A Comparative Analysis of the Kinetic Mechanism and Peptide Substrate Specificity of Human and *Saccharomyces cerevisiae* Myristoyl-CoA:Protein N-Myristoyltransferase*

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Human myristoyl-CoA:protein N-myristoyltransferase (hNmt) catalyzes the transfer of myristate from CoA to the amino-terminal Gly residue of a number of cellular proteins involved in signal transduction pathways, to structural and nonstructural proteins encoded by retroviruses, hepadnaviruses, picornaviruses, and reoviruses, as well as to several transforming tyrosine kinases. hNmt has been purified 230-fold from an erythroblastoma cell line. The monomeric enzyme has no associated methionyl aminopeptidase activity. To determine the enzyme's kinetic mechanism, we examined the effect of covariation of subsaturating concentrations of myristoyl-CoA and peptide substrate on initial velocity. Double-reciprocal plots excluded a double displacement (ping-pong) mechanism. Product inhibition studies indicated that CoA was a noncompetitive inhibitor against myristoyl-CoA and a mixed-type inhibitor against peptide substrates. Together these results are consistent with a sequential ordered mechanism where, in a typical catalytic cycle, myristoyl-CoA binds to apoenzyme before peptide followed by release of the CoA and then myristoylpeptide products. This kinetic mechanism is identical to that described for *Saccharomyces cerevisiae* N-myristoyltransferase (Nmt1p) and emphasizes the impact that regulation of myristoyl-CoA pool size and accessibility may have in modulating protein N-myristoylation in these two species. Comparative studies of the peptide substrate specificities of hNmt and Nmt1p using a panel of 12 octapeptides revealed distinct differences in their tolerance for amino acid substitutions at positions 3, 4, 7, and 8 of parental peptides derived from the amino-terminal sequences of known N-myristoylproteins. This finding contrasts with our recent observation that the acyl-CoA substrate specificities of hNmt and Nmt1p are highly conserved and suggests that these differences in peptide recognition provide an opportunity to develop species-specific enzyme inhibitors.

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(**EC 2.1.3.97** is a 416-residue protein encoded by a single copy gene (Duronio et al., 1992a) that catalyzes the transfer of myristate from CoA to the amino-terminal Gly residue of proteins with diverse functions (reviewed in Rudnick et al., 1993a)). In addition, hNmt acylates structural and nonstructural proteins encoded by the genomes of several enveloped and nonenveloped viruses (reviewed in Chow and Moscufo (1992)). These include, for example, the Pr55	extsuperscript{env} polyprotein precursor and nef proteins of human immunodeficiency virus I (HIV-I) (Gottlinger et al., 1989: Bryant and Ratner, 1990), hepatitis B virus L protein (Peraing et al., 1987), and the VP4 capsid protein of poliovirus (Chow et al., 1987). The functional contribution of the tetradeacanoyl moiety is quite varied, ranging from a critical role in regulating protein-protein and protein-membrane interactions to no apparent effect associated with its loss.

hNmt is a potential therapeutic target in some viral infections, e.g. blockade of N-myristoylation of HIV-I Pr55	extsuperscript{env} by site-directed mutagenesis of its Gly	extsuperscript{2} codon or selective incorporation of a variety of myristic acid analogs blocks viral assembly in acutely and chronically infected T lymphocyte cell lines (Gottlinger et al., 1989; Bryant and Ratner, 1990; Bryant et al., 1989, 1991; Devadas et al., 1992). Regulation of hNmt activity may also be useful in certain neoplastic states since a number of transforming tyrosine kinases are N-myristoylated: src (Schultz et al., 1985; Buss and Sefton, 1985), yes (Sukegawa et al., 1987; Sudol et al., 1988), fyn/syn (Semba et al., 1986; Kypka et al., 1988; Cheng et al., 1988), jgr (Katamine et al., 1988; Notario et al., 1989), and hck (Quintrell et al., 1987; Lock et al., 1991). Species-specific inhibitors of yeast Nmts that have little effect on hNmt may also be useful in treating systemic fungal infections. *Candida albicans* and *Cryptococcus neoformans* each produce a few N-myristoylproteins (Wiegand et al., 1992; Langner et al., 1992). Incorporation of some but not all oxatetradecanoic acids into these proteins is associated with cell death (Langner et al., 1992). Moreover, genetic studies have directly established that the *Saccharomyces cerevisiae* NMT1 gene is essential for vegetative growth (Duronio et al., 1989, 1991a, 1992a, 1992b, 1992c; Johnson et al., 1993).

