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Purification and partial sequencing of myristoyl-CoA:protein *N*-myristoyltransferase from bovine brain

R. A. Jeffrey McILHINNEY,*‡ Kate McGLONE* and Antony C. WILLIS†

*M.R.C. Anatomical Neuropharmacology Unit, Mansfield Road, Oxford OX1 3TH, U.K.
and †M.R.C. Immunochemistry Unit, Department of Biochemistry, South Parks Road, Oxford OX1 3QU, U.K.

The enzyme myristoyl-CoA:protein *N*-myristoyltransferase (NMT; EC 2.3.1.97) catalyses the transfer of myristic acid to the N-terminal glycine residue of cell and viral proteins. In this report the purification and partial sequencing of this enzyme from bovine brain is described. Using a combination of ammonium sulphate precipitation, chromatography on DEAE-Sepharose and affinity chromatography on CoA–agarose the enzyme was purified some 40-fold. Size-exclusion chromatography of this material in the presence of myristoyl-CoA yielded two peaks of enzyme activity with apparent molecular masses of 66 kDa and 43 kDa. Chromatography of the CoA-affinity-purified material on MONO-S followed by size-exclusion chromatography in the presence of myristoyl-CoA resulted in the isolation of the large form of the enzyme purified 3000-fold. Analysis by SDS/PAGE of this material showed a major 60 kDa silver-stained band. Similar analysis of the 43 kDa enzyme fraction from the same separation showed that this fraction contained several proteins including a major component with an apparent molecular mass of 49 kDa. Attempts at N-terminal sequencing of the 66 kDa form of the enzyme were unsuccessful and therefore this material was digested with trypsin and the resulting peptides separated by reverse-phase h.p.l.c. N-terminal protein sequencing of these peptides yielded sequences which show sequence similarity to those of yeast *N*-myristoyltransferase.

INTRODUCTION

A large number of cellular and viral proteins are now known to be modified by the attachment of myristic acid, via an amide bond, to their N-terminal glycine residue (Towler et al., 1988a; Schmidt, 1989; McIlhinney, 1990). Cell proteins which are modified in this way include the α-subunit of the coupling proteins G_o and G_i (Schulz et al., 1987), myristoylated alanine-rich protein kinase C substrate (MARCKS) (Stumpo et al., 1989), calcineurin B (Aitken et al., 1982) and the catalytic subunit of cyclic AMP-dependent protein kinase (Carr et al., 1982). For some of these proteins there is clear evidence that the attached fatty acid is important for the functioning of the protein, thus the N-myristoylation of the α-subunit of the G_o protein increases its affinity for $\beta\gamma$ subunits (Linder et al., 1991) and myristoylation of both $p60^{src}$ and MARCKS is essential for their membrane-binding activity (Cross et al., 1984; Graff et al., 1989). In addition the prevention of the N-myristoylation of $p60^{src}$ renders the protein transformation defective (Cross et al., 1984). Myristoylated viral proteins include both regulatory and structural proteins from a variety of virus families including human immunodeficiency viruses (HIV), the picorna and rotaviruses (Chow et al., 1987; Clark and Desselberger, 1988; Gottlinger et al., 1989). Interest in this modification of proteins has been stimulated by the finding that prevention of the attachment of the myristate, by mutation of the N-terminal glycine residue of the substrate protein, results, for both HIV and polio virus, in defective viral replication (Marc et al., 1989; Moscufo et al., 1991). In addition heteroatom-substituted analogues of myristic acid and inhibitors of myristoylation have been reported to prevent replication of HIV and other viruses (Shoji et al., 1988; Bryant et al., 1989; Bryant and Ratner, 1990; Saermark et al., 1991). Consequently there is considerable interest in the enzymology of this reaction since it appears to offer potential for the development of novel anti-viral compounds.

The enzyme which catalyses the transfer of myristic acid to the N-terminal glycine residue of its target proteins is myristoyl-CoA:protein *N*-myristoyltransferase (NMT; EC 2.3.1.97). The enzyme has been purified from yeast (Towler et al., 1987) and the mechanism of this enzyme is well characterized (Rudnick et al., 1990, 1991). However, little is known about the mammalian enzyme, which has not been purified. Studies on partially purified mammalian NMT have shown that it has some of the characteristics of the yeast enzyme, but exhibits subtle differences in peptide substrate specificities (Towler et al., 1988b). Given the potential use of inhibitors of NMT as anti-viral agents the purification of the mammalian enzyme seemed a worthwhile objective. In our preliminary studies brain had been identified as the best tissue source for the enzyme (McIlhinney and McGlone, 1990) and in this report we describe the purification and partial sequencing of bovine brain NMT.

