 pmol myristoylpeptide/min.

NMT inhibitor assay

NIP_{71} was assayed as described by King and Sharma [18]. Briefly, inhibitor protein was incuated with NMT in the presence of peptide substrate, as described in the figure legends. Reactions were initiated by the addition of either [^{3}H]- or [^{14}C]myristoyl-CoA and incubated for 0–30 min. Results are expressed as units/mg, where 1 unit of inhibitor protein inhibits the formation of 1 pmol myristoylpeptide/min.

NMT purification

All procedures were performed at 4°C, unless otherwise stated. Fresh bovine brains (3 kg) were obtained from the local abattoir and transferred to the laboratory in packed ice. Brains were washed to remove the membrane and blood vessels and homogenised in 2 volumes of buffer A [40 mM Tris/HCl, pH 7.4, containing 1 mM EDTA, 1 mM EGTA, 10 mM 2-mercaptoethanol, 1 mM benzamidine, 20 μg/ml soybean trypsin inhibitor, 1 mM phenylmethylsulphonyl fluoride] using a Waring blender (2 × 15 sec). The homogenate was adjusted to pH 7.4 and then centrifuged at 8000g for 25 min. The resulting supernatant was then centrifuged

at 100,000g for 1 h. The supernatant obtained represented the soluble (cytosolic) fraction of the cell. The pellet was resuspended in 3 volumes buffer A and used in the purification of NIP_{71} [18].

The cytosolic fraction was applied to a DEAE-Sepharose CL-6B column (4 × 20 cm) equilibrated with buffer A. After application, the column was washed with 2 column volumes of buffer A, followed by the stepwise elution of NMT activity with buffer A containing 400 mM NaCl. NMT activity was dialysed overnight against buffer A and applied to a phosphocellulose column (4 × 20 cm), equilibrated in buffer A. The column was washed with 3 column volumes of buffer A and the NMT was eluted with 2 column volumes of buffer B [40 mM Tris/HCl, pH 7.4 containing 0.1 mM EGTA, 10 mM 2-mercaptoethanol and 10% (w/v) sucrose] containing 1 M NaCl. Phosphocellulose-bound NMT activity was then applied to a hydroxylapatite column (1.4 × 8 cm), equilibrated in buffer C [25 mM potassium phosphate buffer, pH 7.4, containing 0.1 mM EGTA, 10 mM 2-mercaptoethanol, 10% (w/v) sucrose]. The column was washed with 2 column volumes of buffer C and the NMT was eluted with a linear phosphate gradient (25–500 mM potassium phosphate, pH 7.4), containing 0.1 mM EGTA, 10 mM 2-mercaptoethanol, 10% (w/v) sucrose. NMT eluted as a shoulder and a main peak. Peak NMT activity was pooled, concentrated by ultrafiltration through an Amicon PM-10 membrane, and dialysed overnight against buffer D (50 mM potassium phosphate, pH 7.0, containing 0.1 mM EGTA, 10 mM 2-mercaptoethanol) containing 10% (w/v) sucrose.

Dialysed NMT was further purified by f.p.l.c. using mono S column chromatography. One to five mg of protein were loaded onto the mono S column using a 10 ml Pharmacia Superloop. The column, developed at a flow rate of 1 ml/min buffer D, was washed for 2 min and the NMT eluted by a salt gradient in buffer D (0–240 mM NaCl in 6 min, 240–480 mM NaCl in 48 min), followed by washing with 1 M NaCl. One-ml fractions were collected into tubes containing 150 µl 40% (w/v) sucrose. NMT eluted as a doublet of activity at 305 mM and 350 mM NaCl (Fig. 1a). The highest NMT activity was pooled, dialysed overnight against 10 volume buffer D containing 10% sucrose and applied on Mono Q f.p.l.c. (Fig. 1b). The column, developed at 1 ml/min buffer D, was washed with buffer D and the NMT activity eluted by a salt gradient (0–400 mM NaCl in 40 min) in buffer D, followed by washing with 1M NaCl in buffer D. Fractions were collected as described above. Peak activities were pooled and stored at –80°C in 1-ml aliquots. Protein concentration was determined by the dye-binding assay according to Bradford [27] using bovine serum albumin as protein standard.

