Identification and Characterization of Multiple Forms of Bovine Brain N-Myristoyltransferase*

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N-Myristoyltransferase (NMT) catalyzes the co-translational addition of myristic acid to the N-terminal glycine of many cellular, viral, and fungal proteins which are essential to normal cell functioning and/or are potential therapeutic targets. We have found that bovine brain NMT exists as a heterogeneous mixture of interconvertible high molecular mass multimers involving ~60-kDa NMT subunit(s). Gel filtration chromatography of partially purified NMT at low to moderate ionic strength yields NMT activity eluting as 391 ± 52 and 126 ± 17 kDa peaks as well as activity which profiles the protein fractions and likely results from NMT nonspecifically associating with background proteins and/or column matrix. Chromatography in 1 M NaCl causes 100% of this activity to elute as a single peak of ~391 kDa. Subsequent treatment of the ~391 kDa activity peak with an N peptide reaction product (i.e. N-myristoyl-peptide) results in ~75% of the activity re-eluting as a ~126-kDa peak in 1 M NaCl. Rechromatography also yields small amounts of a ~50-kDa NMT monomer which increases with prior storage at 4 °C. Up to 5 NMT subunits were identified by SDS-polyacrylamide gel electrophoresis and specific immunoblotting with a human NMT peptide antibody and by cofactor-dependent chemical cross-linking with an 125I-peptide substrate of NMT. The prominent 60-kDa and minor 57-, 53-, 49-, and 47-kDa NMT immunoblotted subunits co-migrate with five of nine silver-stained proteins in an enzyme preparation purified >7,000-fold with ~50% yield by selective elution from octyl-agarose with the myristoyl-CoA analog, S-(2-ketopentadecanal)-CoA. Storage at 4 °C also leads to conversion of the larger NMT subunit(s) into 49 and 47 kDa forms with no loss of NMT activity. These results identify two interconvertible forms of NMT in bovine brain that result from NMT subunit multimerization and/or complex formation with other cellular proteins. The data also identify a fully active NMT monomer which arises from subunit proteolysis. This study thus reveals a previously unappreciated level of NMT complexity which may have important mechanistic and/or regulatory significance for N-myristoylation in mammalian cells.

N-Myristoylation is the co-translational covalent attachment of myristic acid in amide linkage to the N-terminal glycine residue of a number of mammalian, viral, and fungal proteins (1-4). For many proteins synthesized on free polyribosomes, co-translational N-myristoylation is required for their proper subcellular compartmentalization and subsequent biological function. For example, N-terminal myristate, in conjunction with N-terminal basic residues, is instrumental in effecting interactions of the protein tyrosine kinase, p60src, with the plasma membrane (5) which in turn is essential for p60src-mediated cellular transformation (6). N-Myristoylation is also required for the plasma membrane association of the polyprotein precursor of the human immunodeficiency virus internal structural polyprotein, p55, from which site it directs the assembly of virus capsids and their budding from infected cells (7, 8). N-Myristoylation of certain proteins of Candida albicans and Cryptococcus neoformans, pathogenic fungi which affect immunocompromised patients, is essential for their vegetative growth (9, 10). The central role for myristate in these processes has made N-myristoylation a chemotherapeutic target for antiviral (11-14) and anti-fungal (9, 15, 16) therapies. N-Myristoylation results from the catalytic transfer of myristic acid from myristoyl-CoA to appropriate protein substrates by myristoyl-CoA:protein N-myristoyltransferase (NMT)1 (EC 2.3.1.97). NMT has been purified to homogeneity from the yeast Saccharomyces cerevisiae (17), extensively characterized (1, 17-25), and shown to be a ~53-kDa monomer. The hNMT gene has been isolated by functional complementation of a yNMT temperature-deficient mutant and predicts a ~48-kDa protein having 44% homology with the yeast enzyme (26).

In contrast to yNMT, NMTs purified from several mammalian sources exhibit varying molecular masses, charge heterogeneity, and/or peptide substrate specificities. For example, while native NMT partially purified from human erythroleukemia cells (27) or purified to near homogeneity from bovine spleen (28) exhibits apparent molecular masses by gel filtration consistent with a monomeric 48-58-kDa enzyme, the enzyme from murine leukemia L1210 cells (29) and bovine brain (30-34, 52) display apparent molecular masses up to 390 kDa. Furthermore, at least two distinct forms of NMT have been isolated from L1210 cells (29) while chromatofocusing (30) and ion-exchange chromatography (32, 34) have resolved as many as four separate forms of the enzyme from bovine brain. Assuming that there is a single mammalian gene (26), these data suggest the existence of homo- and/or heteromultimeric NMT complexes. The functional significance and molecular basis for multiple high molecular mass NMTs and NMT subunits ranging in size from 48 to 67 kDa remains unexplained (27, 28, 33, 35).