The ability to exploit Nmt as a therapeutic target requires information about its substrate specificities and kinetic mechanism. To date, the 455-residue, monomeric (Towler et al., 1987b), cytosolic (Knoll et al., 1992) *S. cerevisiae* enzyme...
Experimental Procedures

Purification of Human Nmt—hNmt was prepared from an erythroblast leukemia (HEL) cell line (ATCC TIB 180) using DEAE-Sepharose, S-Sepharose, and hydroxylapatite chromatography as described in Kishore et al. (1993). hNmt activity eluted from the HTP column in two well resolved peaks (Kishore et al., 1993). Hydroxylapatite pools 1 and 2 (HTP1 and HTP2) had modest differences in their specific activities (specific activity of HTP1 was twice that of HTP2 (Kishore et al., 1993). However, HTP1 and HTP2 had indistinguishable kinetic properties when tested against GARASVLS-NH2, a known hNmt substrate representing residues 1-8 of the Pr55^Gag polyprotein precursor of HIV-1 (Kishore et al., 1993). Moreover, HTP1 and HTP2 had no demonstrable differences in their acyl-CoA substrate specificities (as determined by an analysis of a panel of 51 CoA derivatives included in CoA derivatives of C8:0-C16:0, ω-phenyl-containing fatty acids (overall chain length, C12-C15) plus selected tetradeconoyl, tetradeconoyl, and oxatetradecanoic acids (Kishore et al., 1993). Finally, both HTP1 and HTP2 were free of contaminating protease and thioesterase activities (Kishore et al., 1993).

The overall yield of hNmt activity was 25% relative to the unfractionated cell lysate. The -fold purification was 230. Analysis of Coomassie- or silver-stained SDS-polyacrylamide gels containing active functions from each purification step did not allow us to accurately estimate what percent of the HTP1 or HTP2 preparation was hNmt. The HTP1 was used out the course of the C4 reverse-phase HPLC purification of its slightly higher specific activity. The enzyme could be stored at 4 °C for periods up to 9 days in 30 mM MES, pH 6.5, 1 mM DTT, 1 mM EDTA without loss of activity or at ~80 °C for up to 9 months without measurable changes in specific activity.

Purification of S. cerevisiae Nmt1p from E. coli—Details of the host-vector system as well as the protocol for purifying this enzyme using P11 phosphocellulose and Mono-S chromatography can be found in our earlier papers (Rudnick et al., 1990, 1991, 1992a). The purity and integrity of the enzyme preparation was established by several criteria: (i) the presence of a single band on denaturing SDS-polyacrylamide gels incubated with Coomassie and silver stains; (ii) a single pl 8.15 isofrome after isoelectric focusing; and (iii) an atomic mass determined by electrospray mass spectroscopy (52,692 ± 3 Da) that was consistent with what is predicted from the sequence of the NMT1 gene after its introduction into the prokaryotic expression vector (cf. Rudnick et al., 1993).

Purification of Triligated Myristoyl-CoA—[1H]Myristoyl-CoA was enzymatically generated in a preparative reaction mixture composed of 150 μl of Triton X-100 (0.3%), 1.35 ml of 20 mM Tris-HCl, pH 7.5, containing 1 mM DTT and 0.1 mM EDTA, 100 μl of 49 mM ATP, 150 μl of 25 mM CoA in 1 mM DTT, 60 μl of 100 mM DTT, 450 μl of 50 mM HEPES pH 7.5, 0.1 μM and Pseudomonas acyl-CoA synthetase (1 unit/ml in HEPES buffer), and 600 μl of [9,10,13-3H]Myristic acid (1 μCi/ml in ethanol, specific activity = 99.3 Ci/mmol). Following a 1-2 h incubation at 30 °C, [1H]myristoyl-CoA was purified by solid-phase extraction using C8 reverse-phase syringe cartridges in accordance with the manufacturer’s instructions (C8 SPICE cartridge, Anachem, IL). After loading the cartridge with 200 μl of reaction mixture, the cartridges were collected using a stepwise elution protocol consisting of 4 ml each of 50 mM ammonium acetate, pH 5.3, containing 0, 20, 40, and 100% acetonitrile. Control experiments established that CoA, ATP, and other salts eluted in the 0% acetonitrile fraction, [1H]myristoyl-CoA in the 40% fraction, and [3H]myristic acid in the 100% fraction. The 40% fraction was then dried by centrifugal evaporation and resuspended in a small volume of 0.15% Triton X-100. The concentration of [1H]myristoyl-CoA was determined by counting the amount of radioactivity in a small aliquot. The sample volume was then adjusted by adding water and 0.15% Triton X-100 to give a final concentration of ~0.4 μg/ml for TLC/Triton X-100. The radiochemical purity of the sample was assessed by C4 reverse-phase HPLC and found to be >95% with less than 1% residual [3H]myristic acid.