MATERIALS AND METHODS

Materials

CoA–agarose (Type V), activated CH-Sepharose 4B, DEAE-Sepharose Fast Flow, MONO-S HR 5/5 and Superose-12 HR 10/30 columns were purchased from Pharmacia LKB. Iodoacetamide, 1,10-phenanthroline, phenylmethanesulphonyl fluoride, dithiothreitol, Triton X-100, LiCoASH, myristoyl-CoA, leupeptin, aprotinin and peptstatin A were obtained from Sigma. [1-^{14}C]Myristic acid (58 μCi/mmol), [1-^{14}C]palmitic acid (58 μCi/mmol) and [9,10-^{3}H(N)]myristic acid (60 Ci/mmol) were purchased from New England Nuclear. P81 phosphocellulose paper was bought from Whatman. Trypsin of sequencing grade was obtained from Promega. All other reagents were either

Abbreviations used: NMT, myristoyl-CoA:protein *N*-myristoyltransferase; MARCKS, myristoylated alanine-rich protein kinase C substrate; HIV, human immunodeficiency viruses.

‡ To whom correspondence should be addressed.

obtained from FSA Ltd. or BDH and were of the highest quality available.

SDS/PAGE

SDS/PAGE was performed using 10% (w/v) gels and the discontinuous buffer system of Laemmli (1970). Samples were dissolved in 62.5 mM Tris/HCl containing 2% (w/v) SDS, 10% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol and 0.01% (v/v) Bromophenol Blue. Proteins were visualized by silver staining as described by Molnar et al. (1991). Gels for autoradiography were impregnated with 2,5-diphenyloxazole as described by Laskey and Mills (1975), dried and exposed using Kodak X-OMAT AR X-ray film. Marker proteins used were myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase b (94 kDa), BSA (66 kDa), ovalbumin (45 kDa) and carbonic anhydrase (29 kDa).

Protein determinations

Protein was measured using the Bio-Rad dye-binding assay and BSA as a standard.

Preparation of the Co-A affinity column

LiCoA-SH (75 mg) was dissolved in 0.1 M $NaHCO_3$ (10 ml) and added to 2 g (8 ml) of activated CH-Sepharose 4B (Pharmacia) which was prepared by following the manufacturer's instructions. The resultant slurry was mixed by rotation for 16 h at 4 °C. The Sepharose was pelleted by centrifugation (1000 g_{av} for 5 min) and the extent of CoA-SH coupling determined by titration of the supernatant with 5,5′-dithiobis(2-nitrobenzoic acid) and monitoring the absorbance at 410 nm. Usually greater than 80% of the CoA-SH was coupled and the substitution of the final product was between 4 and 5 μmol of CoA/ml of Sepharose. The CoA–Sepharose was washed with 50 mM Tris/HCl, pH 7.5 (100 ml), under vacuum in a Buchner Flask and any remaining reactive groups blocked by incubating the gel with 0.1 M Tris/HCl, pH 7.5 (20 ml), for 4 h at room temperature. The CoA–Sepharose was then washed again with 50 mM Tris/HCl (200 ml) and stored in 50% (v/v) glycerol in Tris/HCl containing 0.05% NaN_3 at −20 °C.

Preparation of [^{14}C]myristoyl-CoA

[^{14}C]Myristic acid (58 mCi/mmol; 50 μCi) was dried under N_2 in an Eppendorf microfuge tube. Fresh stocks of *N*-hydroxysuccinimide (4 mg/ml) and dicyclohexylcarbodiimide (8 mg/ml) were prepared in anhydrous ethyl acetate. Equal volumes (50 μl) of each of these were added to the Eppendorf tube containing the [^{14}C]myristic acid and the contents mixed thoroughly by vortex mixing. The reaction was left overnight at room temperature and the small precipitate of dicyclohexylurea formed was pelleted by centrifugation (10000 g_{av} for 5 min). The pellet was washed with a further 100 μl of ethyl acetate and the combined supernatants dried under N_2 in a clean Eppendorf microfuge tube. The residue was dissolved in anhydrous tetrahydrofuran (50 μl) and 40 μl of a solution containing 2 mg of LiCoA-SH dissolved in 60 μl of 0.25 M $NaHCO_3$ was immediately added. The reagents were mixed well as before and the reaction was allowed to proceed overnight at room temperature. It was terminated by the addition of 100 μl of ice-cold 10% (w/v) perchloric acid. The reagents were kept on ice for 20 min and the precipitated myristoyl-CoA was recovered by centrifugation (10000 g_{av} for 15 min). The pellet was extracted once with a further 100 μl of 10% (w/v) perchloric acid, four times with acetone (100 μl) and three times with diethyl ether (100 μl). The final pellet was carefully dried under N_2 and dissolved in 200 μl of 10 mM sodium acetate, pH 6.0. After determining the yield of the reaction (routinely between 40% and 70%) the volume was adjusted to give a concentration of 0.2 mM myristoyl-CoA which is equivalent to 130000 d.p.m./5 μl. Aliquots (200 μl) of this solution were frozen and stored at −20 °C until use.