To begin to address these questions, we have examined the

1 The abbreviations used are: NMT, N-myristoyltransferase; bNMT, bovine brain NMT; hNMT, human NMT; yNMT, yeast NMT; PAGE, polyacrylamide gel electrophoresis; MOPS, 2-(N-morpholino)ethanesulfonic acid; CAPS, 3-(cyclohexylamino)propane sulfonic acid.
hydromechanical properties and subunit structure of bovine brain NMT (bNMT). We have determined that the bNMT exists in vitro as a dimeric subunit complex which can be intercon
verted by manipulation of ionic strength and/or by treatment with an N-myristoyl-peptide product of the NMT reaction. We have also identified a subunit heterogeneity for bNMT which arises in part from proteolysis of larger subunit precursor(s) and which apparently accounts for the accumulation of a fully active monomeric form of the enzyme. Our data thus provide in one system an explanation for the disparity of data concerning the native NMT structure(s) including physiologically relevant processes which may account for the enzyme quaternary struc
ture and activity. We also provide information for circumvent
ing problems encountered during the manipulation and purifi
cation of NMT from complex tissues such as bovine brain.

EXPERIMENTAL PROCEDURES

Purification of bNMT—All manipulations were carried out at 4°C. Fresh or frozen (−80°C) bovine brain (200 g) was homogenized in 600 ml of homogenization buffer containing proteolytic enzyme inhibitors and bNMT was partially purified by 35−70% (NH4)2SO4 fractionation and batch elution from DEAE-Sepharose Fast Flow (Pharmacia Biotech Inc.) with 130 mM NaCl as described previously (36). The resulting NMT activity was concentrated by ultrafiltration with a YM10 mem
brane (Amicon, Beverly, MA) to 3−4 ml, adjusted to 18% sucrose, applied in two separate loadings to a Sephacryl S-200 HR (or Sephacryl S-300 HR) column (2.5 × 97 cm) (Pharmacia) equilibrated in gel filtra
tion buffer (50 mM potassium phosphate (pH 7.4)) buffer with 1 mM EDTA, 20 μg/ml soybean trypsin inhibitor (Sigma), 2 μg/ml leupeptin (Boehringer Mannheim), 2 μg/ml aprotinin (Boehringer Mannheim), and 0.02% sodium azide containing 1 mM NaCl, and eluted at 8−10 ml/h into 5.8-ml fractions. Assays for enzyme activity revealed a single symmetrical NMT peak nearly coincident with the first eluting protein peak (monitored by absorption at 280 nm). The leading fractions of the active peak were pooled so as to maximize the recovery of enzyme activity and minimize retention of lower molecular weight contaminating pro
teins and concentrated by ultrafiltration to 1−2 ml. The ionic strength was then reduced by repeated dilution with gel filtration buffer followed by ultrafiltration (total of three times) to give a concentrated enzyme solution (<0.05 mM NaCl). To this concentrated enzyme was added 10 mM of an N-myristoylated peptide corresponding to residues 2−10 of p60
(N-myristoyl-GSSKSKPKD), and the mixture was incubated on ice for 10 min. The enzyme mixture was adjusted to a final concentration of 1 mM NaCl and 18% sucrose, diafiltrated by centrifugation, applied to a Sephacryl S-100 HR column (2.5 × 96 cm) equilibrated in gel filtration buffer containing 1 mM NaCl, and eluted and analyzed as described above. The eluting fractions from the resulting major enzyme activity peak (peak II) were pooled so as to maximize the recovery of enzyme activity at the expense of higher molecular weight contaminating proteins and is referred to as the Sephacryl S-100 HR NMT fraction. Final purification was achieved by applying peak II to a column of octyl-agarose (~1.5 unit/ml resin) (Sigma) equilibrated in 50 mM potassium phosphate (pH 7.4) buffer containing 1 mM EDTA, 1.6 μg/ml leupeptin, 1.6 μg/ml aprotinin, 0.02% sodium azide, and 1 mM NaCl, followed by immediately washing (10−20 ml/min) with 8 column volumes of equilibration buffer to remove unadsorbed protein and by washing (5 ml/min) with 10 column volumes of equilibration buffer containing 1 mM S-(2-ketopen
tadecyl)-CoA (prepared as we have previously described (36)) to elute the NMT activity. The fractions containing NMT activity were concen
trated to ~50 μl by ultrafiltration and centrifugation in a Centrifloc 10 (Amicon). The concentrated enzyme was resuspended in 1 ml of equil
ibration buffer containing 1 mM myristoyl-CoA and reconstituted to ~50 μl as before and is referred to as the octyl-agarose NMT fraction. This octyl-agarose step was routinely performed using up to 5 units of NMT activity and ~100 μg of protein.

