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Metabolic Activation of 2-Substituted Derivatives of Myristic Acid To Form Potent Inhibitors of Myristoyl CoA:Protein *N*-Myristoyltransferase†

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ABSTRACT: The importance of myristoylation for the proper biological functioning of many acylated proteins has generated interest in the enzymes of the myristoylation pathway and their interactions with substrates and inhibitors. Previous observations that *S*-(2-oxopentadecyl)-CoA, a nonhydrolyzable methylene-bridged analogue of myristoyl-CoA, was a potent inhibitor of myristoyl-CoA:protein *N*-myristoyltransferase (NMT) [Paige, L. A., Zheng, G.-q., DeFrees, S. A., Cassady, J. M., & Geahlen, R. L. (1989) *J. Med. Chem. 32*, 1665] prompted a closer examination of the effect of substituents at the 2-position on the interactions of myristic acid and myristoyl-CoA analogues with NMT. As an initial approach, three myristic acid derivatives bearing different substituents at the 2-position, 2-fluoromyristic acid, 2-bromomyristic acid, and 2-hydroxymyristic acid, were selected for study. Both 2-bromomyristic acid and 2-hydroxymyristic acid were available commercially; 2-fluoromyristic acid was prepared synthetically. All three compounds were found to be only weak inhibitors of NMT in vitro. Of the three, 2-bromomyristic acid was the most potent (K_i = 100 μM). In cultured cells, however, 2-hydroxymyristic acid was by far the more effective inhibitor of protein myristoylation. Neither 2-hydroxymyristic acid nor 2-bromomyristic acid significantly inhibited protein palmitoylation in cultured cells, indicating that inhibition was not occurring at the level of acyl-CoA synthetase. Activation of the 2-substituted myristic acid derivatives to their corresponding acyl-CoA thioesters by acyl-CoA synthetase resulted in inhibitors of greatly increased potency. The 2-substituted acyl-CoA analogues, 2-hydroxymyristoyl-CoA, 2-bromomyristoyl-CoA, and 2-fluoromyristoyl-CoA, were synthesized and shown to be competitive inhibitors of NMT in vitro (K_i's = 45, 450, and 200 nM, respectively). These data suggested that the enhanced inhibitory potency of 2-hydroxymyristic acid seen in cells was most probably a result of its metabolic activation to the CoA thioester. The presence of substituents at the 2-position also affected the ability of the acyl group to be transferred by NMT to a peptide substrate. Of the three acyl-CoA analogues, only 2-fluoromyristoyl-CoA served as a substrate for NMT.

The covalent attachment of myristate to the amino terminus of proteins was first described for the catalytic subunit of cAMP-dependent protein kinase (Carr et al., 1982) and the B subunit of calcineurin (Aitken et al., 1982). Since these initial discoveries, several additional proteins of both cellular and viral origins have been shown to be covalently modified with myristate [for recent reviews, see Towler et al. (1988a) and Schultz et al. (1988)]. It is the identity of these proteins that has engendered much of the current interest in protein myristoylation. Among others, these proteins include oncogene and protooncogene products such as pp60$^{v\text{-}src}$, pp60$^{c\text{-}src}$, and other members of the *src* family of protein–tyrosine kinases (Marchildon et al., 1984; Towler et al., 1988b), as well as structural proteins encoded by several classes of mammalian retroviruses (Paul et al., 1987; Henderson et al., 1983; Chow et al., 1987; Persing et al., 1987; Streuli & Griffin, 1987). For many of these proteins, myristoylation is essential for proper trafficking and biological function. For instance, nonmyristoylated variants of pp60$^{v\text{-}src}$ retain protein–tyrosine kinase activity but are no longer able to associate with the plasma membrane and are defective in their ability to transform cells (Cross et al., 1984). Likewise, nonmyristoylated variants of the structural proteins of both the human immunodeficiency virus and the Moloney murine leukemia virus do not associate with the plasma membrane and consequently fail to assemble into viral particles (Rein et al., 1986; Göttlinger et al., 1989).

The importance of myristoylation for the proper biological functioning of many acylated proteins has generated interest in the enzymes of the myristoylation pathway and their interactions with substrates and inhibitors. Protein myristoylation is a cotranslational event that occurs following the

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removal of the initiator methionine residue to expose an amino-terminal glycine (Wilcox et al., 1987; Dechaite et al., 1988). Myristoyl-CoA:protein N-myristoyltransferase (N-myristoyltransferase or NMT)[1] (Towler & Glaser, 1986) then catalyzes the transfer of myristate from myristoyl-CoA to the newly exposed glycine residue forming a stable amide linkage. While there is no strict consensus sequence for myristoylated substrates, not all proteins with an amino-terminal glycine are acylated (Tsunasawa et al., 1985). Studies on the myristoylation of both protein (Pellman et al., 1985; Kaplan et al., 1988) and peptide substrates (Towler et al., 1987a) indicated that amino acids present within the first 7–10 residues contain the essential information for substrate recognition. In studies using synthetic octapeptide substrates that were variations on the amino-terminal sequence of cAMP-dependent protein kinase, Towler et al (1987b) showed that residues 1, 2, and 5 played important roles in enzyme–substrate interactions. In particular, a serine residue at position 5 promoted high-affinity binding (Towler et al., 1988b).

Less is known regarding the structural features of compounds that interact with high affinity at the acyl-CoA substrate-binding site. N-Myristoyltransferase shows a marked substrate specificity for myristoyl-CoA. Towler et al. (1987a) and Glover et al. (1988) showed that shorter chain length acyl-CoAs (C10:0 and C12:0) could also serve as substrates in vitro and that C16:0 and C18:0 acyl-CoAs were bound but did not serve as acyl group donors. Studies with CoA thioesters of unsaturated fatty acids and with heteroatom-substituted myristic acid analogues indicated that the length and conformation of the acyl chain were important determinants of substrate specificity (Heuckeroth et al., 1988). These compounds, in general, are not bound with an affinity greater than that of the natural substrate. We showed previously that S-(2-oxopentadecyl)-CoA (**1**), a nonhydrolyzable, methylene bridged analogue of myristoyl-CoA, was a potent inhibitor of NMT (K_i = 24 nM), indicating that structural modifications could be made in the substrate to promote high-affinity interactions at this site (Paige et al., 1989a).

In this study, we have further examined the interactions of substrates and inhibitors with NMT. We have found that various myristic acid analogues bearing substituents at the 2-position are activated by acyl-CoA synthetase to form potent inhibitors of NMT both in vitro and in cultured cells.