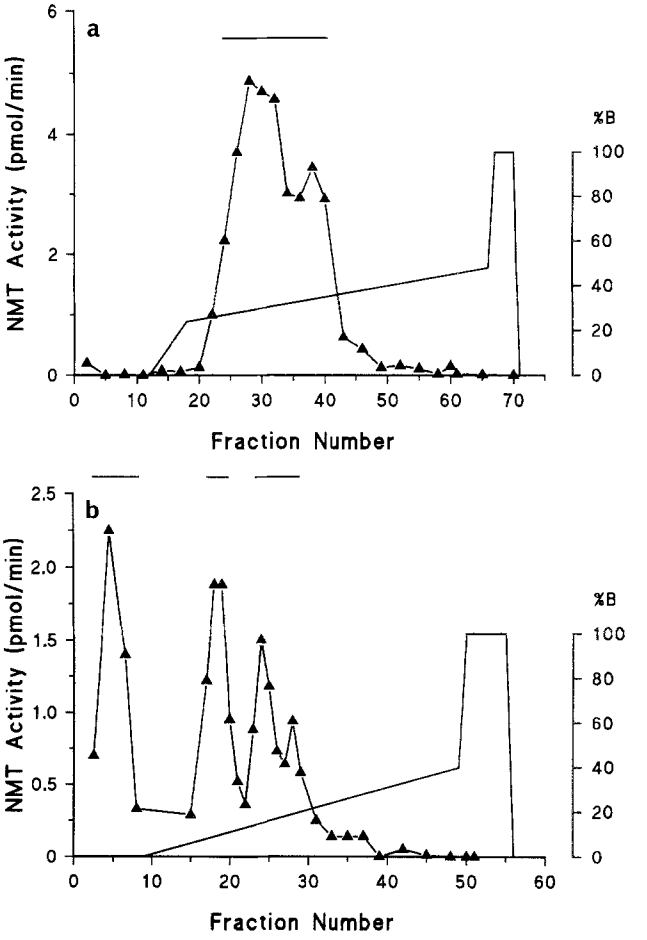

Fig. 1. Elution profile of NMT activity from a) mono S and b) mono Q f.p.l.c.. F.p.l.c. was performed as described in the "Experimental procedures". NMT activity (▲), bar indicates pooled NMT activity.

Results

Purification of NMT activity

NMT was highly purified from bovine brain cytosol by sequential column chromatography on DEAE-Sepharose CL-6B, phosphocellulose, hydroxylapatite and mono S and mono Q f.p.l.c.. Table 1 summarises the results of purification of NMT from 3 kg bovine brain. NMT activity was assayed in one of two ways: either by enzymatically synthesised [^{3}H]myristoyl-CoA [12], or by chemically synthesised [^{14}C]myristoyl-CoA (Amersham) as described in the "Experimental procedures". The assay of NMT with enzymatically synthesised [^{3}H]myristoyl-CoA (0.27 µM, 74.25–87.25 dpm/fmol) resulted in subsaturating concentrations of myristoyl-CoA, and consequently lower catalytic activity values (Table 1), while NMT assayed with [^{14}C]myristoyl-CoA (20 µM, 121.4 dpm/pmol) was at a saturating concentration of substrate (Table 2). Due to the higher specific activity of tri-

Table 1. Purification of NMT·II activity from bovine brain

Purification step	Total Activity (units)	Total protein (mg)	Specific activity (units/mg)	Yield (%)
Cytosol	265.2	9673	0.027	100
DEAE-Sepharose CL-6B chromatography[a]	107.9	3905	0.028	41
Phosphocellulose chromatography	146.7	111	1.32	55
Hydroxyapatite chromatography	134.0	4.92	27.24	51
F.P.L.C. Mono S	50.9	1.51	33.71	19
F.P.L.C. Mono Q				
(I)	14.3	0.430	33.29	5.4
(II)	16.0	0.055	290.90	6.0
(III)	16.1	0.447	35.99	6.7

Details of the purification of NMT from bovine brain (3 kg) are described in the "Experimental procedures". During purification NMT activity was assayed by using enzymatically synthesised, subsaturating [^{3}H]myristoyl-CoA (0.27 μM). [a]The enzyme activity was not increased due to unidentified inhibitor.