Analytical Procedures—SDS-PAGE (37) was performed on 10% poly
acrylamide gels (Integrated Separation Systems, Inc., Hyde Park, MA), and proteins were visualized by Pro-Blue or silver staining (Integrated Separation Systems, Inc.). Protein concentrations were determined by the BCA (Pierce) or colloidal gold (Integrated Separation Systems, Inc.) procedures with bovine serum albumin standard curves. Synthetic pep
tides were purchased from Peptide Technologies (Washington, D.C.). Peptides containing the N-terminal cysteine to SulfoLink Coupling Gel (Pierce) and eluting as a major peak were purified by fast protein liquid chromatography (Pharmacia). Gel filtration cali
tration curves were constructed using Low and High Molecular Weight Gel Filtration Kit proteins (Pharmacia) by plotting the logarithm of molecular weights against Kav (39).

NMT Assay—The assay for NMT activity was similar to that previ
ously described (38) and included 100 μM GSSKSKPKDSPQRH, 10 nM GSSKSKPKDSPQRHY, 100 μM myristoyl-CoA, 0.25% bovine serum albumin, and 40 mM HEPES (pH 7.6) buffer in a final volume of 0.1 ml. The radioidi
nated N-myristoyl-peptide product was isolated by C18 reverse phase high performance chromatography and quantified in a gamma counter. One unit of enzyme activity is defined as 1 nmol of peptide N-acylated/min at 37°C.

Cofactor-dependent Chemical Cross-linking—Cofactor-dependent chemical cross-linking was accomplished by incubating 40−50 μg of protein from the Sephacryl S-100 HR NMT fraction with ~10 nM GSSKSKPKDSPQRH, 10 nM GSSKSKPKDSPQRHY (2 × 10−6 to 1 × 10−4 counts/min) in the presence of 4, 10, or 20 μM S-(2-ketopentadecyl)-CoA or in the presence of 10 μM S-(2-ketopentadecyl)-CoA plus 5, 10, or 50 μM GSSKSKPKDSPQRHY in 16 mM HEPES (pH 7.4) buffer in a final volume of 0.020 ml. After 5 min at room temperature, cross-linking was initiated by the addition of 5 μl of 12.5 mM 1-ethyl-3-(3-dimethylaminopropyl)carbo
midimide (Sigma) in 1 M MOPS (pH 6.0) buffer, and the mixture was incubated for 30 min at room temperature. Cross-linking was termi
nated by the addition of 0.050 ml of SDS-sample buffer (37) and boiling for 5 min. Denstometry analysis of autoradiograms was performed using a Laser Scan and the GelScanXL (v. 2.1.2) software (Pharmacia).

Immunoblotting—Rabbit antiserum to a peptide corresponding to residues 27−38 (i.e. KTMEAEKRSYQ) encoded by the hNMT gene (26) was prepared by Research Genetics (Huntsville, AL) using the multiple antigen peptide resin technology (40). Peptide specificity of the antiserum was determined by enzyme-linked immunosorbent assays (41). The serum immunoglobulin fraction was isolated using Immunopure Peptide A/G (Pierce). Affinity purification of the hNMT peptide antibody was accomplished by applying the immunoglobulin fraction to an affinity column containing the peptide antigen immobilized through an N-terminal cysteine to SulfoLink Coupling Gel (Pierce) and eluting as recommended by the manufacturer. Monoclonal antibodies were produced by SDS-
PAGE, electroblotted to nitrocellulose membranes, and the monoclonals were blocked with 1% bovine serum albumin. After incubation with primary antibody, immuneoreactive bands were visualized using goat anti-rabbit secondary antibodies conjugated to alkaline phosphatase and 5-bromo-4-chloro-3-indyl phosphate/nitro blue tetrazolium was used as substrate (Promega Corp., Madison, WI).

Deglycosylation of N-Linked or O-Linked Oligosaccharides—Fetuin (20 μg/ml, Sigma) or the Sephacryl S-100 HR NMT fraction (11 μg of protein) were denatured and deglycosylated with N-glycosidase, neurami
nidase, and O-glycosidase essentially as recommended by the Gen
zyme Company (Cambridge, MA) and included leupeptin (5 mg/ml) and aprotinin (10 mg/ml) with incubations up to 18 h at 37°C. The effect of deglycosylation on the electrophoretic migration of fetuin was visualized by staining with Pro-Blue while that on NMT was assessed by immunoblotting.