Materials and Methods

Materials

Pseudomonas acyl-CoA synthetase, ATP, lithium CoA, myristoyl-CoA, and CM-Sepharose were purchased from Sigma. [^{3}H]Myristic acid (39.3 Ci/mmol), [^{3}H]palmitic acid (30 Ci/mmol), and ^{125}Na I (17 Ci/mmol) were obtained from Du Pont New England Nuclear. IODO-GEN was purchased from Pierce. Myristic acid and 2-bromomyristic acid were obtained from Aldrich and 2-hydroxymyristic acid was from Fluka. These were used without further purification. All other reagents and chemicals used for the synthesis of 2-fluoromyristic acid and 2-substituted myristoyl CoA analogues were purchased from Aldrich unless noted otherwise. The peptide substrates Gly-Asn-Ala-Ala-Ala-Ala-Arg-Arg(NH_2) and Gly-Asn-Ala-Ala-Ala-Ala-Arg-Arg-Tyr(NH_2) were prepared by the Purdue University Peptide Synthesis Facility. HPLC separations were performed on a Vydac reversed-phase C_{18} column (4.6 mm × 25 cm) purchased from Rainin.

Methods

Assay of N-Myristoyltransferase. NMT was partially purified from mouse brain and assayed as described previously (Paige et al., 1989b). Briefly, the assay measures the transfer of [^{3}H]myristic acid from [^{3}H]myristoyl-CoA to the amino-terminal glycine of a synthetic peptide substrate (GNAAAARR). The resulting [^{3}H]myristoylpeptide is isolated by ion-exchange chromatography on CM-Sepharose. Standard reactions contained 0.2 mM peptide, 0.3 μM [^{3}H]myristoyl-CoA, 10 mM EDTA, 15 mM Tris-HCl, pH 7.8, and 0.5 mM dithiothreitol. The [^{3}H]myristoyl-CoA was synthesized from [^{3}H]myristic acid and lithium CoA by using *Pseudomonas* acyl-CoA synthetase. The acyl-CoA synthetase was then heat denatured to eliminate the carryover of any activity to the NMT reaction. Any acyl-CoA synthetase activity that might have contaminated the NMT preparation was inhibited by the inclusion of 10 mM EDTA in the transferase assay. Reactions utilizing ^{125}I-GNAAAARRY as the acyl acceptor contained 5 μM acyl-CoA and were terminated by the addition of 0.1 mL of methanol and 10 μL of 100% trichloroacetic acid. Aliquots (50 μL) from each reaction were analyzed by reversed-phase HPLC by using a gradient of 0–70% acetonitrile. Myristoylation of the labeled peptide was quantified by γ counting.

Radioiodination of GNAAAARRY. The tyrosyl residue in the peptide substrate GNAAAARRY was iodinated in a 0.5-mL reaction containing 1 mg of peptide, 100 μg of IODO-GEN, 50 mM Tris-HCl, and 2 mCi Na^{125}I for 15 min at room temperature. NaI was added to a concentration of 2 mM and the reaction was allowed to continue for an additional 15 min. The reaction was applied to a Sep-Pak C_{18} cartridge (Waters) equilibrated in 0.1% trifluoroacetic acid (TFA). The column was washed with 15 mL of 0.1% TFA. Labeled peptide was eluted with 2 mL of 100% acetonitrile and 0.1% TFA and was blown dry under a stream of nitrogen.

Protein Myristoylation in Cultured Cells. The T lymphoma cell line LSTRA was originally induced in Balb/c mice by the Moloney murine leukemia virus (Glynn et al., 1964). LSTRA cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 12 mM $NaHCO_3$, 100 IU/mL penicillin G, 100 μg/mL streptomycin, 1 mM sodium pyruvate, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, and 12.5 mM Hepes, pH 7.4. Fatty acid analogues were added at varying concentrations as potassium salts complexed with bovine serum albumin (Pande et al., 1971) to 3 × 10^6 cells in a volume of 1.0 mL. Cells were preincubated with analogue for 15 min prior to the addition of 0.05 mCi [^{3}H]myristic acid. Following a 4-h labeling period, cells were harvested, washed with phosphate-buffered saline, and then resuspended in 5 mM Hepes, pH 7.4, 5 mM $MgCl_2$, and 10 μg/mL aprotinin at 0 °C and allowed to swell for 5 min. The cell suspension was made 1% in Triton X-100, incubated an additional 10 min, and centrifuged at 1300*g* for 10 min at 0 °C. The supernatant fraction was recovered and protein concentrations were determined by using the BCA protein assay reagent (Pierce). Proteins were separated by SDS–polyacrylamide gel electrophoresis using 10% polyacrylamide gels and the buffer systems described by Laemmli (1970). Radiolabeled proteins were identified by fluorography (Soltysiak et al., 1984).

Synthesis of 2-Fluoromyristic Acid (**2**). *Route i.* To a solution of 19.06 g (0.11 mol) of N-bromosuccinimide in 100 mL of dry sulfolane under argon was added 200 g of hydrogen

[1] Abbreviations: NMT, myristoyl-CoA:protein N-myristoyltransferase; Tris, tris(hydroxymethyl)aminomethane; TFA, trifluoroacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; HMA, 2-hydroxymyristic acid; BMA, 2-bromomyristic acid; FMA, 2-fluoromyristic acid.

fluoride in pyridine, followed by the slow addition over 5 min of 17.6 mL (13.64 g, 0.1 mol) of 1-tetradecene. The solution was allowed to stir at room temperature for 1 h. The reaction mixture was then poured into 500 mL of ice water and extracted with ether (3 × 200 mL). The orange ether layers were first washed with water and then a saturated sodium bicarbonate solution until the aqueous layer was neutral. The ether layer was then separated, dried, and evaporated. The resulting orange oil was vacuum-distilled by using a Claisen fractional distillation head. The fraction boiling from 106 to 126 °C (0.1 mmHg) was collected to give 16.52 g (56% yield) of 1-bromo-2-fluorotetradecane (**5**): ^{1}H NMR ($CDCl_3$) δ 0.88 (t, 3 H, CH_3), 1.26 (m, 20 H, alkyl CH_2's), 1.71 (m, 2 H, 3-CH_2), 3.46 (ddd, 2 H, CH_2Br), 4.62 (m, 1 H, CHF); CIMS (methane) m/z (relative intensity) 295 (9, $M^+ - 1$ for ^{81}Br), 293 (9, $M^+ - 1$ for ^{79}Br), 277 (51), 275 (66), 273 (26); HRMS (CI) calcd for $C_{14}H_{27}BrF$ m/z 293.1281, found 293.1278.

A suspension containing 16.30 g (0.055 mol) of **5**, 8.27 g (0.055 mol) of anhydrous sodium iodide, 9.06 g (0.11 mol) of anhydrous sodium acetate, and 250 mL of dry dimethylformamide was heated at 120–130 °C in an oil bath for 44 h. The dark brown solution was cooled to room temperature and 240 mL of water was added. The aqueous layer was then extracted with ether (1 × 320 mL, then 2 × 80 mL). The ether extract was washed with water, dried, and evaporated, giving a yellow oil, which was purified by silica gel flash column chromatography and recrystallized from ether to yield 7.56 g (50% yield) of 1-*O*-acetyl-2-fluorotetradecane (**6**) as colorless plate crystals: mp 29–30.5 °C; ^{1}H NMR ($CDCl_3$) δ 0.88 (t, 3 H, CH_3), 1.26 (m, 22 H, alkyl CH_2's), 2.10 (s, 3 H, CH_3CO), 4.16 (m, 2 H, 3-CH_2), 4.65 (m, 1 H, CHF); CIMS (methane) m/z (relative intensity) 275 (56, $M^+ + 1$), 273 (43), 255 (100). Anal. Calcd for $C_{16}H_{31}O_2F$: C, 70.03; H, 11.38. Found: C, 69.79; H, 11.09.