In Vitro Nmt Assay—This assay is described in our earlier publications (Towler and Glaser, 1986; Kishore et al., 1991; Rudnick et al., 1992a). It involves initial enzymatic generation of [1H]myristoyl-CoA using [9,10,13-3H]myristoyl-CoA and Pseudomonas acyl-CoA synthetase (EC 6.2.1.3, Sigma). Triligated myristoyl-CoA is then added (either directly from the synthetase reaction or after purification) to tubes containing an octapeptide substrate and sufficient buffer to give a final concentration of 50 mM HEPES pH 7.5, 1 mM DTT, and 0.1% Triton X-100. Assays were initiated with either E. coli-derived Nmt1p or hNmt (HTP1). After a 10 min incubation at 25 °C, the reaction was terminated by adding ice-cold methanol. The
tritiated acyl peptide was separated from \(^{1}H\)myristoyl-CoA by reverse phase HPLC using a Vydac C4 column (15 cm × 0.46 cm) and acetonitrile-water:0.05% trifluoroacetic acid or acetonitrile:50 mM ammonium acetate, pH 5.3 (gradient for each buffer system = 35-45% acetonitrile with a slope of 1.67%/min). The amount of labeled acyl peptide produced was quantitated with an in-line scintillation counter (Packard, Radiochemical). pH Dependence of hNmt and Nmtlp Activity—Parallel in vitro enzyme assays employing partially purified hNmt and purified recombinant Nmtlp were conducted as described above except that 75 mM bis-tris propane, pH 6-9.5, was substituted for HEPES. NaCl (50 mM) was also added to control ionic strength changes in the buffer at the different pH values. Purified myristoyl-CoA (0.49-3.1 mM) was used to eliminate potential buffering effects from components of the acyl-CoA synthetase reaction. GARASVLS-NH\(_2\) (final concentration = 35 \(\mu\)M) was employed as the peptide substrate.

Kinetic Studies—Initial velocity studies were carried out in parallel using partially purified hNmt (0.3 \(\mu\)g of protein) and purified Nmtlp (5 ng). Each combination of substrate concentrations was assayed in triplicate, except for the CoA inhibition studies, which were done in duplicate. Each assay was evaluated for substrate depletion, and the results were discarded if substrate utilization exceeded 15%. Each study was repeated once. All kinetic analyses used [\(^{1}H\)]myristoyl-CoA directly from the acyl-CoA synthetase reaction, except for the CoA product inhibition studies, which employed purified [\(^{1}H\)]myristoyl-CoA. To characterize the kinetic profiles of hNmt and Nmtlp, initial velocities were examined by covariation of subsaturating concentrations of myristoyl-CoA (0.74-4.3 \(p\)M) and GNAAAAAR-NH\(_2\) (24-191 \(p\)M). Similarly, CoA product inhibition patterns against variable concentrations of myristoyl-CoA (0.49-3.1 \(p\)M) were collected using 26 \(p\)M GARASVLS-NH\(_2\) and a range of CoA concentrations (0-1200 \(p\)M). CoA product inhibition with GARASVLS-NH\(_2\) as the varied substrate (4.3-35 \(p\)M) employed 1 \(p\)M myristoyl-CoA and variable CoA concentrations from 0 to 600 \(p\)M. Inhibition by the non-hydrolyzable analog of myristoyl-CoA, S-(2-oxo)pentadecyl-CoA (Pange et al., 1989), was determined by varying its concentration from 0 to 80 \(nM\) with a fixed concentration of GARASVLS-NH\(_2\) (99 \(p\)M) and a range of myristoyl-CoA concentrations (0.2-3.9 \(p\)M).