Preparation of Recombinant hNMT—cDNAs for hNMT were ob
tained by polymerase chain reaction amplification of a reverse-tran
scribed cDNA template derived from the human breast carcinoma cell line MDA486 (provided by Drs. J. James Battey and Mark Helmi
ch, Laboratory of Biological Chemistry, DTP, NCI, NIH), using synthetic primers and cloned into a pFLAG expression vector (IBI, New Haven, CT). The pFLAG vectors were transformed into Escherichia coli strain BL21 and hNMT was induced with 500 μm isopropl-β-thiogalacto
pyranoside in Luria-Bertani broth containing 100 μg/ml ampicillin. Lysyosome (0.25 mg/ml) (Sigma)-treated bacterial pellets were extracted into buffer containing 0.05 mg/ml ovomucoid protease inhibitor (Sig
ma), and the 18,000 × g supernatant fraction was subjected to SDS-
PAGE and either immunoblotted or stained with Pro-Blue.

RESULTS

Multiple Forms of bNMT—We have investigated the ques
tion of bNMT heterogeneity using size exclusion chromatogra
phy. bNMT which had been partially purified by (NH4)2SO4 fractionation and DEAE-Sepharose ion-exchange chromatogra
phy (36) was applied to a column of Sephacryl S-200 HR equil
ibrated in 50 mM potassium phosphate (pH 7.4) buffer contain
ing proteolytic enzyme inhibitors. Under these conditions the majority of NMT activity eluted as two comparable peaks, the
first close to the column void volume (peak I) followed by a closely eluting second peak (peak II). Meanwhile, the remaining activity eluted in tailing fractions, generally coinciding with the protein elution profile (Fig. 1A). When compared to molecular mass standards, peaks I and II exhibited apparent molecular masses of 391 ± 52 and 126 ± 17 kDa, respectively (Table I). In addition, the general spreading of enzyme activity was suggestive of nonspecific interactions between NMT and other cellular proteins and/or column matrix. Considering that this gel filtration analysis, as well as previously described chromatofocusing (30) and ion-exchange (32, 34) studies, were conducted under conditions of low to moderate ionic strength, we speculated that the trailing of activity might be due to nonspecific ionic interactions.

Reversible Equilibrium between Multimeric Forms of \( bNMT \)—In order to suppress nonspecific interactions, the DEAE-Sepharose purified \( bNMT \) was again subjected to gel filtration analysis as described above except that the Sephacryl S-200 HR column was equilibrated in 50 mM potassium phosphate (pH 7.4) buffer containing 1 M NaCl. In addition to preventing the general spreading of enzyme activity over the latter fractions, 1 M NaCl caused all of the NMT activity to elute as a single symmetrical peak near to the column void volume corresponding to the elution volume of the first of the two major activity peaks (peak I) eluted in the absence of NaCl (Fig. 1B). Similar results were obtained using Sephacryl S-300 HR columns. Our finding of oligomeric forms of \( bNMT \) is consistent with the reported presence of high molecular weight forms of the enzyme in bovine brain (32–34). It is, however, in marked contrast with reports of NMTs having native molecular masses of 48–58 kDa in yeast, human erythroblasts, and bovine spleen (17, 27, 28). We therefore considered the possibility that in addition to blocking nonspecific ionic interactions, the high ionic strength used in our experiments might induce the formation of a single high molecular weight form of the enzyme by promoting hydrophobic interactions between NMT subunits and/or between NMT and other proteins. If true, we speculated that NMT multimerization might be prevented and/or reversed by treatment with amphipathic agents.

To test this hypothesis, the NMT activity (peak I) from the Sephacryl S-200 HR column shown in Fig. 1B was pooled, concentrated, re-equilibrated in 50 mM potassium phosphate (pH 7.4) buffer without NaCl, and treated with 10 mM of a synthetic N-myristoylated peptide corresponding to amino acids 2–10 of p60\( \text{src} \) (i.e. N-myristoyl-GSSKSKPKD). In addition to being amphipathic, the N-myristoyl-peptide is an enzymatic product of the NMT reaction. Upon subsequent rechromatography on a Sephacryl S-100 HR column equilibrated in 50 mM potassium phosphate (pH 7.4) buffer containing 1 M NaCl, only ~25% of the applied activity eluted close to the original peak I while the majority of the applied activity (~75%) eluted with an apparent molecular mass identical with peak II and ~2% of the activity eluted as a third peak of ~50 kDa (peak III) (Fig. 1C). Comparable results were obtained when the experiment was carried out using Sephacryl S-200 HR, Superdex 200, or Superose 12 columns (Table I). When the activity in peak I (Fig. 1C) was again treated with 10 mM N-myristoyl-GSSKSKPKD and rechromatographed on the same Sephacryl S-100 HR col-