[^{3}H]Myristoyl-CoA at a specific activity of 166 μCi/mmol was prepared by drying 500 μCi of [^{3}H]myristic acid (60 Ci/mmol) together with 3 μmol (684 μg) of myristic acid in an Eppendorf microfuge tube. The reaction scheme described above was followed except that the reactant concentrations were increased 3-fold.

NMT assay

NMT activity was determined by measuring the transfer of radioactive myristic acid from myristoyl-CoA to the N-terminal glycine residue of a peptide substrate, which was based on the N-terminal 16 amino-acid residues of p60^{src} (GSSKSKPKDPSQRRRY). In our initial studies the enzymic production of myristoyl-CoA and the assay were performed as described earlier (McIlhinney and McGlone, 1989). However, it was found that with increasing purity this assay became non-linear and appeared to be limited with respect to myristoyl-CoA concentration. To overcome this problem we chemically synthesized lower-specific-activity myristoyl-CoA, as described above, which allowed the use of a higher concentration of myristoyl-CoA in the assay. To facilitate the assay of large numbers of column fractions we adopted the method of King and Sharma (1991) using phosphocellulose paper for the determination of myristoylated peptide. Briefly 10 μg of peptide (5 μl), and ^{14}C-labelled myristoyl-CoA (5 μl; 130000–150000 d.p.m.), were added to 25 μl of assay buffer [0.05 M Tris/HCl, pH 7.5/1% (v/v) Triton X-100/0.5 mM EDTA/0.5 mM EGTA/1 mM dithiothreitol]. NMT was added and the volume adjusted to 60 μl with assay buffer. The reaction was continued for 10 min at 30 °C and 15 μl of the reaction mixture spotted on to 2 cm^2 pieces of phosphocellulose paper. These were washed and processed for scintillation counting as described by King and Sharma (1991). Routinely, all determinations were performed in duplicate and the results obtained using this assay system were in good agreement with those obtained earlier (McIlhinney and McGlone, 1989). The availability of the chemically synthesized myristoyl-CoA and the rapid method for the determination of acylated peptide greatly facilitated the purification.

Purification of NMT

All steps, unless otherwise stated, were performed at 4 °C, and all buffers for dialysis and homogenization contained aprotinin, pepstatin A and leupeptin at concentrations of 1 μg/ml plus dithiothreitol at 1 mM. Bovine brain was obtained from the local abattoir, transported on ice, the cortex dissected and either used immediately, or frozen in liquid nitrogen for storage at −70 °C. Dissected cortex (800 *g*) was cut into 1 cm^3 pieces and 3 vol. of cold Tris/HCl (50 mM; pH 7.5) containing the above protease inhibitors plus 1 mM phenylmethanesulphonyl fluoride, 1 mM iodoacetamide, 0.5 mM EDTA, 10 μM 1,10-phenanthroline and 0.05% Triton X-100. Approx. equal aliquots (500 ml) were homogenized in a Waring blender (maximum speed; three 30 s bursts) and the homogenate centrifuged in a Sorvall GSA rotor at 10000 g_{av} for 2 h. The supernatant was carefully decanted and adjusted to 30% saturation with $(NH_4)_2SO_4$. After stirring at 4 °C for 1 h the precipitate was removed by centrifugation (16000 g_{av} for 1 h) and the supernatant adjusted to 65% satu-

Table 1 Purification of bovine NMT

The yields from both the MON-S and Superose-12 columns were reduced dramatically because the fractions were pooled for purity rather than yield, and only the yield of the high-molecular-mass form of the enzyme is included in the Table.