All kinetic data were analyzed by averaging the replicate initial velocities and generating double-reciprocal (Lineweaver-Burk) plots of the averages. Kinetic parameters (e.g. \(k_{cat}\), \(V_{max}/K_{m}\), \(K_{i}\)) were determined from least-squares analysis of secondary plots. Studies of the Peptide Substrate Specificity of hNmt and Nmtlp—Octapeptides were synthesized on 4-methylbenzhydrylamine resin. Deprotected peptides were purified by reverse phase HPLC and were characterized by amino acid analysis and fast atom bombardment mass spectrometry. The peptide substrate specificities (\(V_{max}/K_{m}\) and \(V_{max}/K_{i}\)) of the orthologous human and S. cerevisiae acyltransferases were evaluated from primary plots (\(v/velocity versus 1/v[peptide]\)) of data collected with a fixed concentration of myristoyl-CoA (0.25 \(p\)M). Studies were carried out in parallel using 0.3 \(p\)M of 230-fold purified hNmt and 5 ng of purified Nmtlp, with the following exceptions: GNAASAR-NH\(_2\), 500 ng of Nmtlp; GNAASAA-NH\(_2\), 1.5 \(p\)M of hNmt, 50 ng of Nmtlp; GCRQSSE-NH\(_2\), 0.6 \(p\)M of hNmt, 50 ng of Nmtlp; GCTLSAED-NH\(_2\), 0.6 \(p\)M of hNmt, 500 ng of Nmtlp.

RESULTS

The Kinetic Pattern of Human and S. cerevisiae Nmt with the Substrates Myristoyl-CoA and GNAAAAAR-NH\(_2\)—To establish the range of enzyme concentration and assay time in which the initial velocity is linear, a broad range of these variables was examined using fixed concentrations of myristoyl-CoA (16 \(p\)M) and an octapeptide derived from the N-myristoylated catalytic subunit of protein kinase A, GNAAAAAR-NH\(_2\) (1 \(p\)M). The dependence of product formation on hNmt concentration was noted at five different assay times (5-35 min). At the shorter assay times (5-15 min), myristoyl GNAAAAAR-NH\(_2\) product formation increased linearly with enzyme concentration, but at assay times >20 min it deviated from linearity because of substrate depletion (data not shown). We established that 0.3 \(p\)M of total protein and a 10-min incubation gave an ample signal with no appreciable depletion of GNAAAAAR-NH\(_2\) or other peptide substrates used in this report.

The pH of the Nmt assay was varied from 6.0 to 9.5, the greatest activity was detected at pH 8.5 with both the human and yeast Nmts. The human and S. cerevisiae enzymes showed similar activities when the assay was supplemented with 100 mM Li\(^+\), Na\(^+\), or K\(^+\) (data not shown). With the assay conditions defined, we were able to investigate the effect of covariation of subsaturating concentrations of myristoyl-CoA and GNAAAAAR-NH\(_2\) on initial velocity. Double-reciprocal plots of 1/velocity versus 1/[GNAAAAAR-NH\(_2\)] at a series of fixed concentrations of myristoyl-CoA, and 1/velocity versus 1/[myristoyl-CoA] for a series of GNAAAAAR-NH\(_2\) concentrations are shown for hNmt in panels A and B of Fig. 1. A series of lines are seen for fits to sets of data points corresponding to fixed concentrations of the second substrate. Comparable results were obtained with Nmtlp (Rudnick et al., 1993b). The intersecting lines mean that for both enzymes the mechanism is likely one in which both substrates combine with enzyme to form a ternary complex (sequential mechanism) since a double displacement (ping-pong) mechanism normally leads to parallel lines. The elimination of the ping-pong mechanism is consistent with previous studies of Nmtlp (see the Introduction).

Secondary plots of the hNmt primary plot intercepts (panels C and D of Fig. 1) yields a \(K_{i}\) of 2.3 \(p\)M for myristoyl-CoA and 430 \(p\)M for GNAAAAAR-NH\(_2\). The corresponding values for S. cerevisiae Nmtlp are 1.4 \(p\)M for myristoyl-CoA and 68 \(p\)M for GNAAAAAR-NH\(_2\) (panels A and B of Fig. 2).