| TABLE I | Gel filtration chromatography of bNMT |
|---|---|---|---|
| Columns | Apparent molecular mass of activity peaks | I | II | III |
| Superdex 200 | 315,000 | 120,000 | 48,500 |
| Superose 12 | 350,000 | 107,000 |
| Sephacryl S-300 HR | 465,000 |
| | 400,000 |
| | 430,000 |
| Sephacryl S-200 HR | 380,000 |
| | 440,000 |
| Sephacryl S-100 HR | 135,000 |
| | 150,000 |
| | 118,000 |
| Mean (± S.D.) | 391,000 ± 52,000 | 126,000 ± 17,000 | 48,500 |
umn as described above, the applied activity eluted with a distribution similar to that shown in Fig. 1C except that the amount of activity in the ~50 kDa peak increased with lengthened time of storage at 4 °C prior to rechromatography (data not shown). Furthermore, when the enzyme in peak II was rechromatographed on a Superdex 200 column equilibrated in Not shown). Furthermore, when the enzyme in peak II was rechromatographed on a Superdex 200 column equilibrated in 10 mM Tris-CAPS (pH 9.4) buffer containing 1 mM NaCl, the NMT activity once again eluted as the original ~391-, ~126-, and ~50-kDa peaks (data not shown). Together these experiments provide evidence for two major forms of native bNMT of ~391 and ~126 kDa as well as a minor ~50 kDa form which accumulates during storage. The experiments also reveal an equilibrium between the ~391 and ~126 kDa forms which is readily manipulated by changes in ionic strength and/or by the presence of an N-myristoyl-peptide. Finally, our ability to manipulate this multimerization phenomenon proved useful in the eventual purification of the enzyme.

Purification of bNMT—The ability to block nonspecific interactions while controlling the elution volume of NMT from gel filtration columns provided a major advance in our purification of bNMT. Following (NH4)2SO4 fractionation and batch elution from a DEAE-Sepharose ion-exchange column, significant purification was accomplished by two consecutive size exclusion columns, initially under conditions favoring the accumulation of peak I and the second under conditions promoting the formation of peak II as described above. This tandem size fractionation removed the majority of low molecular weight contaminants by the first Sephacryl S-200 HR column (see Fig. 1B) followed by the removal of most of the high molecular weight impurities by the subsequent Sephacryl S-100 HR column (see Fig. 1C). The enzyme was thus readily purified ~500-fold over 4 days and exhibited excellent stability when concentrated in the same elution buffer: half-life of ~10 days at 4 °C and ~9 mg protein/ml. Final purification was obtained by applying peak II to an octyl-agarose column and eluting the NMT activity with 1 μM of the myristoyl-CoA analog, S-(2-ketopentadecyl)-CoA (36, 42). The enzyme was thereby purified ~7,000-fold with >50% recovery from the activity observed in the (NH4)2SO4 fraction (Table II) and exhibited up to nine protein bands when analyzed by SDS-PAGE and silver staining (see Fig. 3A, lane 2). The p53 marker indicates the position of a very lightly stained protein which, although seen in the original gel, is not visible in the photograph shown in Fig. 3. A silver stain of the Sephacryl S-100 HR NMT fraction is also shown for comparison (Fig. 3A, lane 1).

Identification of NMT Subunits—Two experimental approaches were used to identify the number and apparent molecular size(s) of the bNMT subunit(s) in the purified NMT fractions. The first approach was based upon the assumption that bNMT follows the same sequential ordered Bi Bi mechanism as described for yNMT and hNMT where initial myristoyl-CoA binding is required before subsequent binding of the acyl acceptor peptide substrate (24, 25). Replacement of the myristoyl-CoA with the high affinity and non-hydrolyzable myristoyl-CoA analog S-(2-ketopentadecyl)-CoA prevents acyl transfer and stabilizes the normally transient ternary complex. Subsequent chemical cross-linking of juxtaposed ε-amino and carboxyl groups by a water-soluble carbodiimide leads to covalent linkages between enzyme and the peptide substrate. Such an analysis was carried out by incubating the Sephacryl S-100 HR NMT fraction with the 125I-peptide substrate used in our standard enzyme assay (i.e. GSSKSKPKDSPQRRELGRG) alone or in the presence of 4, 10, or 20 μM S-(2-ketopentadecyl)-CoA. Cross-linking between GSSKSKPKDSPQRRELGRG and NMT was then accomplished by the addition of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide, and after appropriate incubation the mixtures were analyzed by SDS-PAGE and autoradiography (Fig. 2A). As shown, cross-linking resulted in the radiolabeling of a prominent 75 kDa band (p75) in all lanes, including the control without cofactor analog (lane 1), which varied only slightly in intensity across the gel and which appears to represent a nonspecifically radiolabeled protein. However, in the presence of increasing concentrations of the cofactor analog, a broad new band of 64–66 kDa was also radiolabeled in a dose-dependent manner (lanes 2–4). In some experiments, this latter band was clearly resolved into at least two distinct bands of 64 and 66 kDa (lane 5); also note the bands at 58 and 47 kDa which were similarly weakly radiolabeled in a dose-dependent manner. The specificity of cross-linking was confirmed by dose-dependent competition of radiolabeling with increasing concentrations of the non-radioactive peptide substrate (lanes 5–7). When the specific cross-linking was normalized to the nonspecific radiolabeling of the respective 75 kDa band in each lane by densitometry, cross-linking of the 64–66 (p66 + p64) and 47 (p47) kDa bands increased ~8–fold relative to cross-linking in the control lane (see Fig. 2A, lane 1) and were coordinately decreased to control levels by addition of the non-radioactive peptide competitor (Fig. 2B). These data provide evidence for the presence in this partially purified enzyme mixture of at least four polypeptides which bind the 125I-peptide substrate in a cofactor-dependent manner. From a comparison of the specific activities of the enzyme and polypeptide compositions in the Sephacryl S-100 HR NMT and octyl-agarose NMT fractions (Table II), we estimate that NMT in the cross-linking reactions represents <5% of the total protein; therefore, chemical cross-linking exhibits a level of specificity consonant with that expected for radiolabeling of the NMT subunits. The data also reveal that preparations of the bNMT contain several different sized subunits.