Sample	Protein (mg)	Specific activity (pmol/min per mg of protein)	Total activity (pmol/min)	Yield (%)	Purification
Cytosol	11700	37.1	434070.0	100.0	1.0
35–65% $(NH_4)_2SO_4$	6770	57.8	391306.0	90.1	1.55
0.2 M NaCl	2160	151.9	328104.0	75.5	4.1
0.5 M KCl	50.7	1415.0	71800.0	16.5	38.1
MONO-S	0.28	25200.0	7056.0	1.63	679.2
Superose-12	0.0075	95500.0	716.3	0.17	2574.1

ration with $(NH_4)_2SO_4$. After stirring for a further hour the precipitate which contained the NMT activity was recovered by centrifugation as above, dissolved in Tris/HCl (20 mM; pH 7.5) and dialysed against two changes of 5 litres of this buffer for 18 h. The resulting dialysate was loaded at a flow rate of 30 ml/h on to a DEAE-Sepharose Fast Flow column (300 ml) and eluted with the same buffer until the A_{280} value declined to baseline. The column was then eluted stepwise with 60 mM, 0.2 M and 1 M NaCl in 20 mM Tris/HCl. The protein peaks eluted with each salt concentration were monitored at 280 nm and pooled. Routinely the NMT activity eluted in the 0.1 M NaCl and was concentrated by bringing this fraction to 70% saturation with $(NH_4)_2SO_4$ and recovering the enzyme by centrifugation. The ammonium sulphate precipitate was dissolved in a minimum volume of 50 mM Tris/HCl, pH 7.5, and dialysed against this buffer (5 1; 16 h). The dialysate (50 ml) was rotated with 25 ml CoA–agarose for 4 h, the agarose recovered by centrifugation (1000 g_{av} for 15 min) and packed into a column. The agarose was washed with Tris/HCl until the A_{280} value fell to baseline levels, and then eluted stepwise with 0.1 M, 0.5 M and 1.0 M KCl. NMT activity was recovered in both the 0.1 M and 0.5 M fractions but the specific activity of the 0.5 M fraction was 3–5-fold higher than that of the 0.1 M material and this was therefore used for further processing. This fraction was concentrated in Centriprep 10 S spin concentrators (Millipore), dialysed against 50 mM Tris/HCl, pH 7.5, containing 0.1 M NaCl (5 1; 16 h) at which point it retained its activity for some weeks at 4 °C. Dialysis against lower-ionic-strength buffers caused significant precipitation of proteins and losses of NMT activity.

For final purification, material from the CoA affinity column was dialysed against 20 mM Mes, pH 6.7 (5 1; 18 h) and aliquots (6 mg) loaded on to a MONO-S column. Elution was performed at 1 ml/min at room temperature using a gradient of 0–0.5 M NaCl over 30 min followed by a steep gradient to 1 M NaCl over 5 min. The most active fractions were pooled, concentrated using Ultrafree-MC 10000 concentrators (Millipore), and dialysed against 50 mM Tris/HCl, pH 7.5, containing 0.1 M NaCl. This material was incubated with 200 μM myristoyl-CoA for 5 min at room temperature, centrifuged (10000 g_{av}) for 5 min and loaded on to a Superose-12 column. Elution was performed at 0.5 ml/min and fractions collected every 0.5 min. NMT eluted as two active peaks, the most active fractions of which were pooled and analysed on SDS/polyacrylamide gels.

Protein digestion and sequencing

A sample of purified 60 kDa NMT (32 pmols) was digested overnight with trypsin (1 μg) in a final volume of 200 μl of 75 mM Tris/HCl, pH 8.0, at 37 °C. The relatively high ratio of trypsin to protein was used to ensure digestion of the undenatured protein and therefore a control digestion containing only trypsin was also performed. The products of both digests were separated on an Aquapore RP300 (C8) column (100 mm × 2 mm) equilibrated with 2% (v/v) acetonitrile and 0.1% trifluoroacetic acid. The peptides were eluted with a linear gradient of 2–50% (v/v) acetonitrile over 50 min, followed by a linear gradient of 50–90% (v/v) acetonitrile over 18 min at a flow rate of 0.2 ml/min. The peptides were collected by monitoring at 215 nm and those found only in the NMT digest were subjected to N-terminal sequencing as, separately, was a sample (16 pmol) of undigested NMT.

For N-terminal sequencing all liquid samples were applied to a glass-fibre disc treated with polybrene and then run on either an Applied Biosystems (ABI) 470A or on a 473 protein sequencer with online PTH analyser using the standard 03CPTH or NORM programs respectively from Applied Biosystems.