Table 2. Michaelis constants of bovine brain NMT·II

Peptide	Sequence	Apparent K_m		V_{max}
		(20 μM)	(0.27 μM)	(units/mg)
cAMP-dependent protein kinase	GNAAAAKKRR	>560 ± 87 (11)	64 ± 9 (8)	29,800
pp60^{src}	GSSKSKPKR	187 ± 50 (7)	17 ± 2 (6)	47,600
M2	GNASSIKKK	38 ± 8 (2)	25 ± 5 (2)	17,300
MARCKS	GAQFSKTARR	ND	ND	1,500
Myristoyl-CoA		2.75 (2)		

Michaelis constants were determined for NMT·II at both saturating (20 μM; [^{14}C]myristoyl-CoA) and subsaturating (0.27 μM; [^{3}H]myristoyl-CoA) concentrations of myristoyl-CoA, V_{max} values were determined using saturating (20 μM) concentrations of [^{14}C]myristoyl-CoA as described in the "Experimental procedures". Apparent K_m values were determined using Eadie-Hofstee plots. MARCKS was too poor a substrate to obtain accurate Michaelis constants. Results are expressed as mean ± SEM of 2–11 experiments (performed in duplicate), as indicated in parentheses.

tiated myristate the enzymatically synthesised myristoyl-CoA system was routinely used during the purification procedure (Table 1). The final specific activity of NMT and fold-purification were determined using chemically synthesised myristoyl-CoA (20 μM).

At several stages during purification NMT activity was observed to elute as either broad peaks with shoulders or multiple peaks of enzyme activity. These activities were consistently observed with different NMT preparations. For example, during the phosphocellulose column chromatography step approx 30% (32% ± 4%; mean ± SEM, n = 4) of the total NMT activity consistently failed to bind. This unbound phosphocellulose NMT activity (termed NMT·PU) was not further purified but characterised with respect to NMT protein inhibitor activity (see below). F.p.l.c. on mono S resulted in a broad doublet of NMT activity eluting at 304 ± 7 mM and 350 ± 10 mM NaCl (Fig. 1a). This doublet was routinely pooled and further purified by mono Q column chromatography. Interestingly, after dialysis, the NMT was able to bind to the positively charged mono Q matrix under the same buffer conditions as the negatively charged mono S matrix. Mono Q column chromatography resulted in three enzyme fractions. The first NMT activity (NMT·I; 26.4% ± 8.7%) failed to bind to mono Q at pH 7.0. Reapplication of this activity resulted in it again failing to bind to the mono Q column, indicating that capacity was not a problem. The remaining activity eluted at 96 ± 9 mM NaCl (NMT·II; 36.5% ± 3.5%) and 170 ± 7 mM (NMT·III; 31.7% ± 8.3%) (Fig. 1b).

Characterisation of NMT activity

NMT·II was purified 6330-fold from the homogenate of bovine brain. This value was calculated based on the initial rate of NMT in crude homogenate (8.66 pmol myristoyl-peptide formed/min/mg homogenate) and the final specific activity of NMT·II (using 20 μM [^{14}C]myristoyl-CoA and cAMP-dependent protein kinase-derived peptide substrates) (29,800 pmol myristoylpeptide formed/min/mg protein: Table 2) and the presence of NIP_{71} in the particulate fraction, i.e., the presence of NIP_{71} resulted in 46% inhibition of the homogenate NMT activity [18]. The kinetic constants for NMT·II using subsaturating (0.27 μM) and saturating (20 μM) myristoyl-CoA are summarised in Table 2. NMT·I and NMT·III exhibited similar Michaelis constants for myristoyl-CoA and the peptide substrates (unpublished data). The observed apparent Michaelis constants for the cAMP-dependent protein kinase- and pp60^{src}-derived peptides at subsaturating myristoyl-CoA concentration are in agreement with published results [10, 11, 13]. It was observed that differences in NMT kinetics were observed when the NMT activities were analyzed at saturating (20 μM) and subsaturating (0.27 μM) myristoyl-CoA concentrations (Table 2). Saturating myristoyl-CoA concentrations resulted in an approximate 10-fold increase in the apparent K_m values of NMT for both the cAMP-dependent protein kinase- and pp60^{src}-derived peptides.