Multiple NMT subunits were also identified in the purified enzyme preparations by immunoblotting with an affinity purified hNMT antibody. For this analysis, a polyclonal antibody was raised against a synthetic peptide encoded by the hNMT gene (26). Antibody specificity was confirmed by immunoblotting of bacterially expressed hNMT and by total competition of that immunostaining by preincubation of the antibody with 20 μM of the peptide antigen (data not shown). Immunoblotting of the Sephacryl S-100 HR NMT fraction with this affinity purified antibody revealed a major stained band of 60 kDa and four lesser stained bands corresponding to polypeptides of 57, 53,
Increasing concentrations of the non-radioactive peptide (radioactivity of the same band in each respective lane and plotted as a percentage of the maximum p75 level of radioactivity in the non-specifically labeled 75kDa band (p66) or the 47 kDa band (p47) was normalized to the difference in apparent molecular weights of the upper specifically cross-linked bands and the reduced resolution of the broad lower cross-linked band, most likely resulting from the addition of covalently linked peptide. These data thus reveal an exceptional coincidence of cross-linking, immunoblotting, and silver staining, which is consistent with the identification of up to 5 NMT subunits in the enzyme fractions purified from bovine brain.

Characterization of bNMT Subunit Heterogeneity—In order to exclude the possibility that the apparent size and/or heterogeneity of bNMT subunits is the result of protein glycosylation, the Sepharocl S-100 HR NMT fraction was incubated with N-glycosidase or with neuraminidase followed by O-glycosidase, and the effect of these treatments on bNMT subunit electrophoretic mobility was determined by SDS-PAGE and immunoblotting with our affinity purified hNMT antibody. These analyses revealed no discernible effect on the pattern of 49, and 47 kDa (Fig. 3B, lane 2). Again, the immunostaining of all five bands was blocked by preincubation of the antibody with 20 μM of the peptide antigen (Fig. 3B, lane 1). A similar analysis of the octyl-agarse NMT fraction revealed the same immunostaining pattern (Fig. 3B, lane 3) except for the 53 kDa band which is not seen in Fig. 3B but which was evident in other blots from the same preparation, although at a reduced intensity compared to the blot from the Sepharocl S-100 HR NMT fraction (data not shown). These experiments thus identify five polypeptides in our most purified enzyme fractions with immuno-cross-reactivity to hNMT. Analysis of duplicate aliquots of the octyl-agarse NMT fraction by SDS-PAGE and either immunoblotting or silver staining revealed that the five immunostained bands had electrophoretic mobilities identical with five of the nine silver-stained bands (indicated by triangle markers in Fig. 3, A and B). Also, the pattern of immunoblotted bands (Fig. 3B, lane 2) was notably similar to the pattern of radiolabeled cross-linked bands (see Fig. 2A) except for the difference in apparent molecular weights of the upper specifically cross-linked bands and the reduced resolution of the broad lower cross-linked band, most likely resulting from the addition of covalently linked peptide. These data thus reveal an exceptional coincidence of cross-linking, immunoblotting, and silver staining, which is consistent with the identification of up to 5 NMT subunits in the enzyme fractions purified from bovine brain.

FIG. 3. Silver staining and immunoblotting of bNMT. Aliquots from the Sepharocl S-100 HR NMT (1.5 μg of protein) and octyl-agarse NMT fractions (0.16 μg of protein) were analyzed by SDS-PAGE and either silver stained for protein or immunoblotted for bNMT subunits. Part A, silver stain: lane 1, Sepharocl S-100 HR NMT fraction; lane 2, octyl-agarse NMT fraction. Part B, immunoblot: lane 1, Sepharocl S-100 HR NMT fraction blotted with an affinity purified hNMT peptide antibody which had been preincubated with 20 μM peptide antigen (+); lane 2, Sepharocl S-100 HR NMT fraction blotted with untreated (−) antibody; lane 5, octyl-agarse NMT fraction blotted with an untreated (−) antibody.
enzyme-treated bNMT while the mobility of the fetuin control was quantitatively changed with each treatment (data not shown).