RESULTS

The purification scheme adopted for bovine NMT follows that described for the yeast enzyme (Towler et al., 1987) as far as the CoA-affinity column (Table 1). Only minor changes to the salt concentrations used to elute the columns were needed to maximize the recovery of the bovine enzyme. NMT was eluted from the CoA–agarose column with 0.5 M KCl and this material, purified some 40-fold, was stable at 4 °C for some weeks. However, continued purification of the enzyme proved difficult due to the high loss of enzyme activity which occurred during further chromatography on a variety of column packings. Size-exclusion chromatography of the CoA-affinity-purified bovine NMT resulted in the elution of a broad peak of enzyme activity, with a molecular-mass range of 150 kDa–60 kDa, which co-eluted with the bulk of the eluting proteins (Figures 1a and 1b). In some preparations an additional small peak of NMT activity rather better resolved and eluting with a molecular mass of 66 kDa could be seen, but it always represented a minor fraction of the enzyme activity recovered. Since yeast NMT has been shown to form a stable covalent complex with myristoyl-CoA the effect on the chromatographic behaviour of the enzyme of incubating the CoA-affinity-purified bovine NMT with myristoyl-CoA was examined. The enzyme was incubated for 5 min at room temperature with 200 μM myristoyl-CoA, which is a concentration 10 times the apparent K_m of the enzyme for myristoyl-CoA (King and Sharma, 1991), and a time sufficient for the yeast enzyme to form the stable complex (Rudnick et al., 1990). The result was to cause the enzyme to elute from the

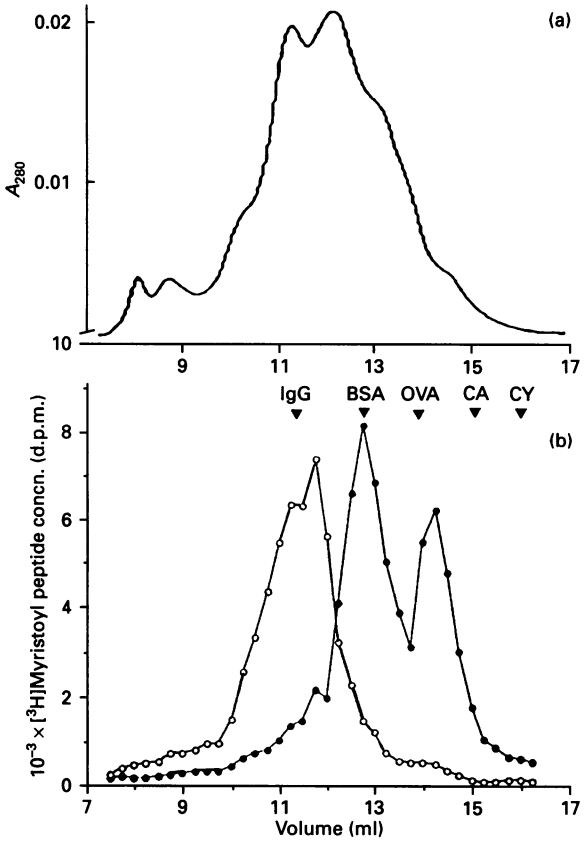

Figure 1 Size-exclusion chromatography of CoA-affinity-purified bovine NMT

NMT (200 μg) was incubated in the presence (**b**; ●) or absence (**b**; ○) of myristoyl Co-A and then subjected to chromatography on a Superose-12 HR 10/5 column. The eluting proteins were continuously monitored at 280 nm (**a**) and aliquots (20 μl) of the fractions (0.25 ml) starting from the void volume (7.5 ml) of the columns analysed for NMT activity as shown (**b**). The indicated elution positions of the marker proteins IgG (150 kDa), BSA (66 kDa), ovalbumin (OVA; 45 kDa), carbonic anhydrase (CA; 28 kDa) and cytochrome *c* (CY; 12.5 kDa) were determined by monitoring their elution at 280 nm following identical chromatography of 20 μg of each protein.

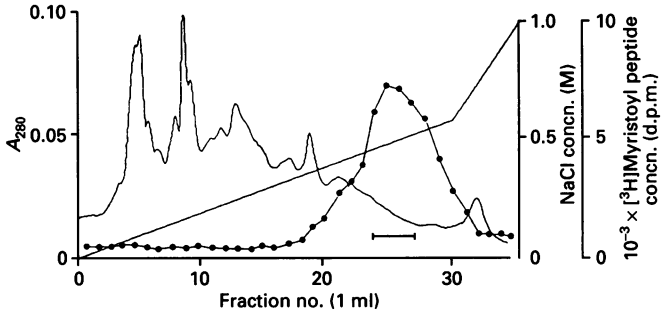

Figure 2 Chromatography of CoA-affinity-purified bovine NMT on a MONO-S column

CoA-affinity-purified bovine NMT (6 mg) was loaded on to a MONO-S HR 5/10 column in 20 mM Mes, pH 6.7. The column was eluted with 10 ml of 20 mM Mes, pH 6.7, and a substantial breakthrough protein peak obtained. This is not shown since this fraction contained no NMT activity. The salt gradient illustrated was then applied and 20 μl aliquots of all the fractions (1 ml) were assayed for NMT activity (●). The enzyme eluted as a broad peak and the most active fractions, shown by the bar, were pooled for further purification. The elution of the proteins was monitored continuously at 280 nm (continuous line).