CoA Product Inhibition—Increasing amounts of either CoA and myristoyl-CoASVLS-NH\(_2\) and GARASVLS-NH\(_2\) were added to enzyme assays in the presence of a fixed subsaturating concentration of second substrate to determine the mode of product inhibition. The graphical analyses of double-reciprocal plots indicated that CoA was a noncompetitive inhibitor against myristoyl-CoA and a mixed-type inhibitor against GARASVLS-NH\(_2\) (Fig. 3). When the slopes and intercepts of primary plots were replotted to determine \(K_{i}\) for CoA, the data points were not linear but hyperbolic. These findings suggest that CoA is a partial noncompetitive inhibitor for myristoyl-CoA and a mixed-type inhibitor (\(\beta = 0^+\)) for GARASVLS-NH\(_2\) (Segal, 1975).

The product inhibition patterns can distinguish different kinetic mechanisms of a two-substrate reaction. A ping-pong reaction mechanism for hNmt was eliminated by graphical analysis of intersecting double-reciprocal plots in which one substrate is varied while the other is at a fixed, subsaturating concentration. The Theorell-Chance mechanism can be elim-

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2 We have suggested that a His residue is important for Nmtlp activity based on the results of chemical modification with dithiobis(pyridylcarboxylic acid) (Towler et al., 1987b). A marked increase in activity is observed when the pH is raised from 6.5 to 7.5 (data not shown). This finding is compatible with the notion that an amino acid with a neutral to slightly basic pK\(_a\) is important for activity. One of the 4 conserved His residues present in S. cerevisiae, C. albicans and human Nmt may also reside at or near the active site of hNmt (see Duronio et al. (1992d) and Rudnick et al. (1992c) for multiple sequence alignments of the primary structures of these orthologous enzymes).

3 The noncompetitive model of inhibition is a simple example of mixed inhibition in which the inhibition constants \(K_i\) and \(K_{i}\) are equal (Segal, 1975). For the purposes of enzyme mechanism characterization, these models are equivalent (Rudolph, 1979).

4 The partial noncompetitive and mixed-type (\(\beta = 0\)) models of inhibition represent a more complex reaction mechanism in which the formation of an ESI complex can form a product with equal or less activity than the ESI complex. This is different from traditional noncompetitive and mixed-type inhibition in which the ESI or ESI complex cannot undergo catalysis (Segal, 1975).
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Fig. 1. Primary and secondary plots to determine peptide and myristoyl-CoA $K_m$ values for human Nmt. To determine true $K_m$ values of GNAAAARR-NH$_2$ and myristoyl-CoA, the concentration of one substrate was varied at a fixed subsaturating value of the second substrate. Shown are double-reciprocal plots of the initial velocity pattern with myristoyl-CoA as the varied substrate (panel A) and with GNAAAARR-NH$_2$ as the varied substrate (panel B). Secondary plots of $1/(V_{max})$ versus $1/[substrate]$ reveals the true $K_m$ for myristoyl-CoA (panel C) and GNAAAARR-NH$_2$ (panel D).

Fig. 2. Secondary plots to determine peptide and myristoyl-CoA $K_m$ values for S. cerevisiae Nmt1p. Analyses comparable with those described in the legend to Fig. 1 were performed.

The peptide $K_m$ values of the peptide derived from the C$_a$ subunit of bovine heart protein kinase A (GNAAAARR-NH$_2$) was similar for both acyltransferases as was its $V_{max}$ (Table I). Addition of an amino-terminal Met residue to this peptide (yielding MGNNAAAARR-NH$_2$) or substituting Ala for Gly$^1$ (ANAAAARR-NH$_2$) produced peptides, which are not substrates. Addition of a ≥10-fold molar excess of ANAAAARR-NH$_2$ inhibited because each product would have to be a competitive inhibitor of one substrate and a noncompetitive inhibitor of the second substrate (Segal, 1975). For random mechanisms, each product is a competitive inhibitor of each substrate, which also does not apply to hNmt. The reaction mechanism that best fits that of human Nmt is an ordered Bi Bi mechanism. Myristoyl-CoA binds first to the enzyme followed by the addition of the peptide/nascent protein. CoA, which is a noncompetitive inhibitor of myristoyl-CoA and peptide, is released first followed by myristoylpeptide.