The effect of storage on NMT subunit activity and heterogeneity was also examined. Immunoblotting of the octyl-agarose NMT fraction at different times during storage at 4 °C revealed a gradual conversion of the higher molecular mass NMT subunits (i.e., 60, 57, and 53 kDa) into the lower molecular mass forms (i.e., 49 and 47 kDa) over several months (Fig. 4A). During the same time period the corresponding NMT activity was not significantly affected (Fig. 4C). However, when a fresh octyl-agarose NMT sample was frozen at −15 °C for 10 days, the typical immunostained pattern of five bands (Fig. 4A, lane 1) was converted to a doublet corresponding to the 47 kDa band and a new slightly faster migrating species of 46 kDa (Fig. 4A, lane 5) while NMT activity was concomitantly reduced by ~30% (Fig. 4C, frozen). Subunit heterogeneity was also examined in a freshly prepared tissue homogenate; the 60 and 47 kDa bands were in approximately the same proportions as seen in the fresh octyl-agarose NMT sample (see Fig. 4A, lane 1), while the other intermediate forms were not observed (data not shown).

The effect of freezing on the NMT subunit pattern was also followed by silver staining (Fig. 4B). This analysis confirmed the loss of the 60 and 49 kDa bands. However, the retention of a 57 kDa silver-stained band after freezing suggested the presence of still another non-NMT protein which co-migrates with the 57-kDa NMT subunit in the octyl-agarose NMT fraction. Together, these data indicate that some or all of the 49- and 47-kDa subunits result from proteolytic processing of larger precursors. Since the same proportion of 60- and 47-kDa subunits were observed in freshly prepared brain extracts as were in the highly purified octyl-agarose NMT preparations and that the conversion of the 60-kDa subunit to the 49- and 47-kDa subunits required several months, it appears that this processing is very slow. It is likely, therefore, that NMT subunit heterogeneity is not totally the result of post-extraction proteolysis but reflects a similar in vivo process, possibly with regulatory significance.

DISCUSSION

We have found that bNMT activity exists as two interconvertible oligomeric complexes which we ascribe to a reversible multimerization of ~60 kDa NMT subunit(s). Evidence for this conclusion includes (i) the conversion of a mixture of ~391 and ~126 kDa forms of NMT (see Fig. 1A) into a single ~391-kDa species by treatment with 1 M NaCl (see Fig. 1B), (ii) the subsequent partial dissociation of this ~391-kDa multimer back into the ~126 kDa form upon treatment with an N-myristoyl-peptide product of NMT (see Fig. 1C), and (iii) the reappearance of both forms of the enzyme when the isolated ~391- and ~126-kDa enzymes are rechromatographed. The data also reveal the accumulation during storage of a fully active monomeric form of the enzyme (i.e., ~50 kDa) presumably resulting from proteolysis of the ~60-kDa subunit. Considering the co-purification of several unidentified proteins with our >7,000-fold purified enzyme preparation, the high molecular weight forms of NMT could also involve other cellular proteins. Nevertheless, assuming that the ~391- and ~126-kDa enzymes represent hexamers and dimers, respectively, of a ~60-kDa NMT subunit, we propose the following model to explain the interrelationships described in this study (Fig. 5). We speculate that dimers made up of two ~60-kDa NMT subunits are formed independent of ionic strength but are dissociated by SDS sample buffer. At low to moderate ionic strength, NMT exists primarily in the form of dimers and hexamers as illustrated by our finding of two prominent high molecular mass forms (i.e., ~126 and ~391 kDa) by gel filtration in the absence of 1 M NaCl (see Fig. 1A). Significant amounts of activity are also found in the later eluting fractions presumably as a result of nonspecific ionic interactions between NMT and background proteins and/or column matrix. Upon treatment with 1 M NaCl, 100% of the NMT activity is converted into a hexamer by blocking nonspecific interactions and by enhancing hydrophobic surface contacts between the dimers (see Fig. 1B). Subsequent treatment with N-myristoyl-CoA and 1 M NaCl dissociates most of the hexamers into dimers due to the binding of the myristoyl moiety to hydrophobic contact sites and/or pockets (i.e., myristoyl-CoA-binding sites) of the dimers thus interfering with dimer-dimer associations. We further suggest that the accumulation of the ~50-kDa monomer during storage reflects the removal of end-to-end subunit contact sites by proteolysis.