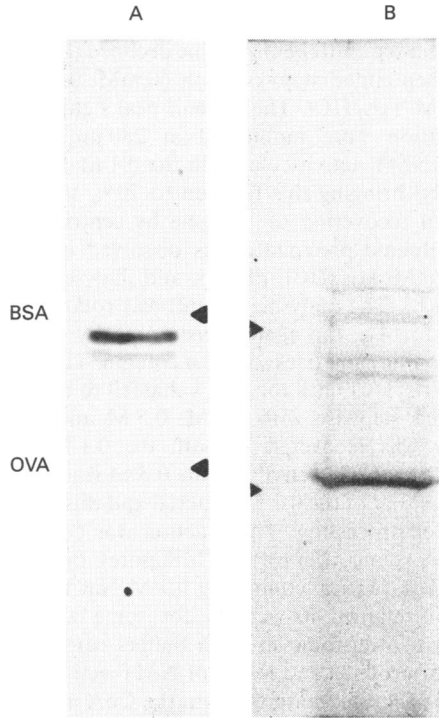

Figure 3 SDS/PAGE of bovine NMT fractions obtained from a Superose-12 column

Following chromatography on a MONO-S column, the NMT was incubated with myristoyl-CoA and loaded on to a Superose-12 column. The most active fractions eluting at positions corresponding to 66 kDa and 43 kDA were pooled, concentrated, and 0.6 μg of each analysed on a SDS/10% (w/v) polyacrylamide gel followed by silver staining. The positions of BSA and ovalbumin (OVA) are shown by arrows.

column in two peaks of activity with apparent molecular masses of 66 kDa and 43 kDa (Figure 1b). Increasing the time of incubation to 20 min, or the temperature of the incubation to 30 °C, had no further effect on the elution pattern (results not shown). However, the relative contributions of these two forms of the enzyme did vary with both enzyme preparation and time of storage at 4 °C. Although the smaller form of NMT was always present, in some preparations the larger form of the enzyme was the major component, whereas after prolonged storage the smaller form predominated.

This enhancement of the chromatographic resolution of the enzyme on size-exclusion columns by myristoyl-CoA was then exploited for further purification. CoA-affinity-purified bovine NMT was subjected to chromatography on a MONO-S column (Figure 2) which resulted in a substantial improvement in enzyme purity and a considerable reduction in yield (Table 1), as noted for yeast NMT (Rudnick et al., 1990). These losses were magnified by the fact that the enzyme eluted as a broad peak from the column and only the most active fractions (indicated by the bar in Figure 2) were pooled for further purification. These were concentrated, incubated with myristoyl-CoA, and subjected to size-exclusion chromatography. As before two peaks of NMT activity eluting at 66 kDa and 43 kDa were recovered containing equal amounts of enzyme activity. The most active fractions of each of these peaks were pooled, concentrated and analysed on SDS/polyacrylamide gels. The larger-molecular-mass form of NMT obtained from the size-exclusion column yielded a single band of molecular mass 60 kDa together with a minor contaminating band at 58 kDa (Figure 3a). The 43 kDa lower-molecular-mass form of NMT yielded a major band of 49 kDa

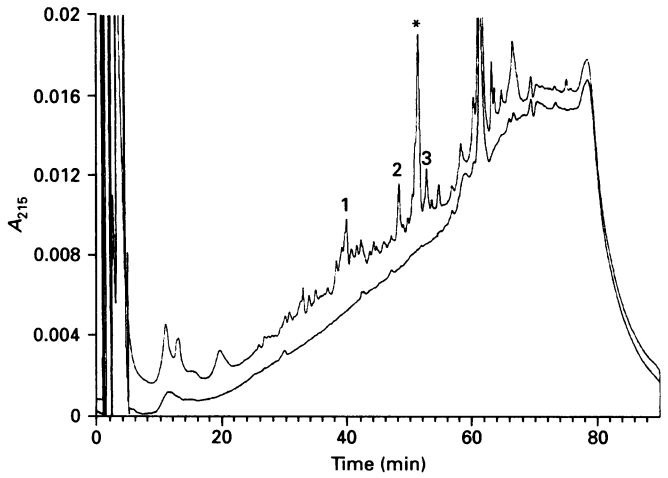

Figure 4 Separation of tryptic peptide fragments of bovine NMT by h.p.l.c.