Inhibitor Studies Using S-(2-Oxo)pentadecyl-CoA—The ability of a non-hydrolyzable analog of myristoyl-CoA, S-(2-oxo)pentadecyl-CoA (Paige et al., 1989), to inhibit human Nmt activity was examined at several different concentrations of analog CoA. The results indicate that S-(2-oxo)pentadecyl-CoA is a competitive inhibitor of myristoyl-CoA (Fig. 4A). The secondary plot revealed a $K_i$ of 56 nM for the compound (Fig. 4B), which is in good agreement with the $K_i$ of 14 nM reported for Nmt1p (Rudnick et al., 1991) and the $K_i$ of 24 nM reported for mouse brain Nmt (Paige et al., 1989).

Peptide ($K_m$)$_{app}$ Determinations—We next compared the peptide substrate specificities of hNmt and Nmt1p using a panel of 12 synthetic peptides. The ($K_m$)$_{app}$ was determined at subsaturating concentrations of myristoyl-CoA (0.25 μM). The results are presented in Table I.
Fig. 3. Analysis of the inhibition of human Nmt by CoA. The effects of increasing CoA concentrations on the activity of human Nmt were examined. hNmt (0.3 µg) was added to assays containing increasing amounts of CoA and substrate at a fixed concentration of second substrate. Analysis of this data by double-reciprocal plots indicated that CoA was a noncompetitive inhibitor with respect to myristoyl-CoA (panel A) and a mixed-type inhibitor with respect to GARASVLS-NH₂ peptide (panel B).

Fig. 4. Analysis of the inhibition of human Nmt by S-(2-oxo)pentadecyl-CoA. The effect of S-(2-oxo)pentadecyl-CoA inhibition against myristoyl-CoA was determined using 100 µM GARASVLS-NH₂ and increasing amounts of S-(2-oxo)pentadecyl-CoA and myristoyl-CoA. Based on an analysis of double-reciprocal plots, S-(2-oxo)pentadecyl-CoA is a competitive inhibitor since the (K_m) app and not (V_max) app of the enzyme was affected (panel A). Replotting the slopes of the reciprocal plot (panel B) revealed a (K_i) app of 56 nM for S-(2-oxo)pentadecyl-CoA.

relative to GNAAAARR-NH₂ had no detectable effect on the amount of myristoyl-GNAAAARR-NH₂ produced under the conditions used for the in vitro enzyme assay, suggesting that the Ala³-containing peptide did not bind to either hNmt or Nmtlp. The results allowed us to conclude that hNmt does not contain an associated methionyl aminopeptidase activity that can remove the initiator Met of nascent substrates and that an amino-terminal glycine residue is essential for substrate recognition by both acyltransferases.

Substitution of an Arg for Ala³ in GNAAAARR-NH₂ produced 2- and 5-fold increases in (K_m) app for hNmt and Nmtlp, respectively, and a 4-fold reduction in peptide catalytic efficiency (V_max/K_m) app for Nmtlp but no significant change in (V_max/K_m) app for hNmt. Earlier studies had indicated that Nmtlp tolerates a variety of amino acids at position 3 of its substrates but that neutral residues are preferred over basic residues, which are preferred over acidic amino acids (Towler et al., 1987b, 1988a, 1988b).

Substituting a serine residue in position 5 of GNAAAARR-NH₂ does not enhance the binding of peptide to hNmt as significantly as to Nmtlp (9-fold versus 135-fold, cf. Table I). The Ser⁵ substitution has a much smaller effect on V_max for hNmt (80% of GNAAAARR-NH₂) than for Nmtlp (V_max = 6% of GNAAAARR-NH₂). The differences in peptide substrate specificities of the two Nmts can be readily seen when the (V_max/K_m) app values obtained with GNAAAARR-NH₂ for each enzyme are divided by the catalytic efficiency obtained with the HIV-I Pr55₅₅ octapeptide, which also contains a Ser⁵ (GARASVLS-NH₂). GARASVLS-NH₂ provides a good reference standard because its peptide catalytic efficiency ((V_max/K_m) app) is the same for human and S. cerevisiae Nmt (61, see Table I). GNAAARR-NH₂ shows an increased catalytic efficiency with Nmtlp compared to GARASVLS-NH₂ ratio of (V_max/K_m) app of GNAAARR-NH₂ divided by (V_max/K_m) app of GARASVLS-NH₂ = 2, whereas the ratio with hNmt is 0.5.