A solution containing 0.5 g (1.8 mmol) of **6** and 0.5 g of KCN in 25 mL of 95% ethanol was refluxed for 17 h. The solution was then evaporated to dryness and the resulting orange solid was extracted with ether (2 × 30 mL). The combined ether layers were washed with water and a saturated NaCl solution, dried with $CaSO_4$, and evaporated to dryness giving 0.418 g (98% yield) of 2-fluorotetradecan-1-ol (**7**) as a white solid: mp 63–65 °C; ^{1}H NMR ($CDCl_3$) δ 0.9 (t, 3 H, CH_3), 1.3 (br s, 20 H, alkyl CH_2's), 1.75 (br m, 2 H, 3-CH_2), 4.1 (m, 2 H, OCH_2), 4.5 (m, 1 H, CHF); CIMS (methane) m/z (relative intensity) 231 (2, $M^+ - 1$), 213 (39), 211 (11), 193 (12); HRMS (CI) calcd for $C_{14}H_{28}OF$ m/z 231.2124, found 231.2101.

A solution of 60 mg (0.26 mmol) of **7**, 5 mL of 20% H_2SO_4, and 102 mg (0.66 mmol) of $KMnO_4$ was stirred at room temperature for 20 h. Sodium bisulfite was added until the solution was clear, and then 20 mL of water was added. The aqueous solution was extracted with ether (4 × 20 mL). The combined ether layers were then extracted with a saturated $NaHCO_3$ solution, which formed a white precipitate. The white precipitate was filtered, combined with the $NaHCO_3$ layer, and acidified with 6 N HCl to pH 1.0. The cloudy solution was then extracted with ether (3 × 50 mL). The ether extract was dried and evaporated to afford a white solid, which, after purification by preparative TLC on silica gel, gave 25 mg (39% yield) of the desired 2-fluoromyristic acid (**2**) as a white solid: mp 74–76 °C; ^{1}H NMR ($CDCl_3$) δ 0.88 (t, 3 H, CH_3), 1.27 (br s, 18 H, alkyl CH_2's), 1.44 (br m, 2 H, CH_2), 1.83 (m, 2 H, 3-CH_2), 4.94 (m, 1 H, CHF); CIMS (methane) m/z (relative intensity) 247 (100, $M^+ + 1$), 85 (56), 83 (69); HRMS (CI) calcd for $C_{14}H_{27}O_2F$ m/z 246.1995, found 246.1995. Anal. Calcd for $C_{14}H_{27}O_2F$: 68.25; H, 11.04. Found: C, 68.30; H, 11.33.

Route ii. To a suspension of 0.32 g (11.2 mmol) of an 80% oil dispersion of NaH in 8 mL of dry DMF was added dropwise 2 g (11.2 mmol) of diethyl fluoromalonate under argon. The suspension was then stirred for 4.5 h after which time, 2.79 g (11.2 mmol) of 1-bromododecane was added and the solution was heated at 91 °C for 18 h. The yellow suspension was then poured into 10 mL of water and extracted with ether (2 × 15 mL). The combined ether layers were washed, dried, and evaporated. A yellow oil resulted, which was used for the next reaction without further purification. A mixture of 3.8 g of the crude diethyldodecylfluoromalonate (**8**), 30 mL of 6 N HCl, and 50 mL of dioxane was refluxed for 72 h. After cooling, the yellow solution was dissolved in 100 mL of petroleum ether (bp 40–60 °C). The organic layer was separated and washed with water (3 × 50 mL) and 10% KOH (2 × 250 mL). The combined aqueous layers were acidified to pH 1.0 with concentrated HCl and extracted with ether (3 × 100 mL). The ether layer was dried, filtered, and evaporated to dryness to yield a green solid. The solid was decolorized with activated carbon and recrystallized from petroleum ether (bp 40–60 °C) to give 1.501 g (54% overall yield) of 2-fluoromyristic acid (**2**) as white needles.

Synthesis of 2-Fluoromyristoyl-CoA (**12**). 2-Fluoromyristic acid (**2**) (73.8 mg, 0.3 mmol) was added to a solution of *N*-hydroxysuccinimide (34.5 mg, 0.3 mmol) in dry ethyl acetate (2 mL). A solution of dicyclohexylcarbodiimide (61.8 mg, 0.3 mmol) in dry ethyl acetate (1 mL) was then added, and the reaction mixture was maintained at room temperature overnight. Dicyclohexylurea was removed by filtration, and the filtrate was evaporated to yield a white solid, which was then recrystallized from ethanol to give 89.6 mg (87% yield) of the *N*-hydroxysuccinimide ester of 2-fluoromyristic acid (**9**) as white crystals: mp 80 °C; ^{1}H NMR ($CDCl_3$) δ 0.88 (t, 3 H, J = 6.6 Hz, CH_3), 1.26 (br s, 18 H, alkyl CH_2's), 1.42 (m, 2 H, 4-CH_2), 1.90 (m, 2 H, 3-CH_2), 2.75 (s, 4 H, CH_2's of succinimide), 4.95 (dt, 1 H, J = 49.2 Hz, 5.1, CHF); HRMS (EI) calcd for $C_{18}H_{30}O_4NF$ m/z 343.2159, found 343.2205. Anal. Calcd for $C_{18}H_{30}O_4NF$: C, 62.95; H, 8.80; N, 4.08. Found: C, 62.52; H, 8.94; N, 4.09.

To a solution of 100 mg (0.13 mmol) of CoA-SH in 3 mL of water was added 22 mg (0.26 mmol) sodium bicarbonate. To this solution was added a solution of 89 mg (0.26 mmol) of **9** in 2 mL of dry tetrahydrofuran. This mixture was stirred at room temperature overnight under N_2 atmosphere. After the reaction was completed, 5 mL of 5% perchloric acid were added to precipitate 2-fluoromyristoyl-CoA, unreacted *N*-hydroxysuccinimide ester, and 2-fluoromyristic acid if any. Complete precipitation of the product was achieved by concentration of the mixture. The precipitate was collected by centrifugation and washed with 8% perchloric acid. The combined precipitates were then extracted with acetone (4 × 5 mL) and with ether (3 × 5 mL) to remove the *N*-hydroxysuccinimide ester and 2-fluoromyristic acid. The white residue was then extracted with an aqueous $NaHCO_3$ solution maintained at pH 5.0 (3 × 4 mL) to dissolve 2-fluoromyristoyl-CoA and to remove any water-insoluble impurities. 2-Fluoromyristoyl-CoA was precipitated by addition of 5% perchloric acid and washed with 0.8% perchloric acid and then with acetone, giving 96.5 mg (75% yield based on CoA-SH) of 2-fluoromyristoyl-CoA (**12**) as a white solid: mp 132–133 °C (dec); ^{1}H NMR (D_2O) δ 0.84 (s, 3 H, CH3), 0.86 (br s, 3 H, alkyl CH_3), 0.97 (s, 3 H, CH_3), 1.25 (br s, 20 H, alkyl CH_2's), 1.87 (m, 2 H, 3-CH_2 of alkyl chain), 2.47 (m, 2 H,