Digestion of the 60 kDa form of bovine NMT and separation of the tryptic fragments were performed as described in the Materials and methods section. The lower trace represents the profile obtained with the control trypsin digest. The fragments indicated by numbers were sequenced and the peptide indicated by the asterisk failed to yield sequence presumably due to N-terminal blockade.

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Bovine                (1b)AIELFSVGQG-AK     (1a)SYQFWDVQLF-K
Human   1 M.NSLPAERIQEIQKAIELFSVGQGPAKTMEEASKRSYQFWDTQPVPKLG   50
Yeast   1 MSEEDKAKKLENLLKLLQLNNDDTSKFTQEQKKAMKDHKFWRTQPVKDFD   50

Bovine                                                (2)ELYTLLN
Human  51 EVVNTHGPVEPDK..DNIRQEPYTLPQGFTWDALDLGDRGVLKELYTLLN  100
Yeast  52 EKVVEEGPIDKPKTPEDISDKPLPLLSSFEWCSIDVDNKKQLEDVFVLLN  100

Bovine    ENYVEDDDNMFR
Human 100 ENYVEDDDNMFRFDYSPEFLLWALRPPGWLPQWHCGVRVVSSRKLVGFIS  150
Yeast 100 ENYVEDRDAGFRFNYTKEFFNWALKSPGWKKDWHIGVRVKETQKLVAFIS  150

Human 151 AIPANIHIYDTEKKMVEINFLCVHKKLRSKRVAPVLIREITRRVHLEGIF  200
Yeast 151 AIPVTLGVRGKQVPSVEINFLCVHKQLRSKRLTPVLIKEITAAVNKCDIW  200

Human 201 QAVYTAGVVLPKPVGTCRYWHRSLNPRKLIEVKFSHLSRNMTMQRTMKLY  250
Yeast 201 HALYTAGIVLPAPVSTCRYTHRPLNWKKLYEVDFTGLPDGHTEEDMIAEN  250

Human 251 RLPETPKTAGLRPMETKDIPVVHQLLTRYLKQFHLTPVMSQEEVEHWFYP  300
Yeast 251 ALPAKTKTAGLRKLKKEDIDQVFELFKRYQSAFELIQIFTKEEFEHNFIG  300

Human 301 ......QENIIDTFVVENANGEVTDFLSFYTLPSTIMNHPTHKSLKAAYS  350
Yeast 301 EESLPLDKQVIFSYVVEQPDGKITDFFSFYSLPFTILNNTKYKDLGIGYL  350

Bovine                                                (3)FDVFNALD
Human 351 FYNVHT.................QTPLLDLMSDALVLAKMKGFDVFNALD  400
Yeast 351 YYYATDADFQFKDRFDPKATKALKTRLCELIYDACILAKNANMDVFNALT  400

Bovine    LMENK
Human 401 LMENKTFLEKLKFGIGDGNLQYYLYNWKCPSMGAE.............KV  450
Yeast 401 SQDNTLFLDDLKFGPGDGFLNFYLFNYRAKPITGGLNPDNSNDIKRRSNV  450

Human 414 GLVLQ
Yeast 453 GVVML
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Figure 5 Comparison of human, yeast and bovine NMT peptide sequences

The upper partial sequences are derived from the bovine protein, the middle sequence is from human NMT and the lower sequence is that of the yeast enzyme. The numbers preceding the bovine peptide sequences refer to the peptides isolated by h.p.l.c. in Figure 4. The underlined human sequences are those identical to the tryptic peptides isolated from bovine NMT. The alignment of the yeast and human NMT sequences follows that described by (Duronio et al., 1992) in which the start of the yeast sequence is offset to permit an optimum alignment with the human sequence. Peptide 1 (Figure 4) yielded two amino acids in each sequenator cycle of which one was reproducibly obtained at a lower yield on each cycle. This was probably due to a contaminating peptide in this peak and the sequence derived from this contaminant is indicated as 1b above. The assignment of valine at position 7 in this sequence is tentative, since valine also occurs at position 7 in the immediately following peptide sequence (1a).

on SDS/polyacrylamide gels, which was contaminated by other proteins (Figure 3b) and consequently the 60 kDa material was chosen for further study. Several unsuccessful attempts were made to obtain an N-terminal sequence from this NMT fraction, suggesting that the N-terminus of the protein is blocked. Therefore a sample of this material was subjected to tryptic digestion, the resulting peptides separated by h.p.l.c. and then subjected to N-terminal sequencing. As shown in Figure 4 the peptide yielded several fragments following treatment with trypsin, and of the four peptides sequenced three yielded sequence information and these are aligned with the sequences of yeast NMT and the recently cloned human NMT (Duronio et al., 1992) in Figure 5.