Table I illustrates another example of differences in the interaction of Nmtlp and hNmt with a Ser⁵-containing peptide. S. cerevisiae produces two functionally interchangeable, essential Arf proteins, Arflp and Arf2p (Stearns et al., 1990). GLYASKLSS-NH₂, a derivative of the amino-terminal sequence of Arf2p (GLYASKLF-NH₂) has a 10-fold higher affinity for Nmtlp than hNmt ((K_m) app = 80 nM vs 706 nM, respectively) and a 9-fold higher catalytic efficiency ((V_max/K_m) app = 750 (hNmt) versus 86 (Nmtlp)).

It is important to note that not all octapeptides containing Ser⁵ are substrates for Nmtlp. GCRQSSSEE-NH₂ and GCTLSAED-NH₂ represent the amino-terminal sequences of human Ga4 and rat Ga₁, respectively. The two peptides are not substrates (V_max < 0.01 pmol/min) for S. cerevisiae Nmtlp.

We have suggested that the reason for the enhanced binding yet slower turnover of GNAASARR-NH₂ relative to its GNAAARR-NH₂ parent may be due to hydrogen bonding of Ser⁵ to Nmtlp, substituting Cys for Ser⁵ in GNAASARR-NH₂ or Thr for Ser⁵ dramatically decreases peptide binding ((K_m) app) but increases (V_max) app (Towler et al., 1988a).
order to the addition of substrates, resulting in a partitioning of the reaction pathway. This partitioning arises from a synergy of substrate binding in which the binding of the preferred first substrate greatly enhances the binding of the second (e.g., see Jance et al. [1992]). While the available data support the binding of myristoyl-CoA before peptide, it does not rule out the possible occurrence of peptide binding first in a small fraction of the catalytic events. Nevertheless, our studies strongly support a model in which myristoyl-CoA binds to hNmt (and Nmtlp) before peptide in a typical catalytic cycle. This model emphasizes the importance of defining how myristoyl-CoA metabolism is regulated in vivo in yeast and mammalian cells. In S. cerevisiae, genetic tools are available to manipulate both the de novo pathway for fatty acid biosynthesis and the enzymes that activate exogenous fatty acids so that their relative contributions to the establishment and maintenance of myristate and myristoyl-CoA pools can be ascertained (e.g. see Duronio et al. [1992b, 1992d]). Recent studies of wild type and peroxisome-deficient mutant mammalian cell lines have provided insights about the pathways for metabolic processing of myristate (Wang et al., 1992).

There are evidently three components that must be recognized by Nmt for it to co-translationally process nascent polypeptides; the acyl chain and CoA moiety of myristoyl-CoA must interact with the apoenzyme for a binary complex to form and a functional peptide binding site to be produced. The myristoyl-CoA-Nmt complex must then recognize structural features in the amino terminus of proteins. Although the acyl chain specificity of Nmtlp and hNmt has been extensively characterized, little is known about the features in CoA that are important for recognition. This reflects, in large part, the lack of CoA derivatives with systematic alterations in the adenine, pantenolate, β-alanine, and β-mercaptoethylamine units. Myristoyl 3'-dephospho-CoA and myristoyl etheno-CoA are substrates for Nmtlp, suggesting that limited perturbations of the adenine moiety do not disrupt interactions between the enzyme and its acyl-CoA (Rudnick et al., 1991; Wagner and Retey, 1991). However, deletion of adenine dramatically perturbs these interactions. For example, the methylene bridge between the acyl moiety and the CoA sulfur atom in S-(2-oxo)pentadecyl-CoA makes it non-hydrolyzable and thus unable to serve as an acyl donor. Removal of the adenine moiety from this potent inhibitor of both Nmtlp and hNmt yields a compound, S-(2-
oxopentadecyl-pentanone, that is 1000-fold less active.\(^6\) Given the number of functional groups available, CoA for interaction with protein toxins via hydrogen bonding and/or van der Waals contacts (cf. the structure of citrate synthetase with bound acetyl-CoA (Remington et al., 1982; Wiegand and Remington, 1986)), it is remarkable that seemingly modest alterations in the acyl chain can have so much impact on acyl-CoA/Nmt interactions and formation of a functional peptide binding site. Nonetheless, a number of observations highlight the extent of conservation of acyl chain recognition between yeast and human Nmt: (i) the acyl chain length specificities are indistinguishable (optimum = C14, while addition or deletion of one methylene is well accommodated, presumably because there is no need to select against odd chain length fatty acids in vivo; cf. Kishore et al., 1993); (ii) surveys of 12 tetradecynoyl-CoAs (Y2–Y13), 10 pure cis iso-...
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