CH_2CONH), 3.10 (m, 2 H, SCH_2), 3.41 (m, 2 H, CH_2N), 3.49 (m, 2 H, CH_2N), 3.65 (m, 1 H, CHOP), 3.87 (m, 1 H, CHOP), 4.07 (s, 1 H, CHOH), 4.29 (m, 2 H, 5′-CH_2 of sugar), 4.64 (m, 2 H, 2′-CH and 4′-CH of sugar), 4.92 (m, 1 H, 3′-CH of sugar), 5.14 (m, 1 H, CHF), 6.26 (m, 1 H, 1′-CH of sugar), 8.46 (s, 1 H, 2-CH of adenine), 8.72 (s, 1 H, 8-CH of adenine); FAB MS, *m/z* (relative intensity) 1018 (0.6, MNa^+), 996 (1.2, MH^+), 587 (3), 508 (1), 489 (7); HRMS (FAB) calcd for $C_{35}H_{62}N_7O_{17}P_3SF$ *m/z* 996.3120, found 996.3170. Anal. Calcd for $C_{35}H_{61}N_7O_{17}P_3SF$: C, 42.21; H, 6.17; N, 9.85. Found: C, 42.11; H, 6.56; N, 9.72.

Synthesis of 2-Bromomyristoyl-CoA (**13**). The *N*-hydroxysuccinimide ester of 2-bromomyristic acid (**10**, 95.2 mg, 78% yield) was prepared from 2-bromomyristic acid (**3**, 92.3 mg) by the same procedure as for the synthesis of **9** and obtained as white crystals from EtOH: mp 52 °C; ^1H NMR ($CDCl_3$) δ 0.88 (t, 3 H, J = 6.6 Hz, CH_3), 1.26 (br s, 18 H, alkyl CH_2's), 1.52 (m, 2 H, 4-CH_2), 2.14 (m, 2 H, 3-CH_2), 2.86 (s, 4 H, CH_2's of succinimide), 4.46 (t, 1 H, J = 7.4 Hz, CHBr); FAB MS, *m/z* (relative intensity) 406 (8, MH^+ with ^{81}Br), 404 (8, MH^+ with ^{79}Br), 352 (4), 291 (4), 289 (5), 225 (10), 211 (12), 209 (16); HRMS (FAB) calcd for $C_{18}H_{31}O_4N^{81}Br$ *m/z* 406.1413, found 406.1416; calcd for $C_{18}H_{31}O_4N^{79}Br$ *m/z* 404.1436, found 404.1434. Anal. Calcd for $C_{18}H_{30}O_4NBr$: C, 53.47; H, 7.48; N, 3.46. Found: C, 53.47; H, 7.69; N, 3.41.

2-Bromomyristoyl-CoA (**13**, 115.6 mg, 84% yield based on CoA-SH) was prepared by the coupling of **10** (105 mg, 0.26 mmol) with CoA-SH (100 mg, 0.13 mmol) by the same procedure as for the synthesis of **12** and obtained as a white solid from acetone: mp 135–137 °C (dec); ^1H NMR (D_2O) δ 0.83 (br s, 6 H, 2 CH_3's), 0.96 (s, 3 H, CH_3), 1.23 (br s, 20 H, alkyl CH_2's), 1.96 (m, 2 H, 3-CH_2 of alkyl chain), 2.49 (m, 2 H, CH_2CONH), 3.10 (m, 2 H, SCH_2), 3.41 (m, 2 H, CH_2N), 3.60 (m, 2 H, CH_2N), 3.71 (m, 2 H, CH_2OP), 4.18 (m, 2 H, 5′-CH_2 of sugar), 4.67 (m, 1 H, 4′-CH of sugar), 4.73 (s, 1 H, CHOH), 4.89 (m, 2 H, 2′-CH and 3′-CH of sugar), 4.92 (m, 1 H, CHBr), 6.26 (d, 1 H, J = 4.7 Hz, 1′-CH of sugar), 8.46 (s, 1 H, 2-CH of adenine), 8.67 (s, 1 H, 8-CH of adenine): FAB MS, *m/z* (relative intensity) 1058 (0.2, MH^+ with ^{81}Br), 1056 (0.2, MH^+ with ^{79}Br) 978 (0.2), 649 (0.5) 584 (0.8), 551 (1.3), 549 (1.5), 508 (1.4), 471 (1.3), 428 (2.0); HRMS (FAB) calcd for $C_{35}H_{62}N_7O_{17}P_3S^{81}Br$ *m/z* 1058.2299, found 1058.2306; calcd for $C_{35}H_{62}N_7O_{17}P_3S^{79}Br$ *m/z* 1056.2319, found 1056.2354. Anal. Calcd for $C_{35}H_{61}N_7O_{17}P_3SBr$: C, 39.78; H, 5.82; N, 9.28. Found: C, 39.40; H, 6.03; N, 9.18.

Synthesis of 2-Hydroxymyristoyl-CoA (**14**). The *N*-hydroxysuccinimide ester of 2-hydroxymyristic acid (**11**, 91.3 mg, 89% yield) was prepared from 2-hydroxymyristic acid (**4**, 73.2 mg) by the same procedure as for the synthesis of **9** and obtained as white crystals from EtOH: mp 61 °C; ^1H NMR ($CDCl_3$) δ 0.88 (t, 3 H, J = 6.6 Hz, CH_3), 1.26 (br s, 18 H, alkyl CH_2's), 1.52 (m, 2 H, 4-CH_2), 1.89 (m, 2 H, 3-CH_2), 2.53 (s, 1 H, exchangeable, OH), 2.86 (s, 4 H, CH_2's of succinimide), 4.56 (dd, 1 H, J = 7.4 Hz, 4.7, CHOH); FAB MS, *m/z* (relative intensity) 342 (4, MH^+), 314 (2, MH^+ - CO), 197 (4), 116 (100); HRMS (FAB) calcd for $C_{18}H_{32}O_5N$ *m/z* 342.2280, found 342.2305. Anal. Calcd for $C_{18}H_{31}O_5N$: C, 63.32; H, 9.50; N, 4.10. Found: C, 63.67; H, 9.37; N, 4.04.