Molecular mass determination by size exclusion on S300-Sephacryl gel filtration appeared to be dependent on the protein concentration of the applied sample, and independent of the salt concentration. High protein concentrations (≥ 1 mg/ml) resulted in the NMT activity eluting at a high apparent molecular mass, i.e., 182,000 ± 27,000 kDa (mean ± SEM, n = 3), lower protein concentrations (~ 0.4 mg/ml and ≤ 0.25 mg/ml) resulted in lower observed apparent molecular masses, i.e., 93,000 ± 5000 kDa (mean ± SEM, n = 4) and 51,300 ± 3,200 kDa (mean ± SEM, n=3), respectively. The

high molecular mass NMT activities were not "dissociated" into lower mass NMT(s) by the presence of 500 mM or even 1M NaCl. Hydroxylapatite and f.p.l.c. mono S purified NMT samples were used in the determination of the apparent molecular mass(es) of NMT by S300-Sephacryl gel filtration; due to the low yields of protein there was insufficient sample to determine to molecular mass of the highly purified NMT·II. However, in light of these results and previous studies [13, 23, 24], it would appear likely that the lowest molecular mass activity represent the apparent molecular mass of the NMT·II catalytic subunit.

NMT/Inhibitor interactions

We have previously reported the purification and characterization of a heat-stable 71 kDa NMT Inhibitor Protein (NIP_{71}) from the particulate fraction of bovine brain [18]. NIP_{71} did not directly interact with myristoyl-CoA or interfere with the binding of myristoyl-CoA to NMT. NIP_{71} possessed no thioesterase, protease or demyristoylase activity [18]. NIP_{71} potently inhibited partially-purified NMT (IC_{50} 19.7 nM). We have further studied the interaction of NIP_{71} with highly purified NMT·II with respect to its mechanisms of action against the myristoyl-CoA and peptide substrates. NIP_{71} inhibited the highly purified NMT·II in a comparable dose-dependent manner as previously reported (IC_{50} 23.7 nM) [18]. The NMT·PU (unbound phosphocellulose) fraction represented 32.2% of the total NMT activity. NMT·PU activity was several fold less sensitive to inhibition by NIP_{71}. Under identical incubation conditions only 36% inhibition occurred at 170 nM NIP_{71}. At this concentration the phosphocellulose bound NMT activity was inhibited by 90%. Additionally, at half-maximal inhibition of the phosphocellulose bound NMT activity (28 nM NIP_{71}) no inhibition whatsoever of the NMT·PU activity was observed, this result would indicate that bovine brain NMT is present in multiple forms.

Analysis of the mechanism of action of NIP_{71} was performed using both Lineweaver Burke and Eadie-Hofstee plots. NIP_{71} inhibited NMT by an apparently mixed inhibitory mechanism with respect to both the myristoyl-CoA and peptide substrates, assuming a similar mechanism for the bovine brain NMT to that observed with the yeast enzyme, i.e., an ordered Bi-Bi mechanism [20, 29], with the myristoyl-CoA binding first (Fig. 2). Calculation of the inhibitory constants was performed using the equations devised by from Michealis-Menten for mixed inhibition, where K_i represents the NIP_{71}·NMT inhibitory constant and K_i' represents the NIP_{71}·NMT·myristoyl·CoA inhibitory constant [30]. These results indicated that NIP_{71} binds to the NMT species some 2–3 times more favourably than the NMT·Myristoyl·CoA species (K_i 34 ± 4 nM vs K_i' 88 ± 15 nM). No difference was observed in the inhibitory constant values using either cAMP-dependent protein kinase- and $pp60^{src}$-derived peptide substrates. Also, similar inhibitory values were obtained using either the saturating or subsaturating myristoyl-CoA concentrations. Physiologically, neither the myristoyl-CoA nor the peptide substrates are likely to exist in the concentrations used in the determination of Michaelis and inhibitory constants. Assaying the NIP_{71} at subsaturating concentrations of peptide (20, 35 and 50 μM) and myristoyl-CoA (0.27 μM) indicated that the NIP_{71} inhibited the NMT activity in a sigmoidal manner (Fig. 3). Little difference was observed in the level of inhibition by NIP_{71} using 20 μM, 35 μM or 50 μM cAMP-dependent protein kinase-derived peptide. Under subsaturating myristoyl-CoA concentration the apparent K_m of NMT for this peptide was 64 μM (Table 2) [13].