In addition to our work reported here and previously (30, 31), this model is consistent with the report that 200 μM myristoyl-CoA promotes the dissociation of a large broad bNMT activity...
peak (i.e. 150–60 kDa by gel filtration) into 66 and 43 kDa forms and that relative amounts of the smaller form increased with prolonged storage (33). This latter study showed that the myristoyl-CoA substrate promotes a dissociation similar to that observed with our N-myristoyl-peptide product. Since N-myristoyl-peptides have been shown to competitively inhibit the binding of myristoyl-CoA to the acyl-CoA-binding site of yNMT (21), it is possible that the dissociation of NMT multimers induced by N-myristoyl-peptide (or myristoyl-CoA) envisioned in our model may reflect a process for regulating bNMT activity.

We have also identified a number of bNMT polypeptides which are immuno-cross-reactive with hNMT but which exhibit apparent molecular masses up to 12 kDa larger than that predicted from the 1248-nucleotide open reading frame assigned to the human enzyme (26). It is unlikely that this difference is due to abnormal migration of the bNMT subunits during SDS-PAGE because of the close homology between bNMT and hNMT as revealed by comparable immunoblotting with an hNMT peptide antibody and partial amino acid sequencing (33) and because hNMT (and yNMT) expressed in bacteria exhibit apparent molecular masses on SDS-PAGE within 2% of their predicted values (19, 43). This assumption is also consistent with the apparent absence in bNMT of covalently linked carbohydrate or other obvious structural features which might account for anomalous electrophoretic mobility (44, 45). Therefore, assuming that the bNMT gene is similar to the gene described for hNMT, then one possible explanation for the apparent size discrepancy is the presence in the hNMT gene of an in-frame methionine start codon 183 nucleotides upstream of the assigned start site which defines an open reading frame encoding a protein 6.7 kDa larger than that predicted for the expected human gene product. We have found that a recombinant hNMT translated from that upstream start site co-migrates with our 57-kDa bNMT subunit. This indicates that either the major 60-kDa species could result from initiation at yet another start site even further upstream or that the 5’ sequences of the bNMT and hNMT genes are different (16, 23).

Our experiments indicate that the subunit heterogeneity of bNMT may in part reflect proteolysis of larger subunit precursor(s). It is unlikely that this cleavage occurs from the C terminus because of our finding that proteolysis did not affect bNMT activity and the fact that the five C-terminal residues of yNMT and at least Leu925 through Lys1016 (of 416 residues) of hNMT are essential for yeast and human NMT catalytic activities (23, 26, 27). Furthermore, since our antibody recognizes amino acids 27–38 of hNMT and must be present in order for the fragments to be immunostained, we conclude that NMT subunit heterogeneity most likely results from cleavage of N-terminal sequences preceding the antibody epitope. The removal of up to 12 kDa of the bNMT N terminus with no apparent effect on enzyme activity is also consistent with the suggestion that the N-terminal domains of NMTs may have in vivo regulatory functions reflecting species-specific requirements (16, 23). The possibility that these N-terminal residues could be responsible for subunit multimerization and/or complex formation with other proteins is consistent with the presumed removal of this 12-kDa N-terminal domain during storage as shown by SDS-PAGE (see Fig. 4) and the parallel accumulation of a monomeric (i.e. ~50 kDa) form of NMT as detected by gel filtration (see Fig. 1C). The N terminus of NMT could also mediate physiologically important protein-protein interactions which (i) determine targeting to specific intracellular compartments (38, 46, 47), (ii) facilitate association with ribosomal N-terminal processing complexes (48), and/or (iii) regulate co-substrate availability through associations with intracellular proteins such as acyl-CoA synthetase or acyl-CoA binding proteins (47, 49–51). It is also possible that the N-terminal proteolysis noted in this study reflects in vivo proc-

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2 C. J. Glover and R. L. Felsted, unpublished observations.
esses designed to release a fully active enzyme from regulatory constraints. The extent to which this latter mechanism may be operational could account for reports of different sized NMT subunits from different mammalian sources (27, 28, 33, 35).

The purification of NMT from mammalian sources has been difficult due to its tendency to lose activity and/or to separate into multiple molecular forms during a variety of fractionation procedures (30, 32–34). These difficulties result in part from nonspecific ionic interactions between NMT and other macromolecules and/or chromatographic supports. They also reflect a dynamic equilibrium between several multimeric and monomeric forms as determined by ionic strength, proteolysis, and/or protein concentration (34). A key to the eventual purification of NMT and its subsequent characterization was the application of conditions (i.e., 1 M NaCl) to block nonspecific ionic interactions while preserving enzyme activity. The purification also depended upon our ability to manipulate the dimeric structure of NMT by its selective elution from octyl-agarose with a high affinity cofactor analog. Further-