2-Hydroxymyristoyl-CoA (**14**, 107 mg, 83% yield based on CoA-SH) was prepared by the coupling of **11** (89 mg, 0.26 mmol) with CoA-SH (100 mg, 0.13 mmol) by the same procedure as for the synthesis of **12** and obtained as a white solid from acetone: mp 147–149 °C (dec); ^1H NMR (D_2O)

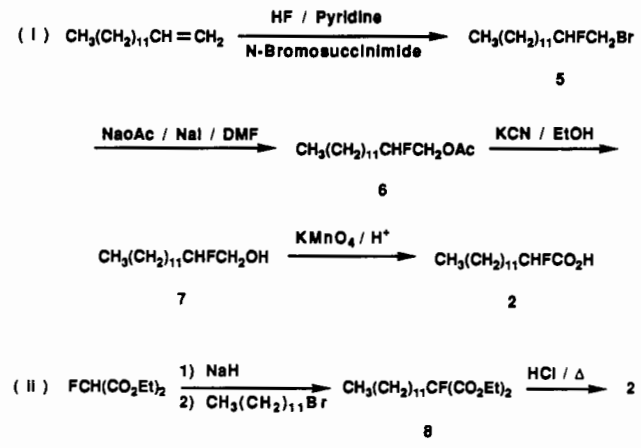

FIGURE 1: Synthesis of 2-fluoromyristic acid (**2**).

δ 0.80 (br s, 6 H, 2 CH_3's), 0.94 (s, 3 H, CH_3), 1.20 (br s, 20 H, alkyl CH_2's), 1.69 (m, 2 H, 3-CH_2 of alkyl chain), 2.48 (m, 2 H, CH_2CONH), 2.94 (m, 2 H, SCH_2), 3.34 (m, 2 H, CH_2N), 3.46 (m, 2 H, CH_2N), 3.68 (m, 1 H, CHOP), 3.95 (m, 1 H, CHOP), 4.19 (s, 1 H, CHOH), 4.33 (m, 2 H, 5′-CH_2 of sugar), 4.60 (m, 1 H, 2-CH of alkyl chain), 4.87 (m, 1 H, 4′-CH of sugar), 4.89 (m, 1 H, 2′-CH of sugar), 4.96 (m, 1 H, 3′-CH of sugar), 6.14 (m, 1 H, 1′-CH of sugar), 8.45 (s, 1 H, 2-CH of adenine), 8.60 (s, 1 H, 8-CH of adenine); FAB MS, *m/z* (relative intensity) 1016 (0.8, MNa^+), 994 (1.3, MH^+), 768 (0.3), 687 (0.6), 607 (1.5), 585 (2.7), 508 (1.8), 487 (11.2); HRMS (FAB) calcd for $C_{35}H_{63}N_7O_{18}P_3S$ *m/z* 994.3163, found 994.3198. Anal. Calcd for $C_{35}H_{62}N_7O_{18}P_3S$: C, 42.30; H, 6.29; N, 9.86. Found: C, 41.47; H, 6.47; N, 9.61.

RESULTS

Synthesis of 2-Fluoromyristic Acid. Our initial studies on the inhibition of NMT by *S*-(2-oxopentadecyl)-CoA (**1**) suggested that substituents located near the 2-position of the acyl chain might enhance the binding of substrate analogues to the enzyme (Paige et al., 1989a). As an initial approach to the study of the interaction of such analogues with NMT, we selected three myristic acid derivatives bearing different substituents at this position: 2-fluoromyristic acid (FMA, **2**), 2-bromomyristic acid (BMA, **3**), and 2-hydroxymyristic acid (HMA, **4**). BMA and HMA were commercially available.

FMA was prepared by two different synthetic routes (Figure 1) (Pattison et al., 1965). The first route (i) involved the fluorobromination of 1-tetradecene to yield 1-bromo-2-fluorotetradecane (**5**). The nucleophilic displacement of the 1-bromo group of **5** with sodium acetate produced 1-*O*-acetyl-2-fluorotetradecane (**6**). Hydrolysis of the acetate gave 2-fluorotetradecan-1-ol (**7**), which was oxidized to yield the desired product **2**. Since the final oxidation step brought a low overall yield (~10%), an alternative route (ii) to FMA was sought. This synthetic approach involved an alkylation of sodium diethyl fluoromalonate to produce the intermediate diethyl 2-dodecyl-2-fluoromalonate (**8**). This intermediate, without further purification, was then hydrolyzed and decarboxylated in a single step to give **2** in an overall yield of 54%.

Inhibition of N-Myristoyltransferase by Fatty Acid Analogues. The 2-substituted fatty acid analogues and the synthetic intermediates obtained in the preparation of FMA were tested at two concentrations for their ability to directly inhibit NMT activity (Table I). All of the compounds tested appeared to be weak inhibitors of NMT. The most potent inhibitors were BMA and HMA. Kinetic analyses indicated that both compounds were competitive inhibitors of NMT with

Table I: Inhibition of NMT by Fatty Acid Analogues[a]

compound	% inhibition 1 mM	0.5 mM
myristic acid	61	27
2-bromomyristic acid	97	86
2-hydroxymyristic acid	82	53
1-bromo-2-fluorotetradecane	53	ND[b]
1-*O*-acetyl-2-fluorotetradecane	49	30
2-fluorotetradecan-1-ol	84	53
2-fluoromyristic acid	67	27
2-fluoropalmitic acid	74	43

[a] Fatty acid analogues were tested for inhibitory activity against NMT by using the in vitro assay as described under Methods. [b] ND, not determined.

Table II: Effect of Activation on the Inhibition of NMT by Fatty Acid Analogues[a]

compound	activity (% of control) +ACS[b]	−ACS[c]
myristic acid	80	108
2-bromomyristic acid	82	98
2-hydroxymyristic acid	6	91
2-fluoromyristic acid	41	87

[a] Fatty acid analogues were preincubated with CoA and acyl-CoA synthetase as described under Methods prior to their addition to the in vitro myristoylation assays. [b] ACS, acyl CoA synthetase. [c] Acyl-CoA synthetase was heat-denatured prior to addition of fatty acid analogues.

respect to myristoyl-CoA, exhibiting K_i values of 100 and 200 μM, respectively.

Inhibition of Protein Myristoylation in Cultured Cells. Despite the relatively low affinity of these compounds for NMT in vitro, those compounds showing the most inhibitory activity were further examined for their effects on protein acylation in cultured cells. The LSTRA cell line, a rapidly growing murine T-cell lymphoma that has previously been shown to incorporate [^{3}H]myristate into cellular proteins (Marchildon et al., 1984), was selected for this analysis. The incubation of LSTRA cells with [^{3}H]myristate or [^{3}H]palmitate led to the ^{3}H-acylation of a distinct subset of cellular proteins (Figure 2). The incorporation of [^{3}H]myristate into proteins was inhibited by cycloheximide and the incorporated label was refractory to cleavage by neutral hydroxylamine as expected for amino-terminal myristoylation (data not shown). Incorporated [^{3}H]palmitate could be removed by treatment with hydroxylamine and inhibitors of protein synthesis had little effect on labeling as expected for ester-linked palmitoylation (data not shown).