Discussion

Purified NMT·II had a specific activity of 47,600 pmol myristoylpeptide formed/min/mg using the $pp60^{src}$ derived peptide as substrate (Table 2), in close agreement with that reported by McIlhinney *et al.*, for pure NMT (95,000 pmol myristoylpeptide formed/min/mg) [22]. Three apparent molecular masses for NMT were observed by size exclusion on S300-Sephacryl gel filtration; 182 kDa, 93 kDa and 51 kDa. The apparent molecular mass was dependent on the protein concentration of the sample applied to the gel filtration column, where high protein concentration resulted in a high apparent molecular mass and lower protein concentrations resulting in subsequently lower apparent molecular masses.

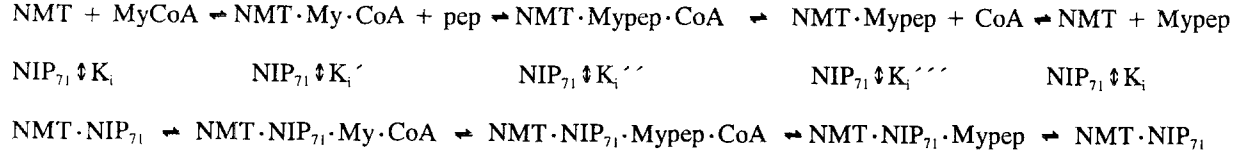

Fig. 2. Schematic sequence of the N-myristoyltransferase reaction catalysed by NMT. Inhibition reactions were performed as described in the "Experimental procedures", using constant cAMP-dependent protein kinase derived-peptide whilst varying the [^{14}C]myristoyl-CoA (0–50 μM, 121.4 dpm/pmol) or using constant [^{3}H]myristoyl-CoA (0.27 μM, 87.25 dpm/fmol) whilst varying the cAMP-dependent protein kinase-derived peptide concentration K_i 34 ± 4 nM and K_i' 88 ± 15 nM were calculated. The apparent mechanism was based on the conclusions of Gordon and coworkers [20]. Abbreviations: MyCoA, myristoyl-CoA; pep, peptide; Mypep, myristoylpeptide.

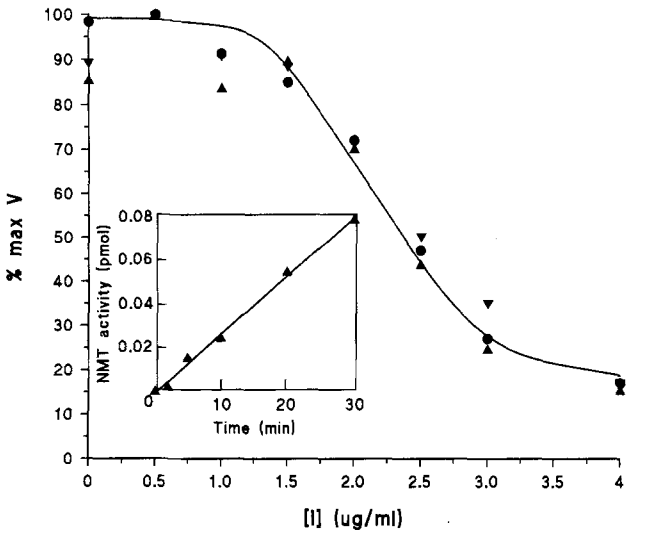

Fig. 3. Inhibition of NMT activity at subsaturating concentrations of myristoyl-CoA and cAMP-dependent protein kinase derived-peptide. NMT activity was assayed as described in the "Experimental procedures". NMT (4.9 μg) was incubated with either 20 μM (▲), 35 μM (▼) or 50 μM (●) cAMP-dependent protein kinase-derived peptide in the presence of increasing NIP_{71} concentrations. The reactions were initiated by the addition of 0.27 μM myristoyl-CoA (87.25 dpm/fmol) and incubated for 30 min. Results are expressed as percent of maximal activity (mean of 2–4 experiments, performed in duplicate). *Insert:* NMT (20.6 μg) was incubated with cAMP-dependent protein kinase-derived peptide (20 μM) in a final volume of 105 μl. The reactions were initiated by the addition of 0.27 μM myristoyl-CoA. Samples (15 μl) of the reaction mixture were assayed at the indicated times as described in the "Experimental procedures". The results are expressed as pmol myristoylpeptide formed/mg (mean ± SEM, n=2).