DISCUSSION

In this report the purification and partial sequencing of an NMT from bovine brain has been described. The purified enzyme had an apparent molecular mass on SDS/polyacrylamide gels of 60 kDa, a specific activity using the $p60^{src}$ peptide substrate of 97000 pmols/min per mg of protein and was purified some 2750-fold from brain cytosol. It should be noted that in earlier experiments (McIlhinney and McGlone, 1990) we observed that removal of membranes from crude brain homogenates resulted in an increase in NMT activity of 3–5-fold due to the elimination of an inhibitor of the enzyme. This means that the enzyme has been purified between 8100 and 12300-fold from crude brain homogenates.

The behaviour of the CoA-affinity-purified NMT during size-exclusion chromatography described here has not been previously reported. The apparent heterogeneity in size of the enzyme, in the absence of myristoyl-CoA, suggests that it is associated with other proteins. Such NMT–protein associations may also be reflected in a heterogeneity in the surface charge of NMT, since the enzyme elutes from MONO-S columns as a broad peak and they could, in part, explain the severe losses of enzyme activity which we experienced during further purification of the enzyme. The NMT–protein associations could be due to either the self aggregation of the enzyme, or the formation of complexes of NMT with either regulatory elements or substrates. The effect of incubating the enzyme with myristoyl-CoA is apparently to break these associations. Since the bulk of the added myristoyl-CoA is removed during the size-exclusion chromatography it is unlikely that this effect of added myristoyl-CoA on NMT is simply due to its breaking non-specific hydrophobic protein interactions. Furthermore once separated in the presence of myristoyl-CoA, the two molecular mass forms of NMT re-chromatograph with molecular masses of 66 kDa and 43 kDa without requiring further additions of myristoyl-CoA. The effects of the added myristoyl-CoA therefore could be due to the formation of a stable myristoyl-CoA–enzyme complex as described for yeast NMT (Rudnick et al., 1990).

The appearance of two forms of NMT of different molecular mass following incubation with myristoyl-CoA was unexpected. While this could be due to the presence of two isoforms of the enzyme we think that this is unlikely since the relative amounts of each form varied with enzyme preparation and time of storage, with the smaller 43 kDa form predominating with prolonged storage at 4 °C. This suggests that the enzyme is converted into the low-molecular-mass form, possibly by proteolysis, during storage. The lower-molecular-mass form, however, remains active and similar active NMT fragments have been described for the yeast enzyme (Rudnick et al., 1990). To date we have been unable to obtain the smaller form of the enzyme in sufficient purity or yield to allow its full characterization and this work is continuing in order to compare the activity and structure of the two different molecular mass forms of the enzyme.

N-terminal sequencing of the 60 kDa form of bovine NMT proved impossible due presumably to the N-terminus being blocked, as is that of the yeast enzyme (Towler et al., 1987). However, N-terminal sequencing of tryptic peptides derived

from this material yielded four sequences, three of which are a perfect match to the recently cloned human enzyme sequence (Duronio et al., 1992) with the other peptide showing partial sequence similarity to the human sequence (Figure 5). The similarities between the sequences derived from the purified bovine enzyme and those deduced from the cloned human enzyme confirm the identity of the material described here as NMT. It is also clear that there is considerable identity between the human bovine sequences and partial sequence similarity between the mammalian and yeast enzymes.

The gene sequence obtained for the human enzyme codes for a protein containing 416 amino-acid residues with an estimated molecular mass of 48 kDa, while the purified bovine enzyme has an apparent molecular mass of 60 kDa. This apparent discrepancy between the predicted size of the cloned human NMT and that found for the larger form of bovine NMT could simply be due to the enzyme behaving anomalously on SDS/polyacrylamide gels. Alternatively a larger form of the mammalian enzyme may be synthesized and in this context it is interesting to note that there are alternative start codons in the cloned cDNA sequence of the human gene which could produce a larger protein which is N-terminally extended by 62 residues (Duronio et al., 1992). The availability of the cloned sequence for human NMT, and the possibility of obtaining antisera to the enzyme mean that the questions above can now be addressed. In addition antisera could be used to localize the enzyme in cells and for a study of the apparent association of NMT with other proteins. The availability of pure enzyme will also greatly facilitate the kinetic characterization of the mammalian enzyme, and this in turn should lead to the more rational design of inhibitors which may prove valuable as anti-viral agents.

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