LSTRA cells were treated with BMA or HMA at concentrations of 0.5 and 1 mM for 15 min prior to labeling with [^{3}H]myristate (Figure 2A) or [^{3}H]palmitate (Figure 2B). Treatment of cells with HMA inhibited the overall incorporation of [^{3}H]myristate into cellular proteins (Figure 2A). Treatment with BMA also appeared to have a general inhibitory effect on myristoylation; however, its potency was much lower. This contrasts with results obtained with the cell-free assay in which the 2-bromo derivative was the more effective inhibitor. Neither of these compounds significantly affected protein palmitoylation at the doses tested. This indicated that the fatty acid analogues were not acting by inhibiting acyl-CoA synthetase, an event that would be expected to affect both myristoylation and palmitoylation to an equal extent, but were more likely interacting specifically with NMT.

Inhibition of NMT by 2-Substituted Myristoyl-CoA Derivatives. The change in the order of potency of HMA and BMA for inhibition of myristoylation in the cell culture versus

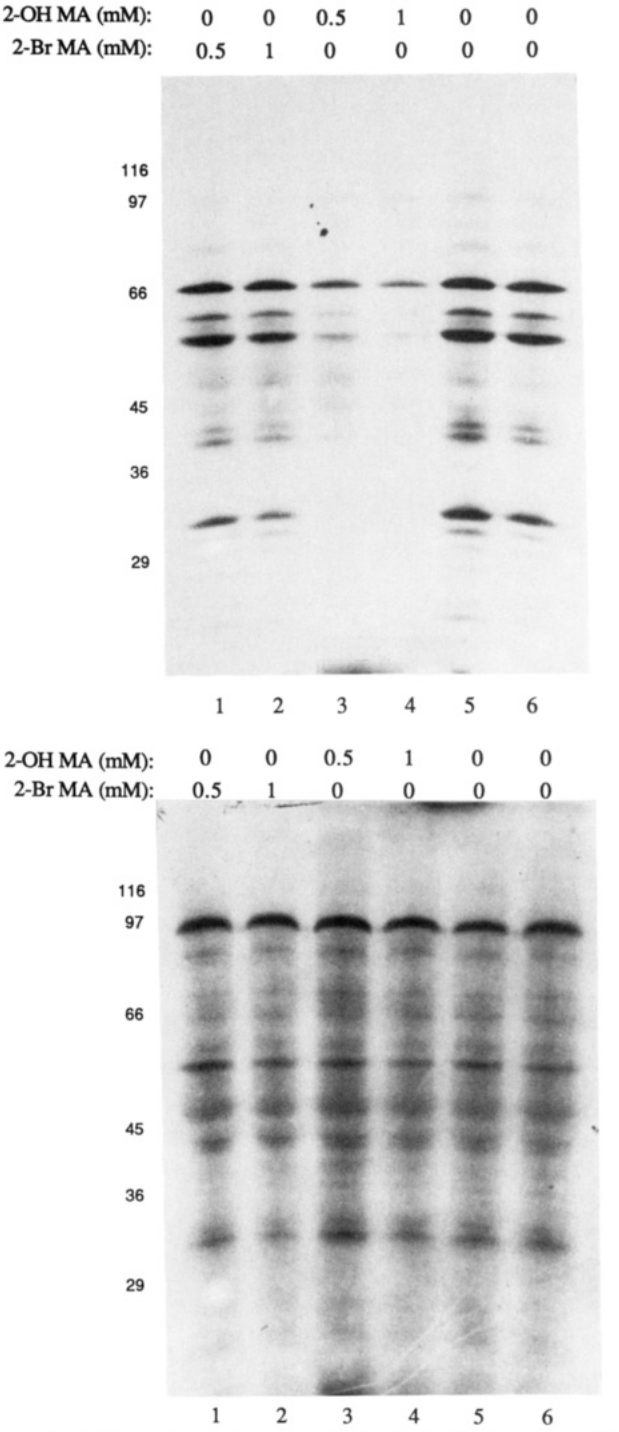

FIGURE 2: Effect of 2-substituted myristic acid derivatives on protein myristoylation and palmitoylation in cultured cells. LSTRA cells were preincubated for 15 min with either 2-hydroxymyristic acid (2-OHMA) or 2-bromomyristic acid (2-BrMA) at the indicated concentrations as described under Methods. Cells were then incubated for 4 h with [^{3}H]myristate (A, top) or [^{3}H]palmitate (B, bottom). Acylated proteins present in cell lysates were separated by SDS–polyacrylamide gel electrophoresis and detected by fluorography. The migration positions of standard proteins of known molecular weights ($\times 10^{-3}$) are indicated.

the cell-free assay suggested the possibility that the 2-substituted fatty acid derivatives were becoming activated by acyl-CoA synthetase in the cell and that the activated forms of these compounds were responsible for the inhibition of NMT. To investigate this possibility, HMA, BMA, as well as FMA, and myristate were preincubated with CoA and *Pseudomonas* acyl-CoA synthetase prior to their addition to the in vitro myristoylation assays. As shown in Table II,

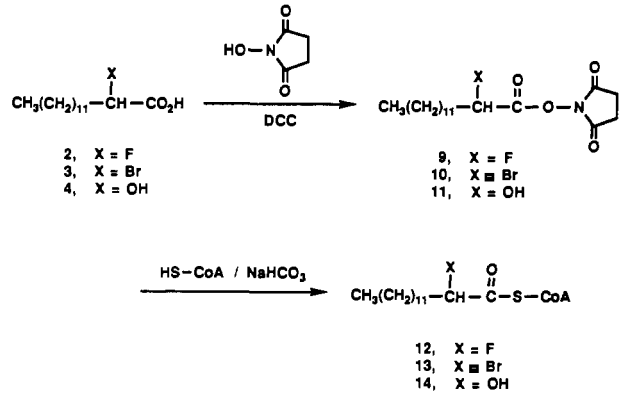

FIGURE 3: Synthesis of 2-substituted myristoyl-CoAs **12–14**.

Table III: Comparison of Inhibition Constants for the Activated and Unactivated Forms of 2-Substituted Fatty Acids[a]

compound	K_i (μM)	compound	K_i (μM)
2-bromomyristic acid	100	2-hydroxymyrisotyl-CoA	0.045
2-hydroxymyristic acid	200	2-fluoromyristoyl-CoA	0.200
2-bromomyristoyl-CoA	0.450		

[a] K_i values were determined by Dixon plots.

preincubation of HMA with acyl-CoA synthetase produced a potent inhibitor of NMT while the preincubation of BMA had little effect. Preincubation of FMA produced an inhibitor of intermediate potency. To control for any inhibitory activity that could be accounted for by the free fatty acid, parallel experiments were run in which the acyl-CoA synthetase was heat denatured prior to its addition to the reactions. The most likely source of inhibitory activity was the CoA thioesters of the fatty acid analogues.