These results are consistent with the theory that NMT is a "sticky" protein aggregating either with itself or with other proteins; the lowest molecular mass (51 kDa) representing a single catalytic subunit and the higher molecular masses representing homogenic or heterogenic multimers (possibly dimers and tetramers). Work from our laboratory and others [13, 22] has demonstrated that the "aggregation" of NMT activity was probably not due to non-specific protein interactions, being dissociated by myristoyl-CoA (200 μM) but not by high salt (1M NaCl). Bovine brain NMT·II demonstrated a ten-fold difference in the observed apparent K_m values with the cAMP-dependent protein kinase-derived and $pp60^{src}$-derived peptide substrates when assayed with either 20 μM myristoyl-CoA or 0.27 μM myristoyl-CoA (Table 2). There could be several possible explanations for such a phenomena. 1) It is possible that the variation represents two enzymes (or active sites within the same NMT): a high affinity, low activity NMT and a low affinity, high activity NMT. For example, assaying NMT activity under saturating (20 μM) myristoyl-CoA conditions resulted in only the low myristoyl-CoA affinity, high activity enzyme being assayed (the low activity, high affinity NMT being effectively swamped). On the other hand, assaying the NMT activity under subsaturating (0.27 μM) myristoyl-CoA conditions resulted in only the high affinity, low activity enzyme being assayed (the low affinity, high activity NMT having insufficient myristoyl-CoA to function efficiently). These enzymes would have a similar affinity for the M2 viral peptide. Such a system has been observed with bovine brain calmodulin-stimulated phosphodiesterase isozymes where the 63 kDa isozyme possessed high affinity for both cAMP and cGMP but low V_{max} whereas the 60 kDa isozyme possessed the reverse, lower affinity for cAMP and cGMP but a higher V_{max} for both the substrates [31, 32]. 2) It is possible that some form of cooperativity, or allosteric effect, exists between the myristoyl-CoA and peptide binding sites, where high myristoyl-CoA inhibits the binding of the peptide substrate or stabilises the NMT into a NMT·Myristoyl·CoA complex. Cooperatively between the myristoyl-CoA and peptide binding sites has been established in the yeast NMT. 3) Alternatively, myristoyl-CoA may allosterically interact with NMT itself. In support of this theory, that myristoyl-CoA interacts with NMT in a specific "conformational" manner in addition to an actual substrate, is the kinetic data reported here as well as the observation, in our laboratory and others [13, 22], that myristoyl-CoA (200 μM) but not high salt (1M NaCl) can dissociated the high molecular mass NMT complexes into lower molecular mass (monomeric) NMT. However, to our knowledge no other kinetic studies of NMT have been performed using the saturating myristoyl-CoA concentrations. Clearly, this will need further study to establish this phenomenon.

Supporting a "conformation" theory is the recent studies from our laboratory demonstrating that bovine spleen NMT can be activated several fold by Tris/HCl, potassium phosphate, imidazole buffers and KSCN at both saturating and subsaturating concentrations of myristoyl-CoA, suggesting that these compounds may "unfold" the structure of NMT into an open, high activity NMT form [24]. Similar results were observed with bovine brain NMT with a 3.5–5 fold stimulation of activity. However, this phenomena was observed only with Tris/HCl (250 mM) and KSCN (100 mM) at subsaturating myristoyl-CoA concentrations (unpublished data). Other buffers and salts, i.e., potassium phosphate, N-tris[hydroxymethyl]methyl-2-aminoethane sulphonic acid (TES), NaCl and KCl, failed to stimulate NMT activity under identical conditions (unpublished data). It is possible that the high myristoyl-CoA concentration performs a similar job to Tris and KSCN, unfolding up the NMT into a high activity enzyme form.

We are at present unable to determine whether our purification has favoured one activity of NMT previously observed [13]. However, in light of recent results (discussed above) it now seems likely that these activities observed in the crude

bovine brain represented aggregation of either homogenous catalytic subunits or a heterogenous subunit composition, the composition of which is yet to be determined. If high molecular mass NMT(s) represent multimeric heterogenous complexes these additional subunits clearly are not essential for enzyme activity. It is possible that they represent regulatory subunits modulating the NMT activity, possibly by cellular localisation in a manner analogous to the G- and M-particles which associate the catalytic subunit of phosphoseryl protein phosphatase type 1 to glycogen and myofibrils, respectively [33].