To test this directly, the CoA thioesters of each of the 2-substituted analogues were synthesized. The synthesis of 2-substituted myristoyl coenzyme A analogues **12–14** is outlined in Figure 3 by using the procedure described by Blecher (1981). The 2-substituted acids **2–4** were condensed with *N*-hydroxysuccinimide in the presence of dicyclohexylcarbodiimide to yield the corresponding *N*-hydroxysuccinimide esters of fatty acids **9–11** as crystalline compounds. Compounds **9–11** were then coupled to the sulfhydryl group of coenzyme A to form the thioesters **12–14**. Each of the three analogues was tested at a range of concentrations for inhibition of NMT in in vitro assays (Figure 4). The order of potency for inhibition of [^{3}H]myristoylpeptide formation corroborated the results obtained with the enzymatically synthesized compounds (Table II). K_i values for competitive inhibition were determined to be 45 nM for 2-hydroxymyristoyl-CoA, 200 nM for 2-fluoromyristoyl-CoA, and 450 nM for 2-bromomyristoyl CoA (Table III).

To determine if 2-hydroxymyristoyl-CoA and the other acyl-CoA analogues were alternate substrates for NMT, the ability of the 2-substituted fatty acid analogues to be transferred to the peptide substrate Gly-Asn-Ala-Ala-Ala-Ala-Arg-Arg-^{125}I-Tyr was examined. For these assays, the acylated peptide product was separated by HPLC as described under Methods. With myristoyl-CoA as a substrate, an acylated peptide product was produced that eluted from a C_{18} column at 58% acetonitrile (Figure 5A) No detectable peptide acylation occurred with either 2-hydroxymyristoyl-CoA or 2-bromomyristoyl-CoA as substrates. Acylated peptide product was formed in reactions containing 2-fluoromyristoyl-CoA as substrate (Figure 5B). The resulting acyl peptide eluted from the column at 60% acetonitrile. The rate of transfer of FMA from the CoA thioester was considerably lower than that for myristate and the extent of acyl peptide

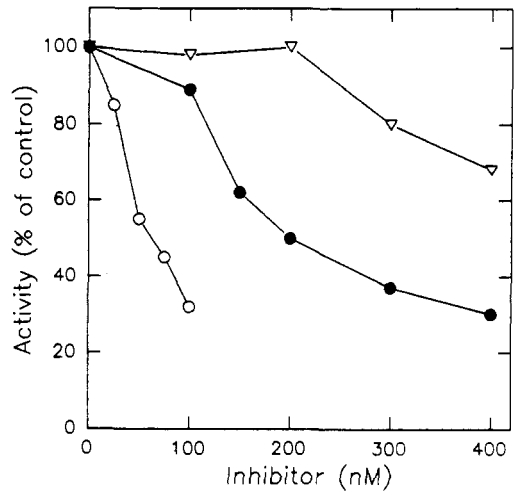

FIGURE 4: Inhibition of in vitro peptide myristoylation by 2-substituted myristoyl-CoA analogues. The effects of varying concentrations of the myristoyl-CoA analogues 2-bromomyristoyl-CoA (▽), 2-fluoromyristoyl-CoA (●), and 2-hydroxymyristoyl-CoA (○) on the in vitro activity of NMT were measured by using the assay described under Methods.

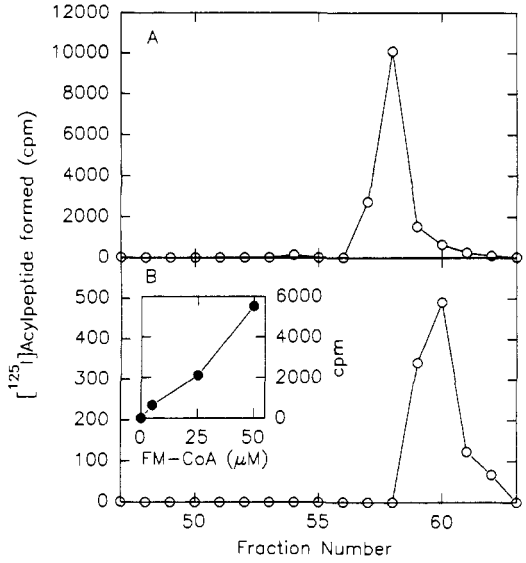

FIGURE 5: Comparison of myristoyl-CoA and 2-fluoromyristoyl-CoA as substrates for NMT. In vitro myristoylation assays were performed by using 5 μM myristoyl-CoA (A) or 2-fluoromyristoyl-CoA (B) as the acyl donor and 0.2 mM ^{125}I-GNAAAARRY as the acyl acceptor. The ^{125}I-acyl peptide products were separated from the unmodified ^{125}I-peptide by HPLC. The elution time for the myristoyl peptide product was 29 min, while the 2-fluoromyristoylpeptide eluted at 30 min. The amount of 2-fluoromyristoyl peptide product formed in the reaction increased with increasing concentrations of 2-fluoromyristoyl-CoA (B, inset).

formation was dependent upon the concentration of the acyl-CoA analogue (Figure 5B, inset).

Discussion

Due to the involvement of myristoylated proteins in both cell transformation and retroviral assembly, there has been considerable interest in the design and development of inhibitors and alternate substrates for *N*-myristoyltransferase. In vitro studies suggest that the first substrate to bind to the enzyme is the acyl donor, the chemical nature of which has considerable influence on the binding affinity of the enzyme for the second substrate. The enzyme will tolerate a number of structural alterations in the acyl donor provided the acyl chain is linear and approximates the chain length of a C14:0

fatty acid (Heuckeroth et al., 1988). Thus, compounds that bind at the acyl donor site are important targets for inhibitor design.

We previously prepared a nonhydrolyzable analogue of myristoyl-CoA [*S*-(2-oxopentadecyl)-CoA] that was bound by NMT with high affinity (K_i = 24 nM) (Paige et al., 1989a). This study suggested that structural analogues of myristoyl-CoA with modifications near the 2-position of the acyl chain might not only be tolerated by NMT but might be bound more tightly than the parent substrate. While compounds such as *S*-(2-oxopentadecyl)-CoA are useful for studies on NMT in vitro, it is unlikely that these highly charged molecules would readily traverse the cell membrane to inhibit the enzyme in intact cells. An alternative approach to the development and delivery of such an inhibitor would be to design an analogue of myristic acid that would be metabolically activated by cellular enzymes (i.e., acyl-CoA synthetase) to generate a potent inhibitor in situ.

2-Bromopalmitate is an inhibitor of β-oxidation and is thought to be activated in cells to form 2-bromopalmitoyl-CoA, an inhibitor of fatty acyl-CoA:carnitine acyltransferase (Chase & Tubbs, 1972). This observation suggests that fatty acid derivatives bearing substituents at the 2-position should serve as suitable candidates for metabolically activated inhibitors. The data presented here indicate that certain 2-substituted derivatives of myristic acid are indeed activated within cells to form potent inhibitors of NMT. This conclusion is based on the following observations: (i) Analogues of myristic acid containing substitutions at the 2-position were only weak inhibitors of NMT in vitro. 2-Bromomyristate was slightly more potent than 2-hydroxymyristate (Table I). (ii) In contrast to the order of potency seen in vitro, 2-hydroxymyristate was a much more potent inhibitor of protein myristoylation in cultured cells than was 2-bromomyristate (Figure 2A). Neither compound significantly inhibited protein palmitoylation at the doses tested, indicating that the inhibition was not at the level of acyl-CoA synthetase (Figure 2B). (iii) When prepared either enzymatically (Table II) or chemically (Figure 4), 2-hydroxymyristoyl-CoA was a much more potent inhibitor of NMT than 2-bromomyristoyl-CoA. Thus, the order of potency of the activated acyl-CoA derivatives as inhibitors of NMT in vitro was consistent with the order of potency of the fatty acid analogues seen in cultured cells. On the basis of these observations, it appears more likely that HMA functions to decrease cellular protein myristoylation through the inhibition of NMT than through the general inhibition of protein synthesis.

The three 2-substituted acyl-CoA derivatives tested in this study were all inhibitors of NMT in vitro (Figure 4 and Table III). However, depending on the nature of the substitution, there was up to a 10-fold difference in inhibitory potency. The most potent inhibitors were those compounds (HMA and FMA) that have the potential to form additional hydrogen-bonding interactions with amino acid residues present within the acyl-CoA binding site on the enzyme.

The presence of substituents at the 2-position also affected the ability of the acyl group to be transferred by NMT to a peptide substrate (Figure 5). The only compound with which acyl peptide formation was detected was FMA. There is some evidence that the chemical nature of the acyl-CoA derivative bound to the transferase might differentially affect the subsequent binding of different protein substrates. Previously reported studies on various heteroatom-substituted myristic acid analogues have suggested that selective protein modification can occur and that the subcellular distribution of proteins acylated with less hydrophobic fatty acid analogues can be altered (Heuckeroth & Gordon, 1989; Bryant et al., 1989). It is not yet known if FMA is also transferred to protein substrates in cultured cells and, if so, what effect this has on the properties of the modified proteins. Efforts to prepare [^{3}H]FMA are currently under way to answer these questions.

Further analyses of these 2-substituted compounds as well as fatty acids containing substitutions at other positions will aid in the characterization of the acyl-CoA binding site on NMT. Characterization of this site and of how structural variations in acyl-CoA derivatives impart altered protein substrate specificity should reveal information extremely useful in the design of compounds for use as antitumor and antiviral agents.

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The Self-Splicing RNA of *Tetrahymena* Is Trapped in a Less Active Conformation by Gel Purification

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ABSTRACT: When the circular form of the self-splicing intervening sequence of *Tetrahymena thermophila* was purified by denaturing polyacrylamide gel electrophoresis by standard methods, the rate of its reaction with tetrauridylate decreased 150-fold at 30 °C and at least 1000-fold at 0 °C. The activity of the self-splicing RNA was restored by heating it to high temperature and letting it renature in the presence of Mg^{2+}. The rate of reaction of tetrauridylate with the self-splicing RNA flanked by exons was also greatly decreased by gel purification. the difference in activation energies for the reaction of native and denatured intervening sequences suggests that a substantial conformational rearrangement of the gel-purified RNA occurs prior to reaction.

Like all RNAs, catalytic RNAs require proper folding of the polynucleotide chain to function. Previous evidence for the importance of structure in reactions of the self-splicing intervening sequence (IVS) from the large rRNA precursor (pre-rRNA) of *Tetrahymena thermophila* is its reduced activity at elevated temperatures and in the presence of denaturants (Cech & Bass, 1986).

In the first step of the self-splicing of pre-rRNA, free guanosine nucleotide causes cleavage at the 5′-splice site and becomes attached to the 5′-end of the IVS. In the second step, the 5′-exon causes cleavage at the 3′-splice site and becomes ligated to the 3′-exon (Kruger et al., 1982). Two reactions have been described that resemble the second step of splicing. In a reaction known as intermolecular exon ligation, an oligonucleotide is incubated with pre-rRNA in the absence of guanosine and cleavage occurs at the 3′-splice site with concomitant ligation of the oligonucleotide to the 3′-exon (Inoue et al., 1985). In a similar reaction called reverse circularization, an oligonucleotide cleaves the linkage between A16 and G414 of the circular form of the IVS (C-IVS) and becomes attached to A16 (Sullivan & Cech, 1985).

Pre-rRNA was prepared by in vitro transcription of plasmid DNA (Inoue et al., 1985), and C-IVS was prepared either by allowing pre-rRNA to splice or by allowing a previously purified linear form of the IVS (L-IVS) to circularize (Sullivan & Cech, 1985). We report here that the usual method of purification substantially reduces the reactivity of the catalytic RNAs and describe a protocol that restores activity to the IVS.

Materials and Methods

Pre-rRNA containing the 413-nucleotide IVS of the large ribosomal RNA precursor of *T. thermophila*, 32 nucleotides of the 5′-exon, and 37 nucleotides of the 3′-exon was prepared by run-off transcription of the plasmid pTTT1A3 using T7 RNA polymerase (Zaug et al., 1986). The transcription reaction mixture contained 40 mM Tris-HCl, pH 8.1, 6 mM $MgCl_2$, 1 mM spermidine, 5 mM dithiothreitol, 0.1 mg/mL bovine serum albumin, 0.8 mM of each NTP (1 Ci/mmol [α-^{32}P]ATP), and 50 μg/mL T7 RNA polymerase. After incubation for 2 h at 30 °C, pre-rRNA was purified by electrophoresis of the reaction mixture in a 4% polyacrylamide–7 M urea gel; elution from the gel slice into 0.25 M NaOAc, 0.1% (w/v) sodium dodecyl sulfate, 1 mM Na_2EDTA, and 10 mM Tris-HCl, pH 8.0; precipitation with ethanol; chromatography using coarse Sephadex G-50 (Pharmacia) in the same buffer as was used for elution from the gel; two additional precipitations with ethanol; and resuspension in water at about 500 nM RNA. To prepare C-IVS or L-IVS, the RNA in the transcription reaction mixture was precipitated with ethanol, resuspended in H_2O, and incubated in 0.1 mM GDP, 100 mM $(NH_4)_2SO_4$, 10 mM $MgCl_2$, and 30 mM Tris-HCl, pH 7.5, for 15 min at 42 °C to give predominantly C-IVS or 30 min at 30 °C to give predominantly L-IVS. Gel-purified C-IVS and gel-purified L-IVS were obtained from these reaction mixtures by the same procedure used to purify pre-rRNA. Native C-IVS was obtained by incubating gel-purified L-IVS for 15 min at 42 °C in 100 mM $(NH_4)_2SO_4$, 10 mM $MgCl_2$, and 30 mM Tris-HCl, pH 7.5, and used immediately. Renatured pre-rRNA and renatured C-IVS were prepared by incubating the gel-purified RNAs in water for 3 min at 95 °C, adding one-fifth volume 500 mM $(NH_4)_2SO_4$, 50 mM $MgCl_2$, and 150 mM Tris-HCl, pH 7.5, and centrifuging the tube to return condensation to the tube's bottom. Cooling time to room temperature was 1–2 min.

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