NIP_{71} potently inhibited NMT, whether crude or highly purified, in a similar dose-dependent manner (IC_{50} values ranged from 19.7–23.7 nM). Gordon and coworkers have extensively characterised the mechanism of action of the yeast NMT enzyme, postulating myristoylation to occur via an ordered Bi Bi mechanism [20, 29]. We have assumed a similar mechanism for mammalian NMT, with myristoyl-CoA binding to NMT in the absence of peptide substrate [18, 20]. The apparent mechanism of inhibition was mixed with respect to both the myristoyl-CoA and peptide substrates, where the NIP_{71} had a higher affinity for the free enzyme than the NMT·myristoyl·CoA complex. Calculation of additional K_i constants involving the enzyme·peptidyl complexes are complicated by several additional rate terms as well as by the effects of product inhibition. Whilst it is useful to calculate such values as K_i and K_i' to obtain an idea of the affinity of binding of the inhibitor to the different enzyme complexes, such values also have their limitations in that they are obtained under very nonphysiological conditions; neither the myristoyl-CoA nor the polypeptide substrate concentration are likely to be so high within the cell. Assaying the activity and interaction of NMT and NIP_{71} at subsaturating concentrations of both substrates produces a more physiological environment. Under such conditions the observed IC_{50} was similar to previous values (21.7 nM vs 28 nM); however, NIP_{71} was observed to inhibit the NMT activity in a sigmoidal manner. Clearly this was not due to any effects of nonlinear kinetics: the rate of myristoylation of the lowest concentration of cAMP-dependent protein kinase-derived peptide substrate was linear over the 30 min incubation time (Fig. 3, insert). The importance of such sigmoidal kinetics in the regulation of NMT activity can clearly be seen. A small change in the concentration or activity of NIP_{71} would result in a large change in the NMT activity, thus a potent and sensitive on-off switch is available to the cell.

The major difference between the forms of NMT (I, II and III) was the catalytic activity values. However, this probably just indicates that NMT·II was more highly purified. It is of interest to note that the specific activities of both NMT·I and NMT·III are similar to each other as well as to the previous mono S f.p.l.c. purified NMT. Recenlty, an NMT activity has been purified from the cortex of bovine brain [22]. Although no characterisation of this enzyme activity has yet been reported, bovine brain NMT showed remarkable peptide sequence homology to the human deduced peptide sequence indicating that other mammalian tissues remain good tools for the study of NMT. McIlhinney *et al.* [22] observed two forms of NMT; however, the lower M_r (43 kDa) NMT was postulated to be the result of proteolysis of the 66 kDa NMT. We have previously observed the existence of multiple activities of NMT from whole bovine brain homogenates [13]. Additionally, during several stages of purification, evidence for multiple activities of NMT was observed. At least one of these NMT activities (NMT·PU) possessed unique properties, failing to bind to phosphocellulose and being insensitive to inhibition by NIP_{71}. Previous work from our laboratory and others [King and Sharma, unpublished results, 11] has indicated that the cerebral cortex did not contain the highest NMT activity. We have observed that hippocampus possesses the highest specific activity, where hippocampus>thalamus>hypothalamus>medulla oblongata>cortex>cerebellum>pituitary(1.00:0.69:0.61:0.52:0.44:0.39:0.17) with respect to NMT activity/mg protein. The presence of multiple activities of NMT may be the result of multiple NMT's expressed in different locations of the brain, McIlhinney *et al.* [22] purifying one specific activity expressed in the cerebral cortex of bovine brain. In conclusion it would appear that NMT in higher eukaryotes (mammalians) appears to be a more complex system than yeasts, involving subunit interactions (NIP_{71}), protein modifications and subcellular compartmentalisation. Recently we have shown that rat liver NMT may play some role in diabetes mellitus and insulin signalling, rat liver NMT having high membrane-associated activity which was elevated in low plasma insulin (insulin-dependent diabetes mellitus) and reduced in high plasma insulin (non-insulin dependent diabetes mellitus) conditions [34, 35]. Studies with animal tissues and models should help elucidate any potential regulatory roles of human NMT in insulin and other actions. Consequently allowing for modes of therapy for these diseases to be developed.

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