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Heteroatom-substituted fatty acid analogs as substrates for *N*-myristoyltransferase: An approach for studying both the enzymology and function of protein acylation

(protein myristoylation/protein–fatty acyl interaction)

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ABSTRACT **Myristoyl-CoA:protein *N*-myristoyltransferase (NMT), the enzyme that transfers the myristoyl (14:0) moiety from myristoyl CoA thioester to nascent proteins, is remarkably specific for both peptide and fatty acyl CoA substrates. To investigate the interaction of NMT with fatty acyl CoA substrates, we have synthesized 10 oxygen- or sulfur-substituted fatty acid analogs. These analogs differ dramatically in hydrophobicity from naturally occurring fatty acids of similar length. As acylpeptides, sulfur-substituted myristic acid analogs migrate on reverse-phase HPLC like 11:0 or 12:0 fatty acids, while oxygen-substituted analogs migrate like 9:0 to 11:0 fatty acids. CoA thioesters of several of these analogs serve as good NMT substrates *in vitro*, implying that NMT selects fatty acyl substrates primarily on the basis of chain length rather than hydrophobicity. Myristelaidoyl (14:1, $\Delta^{9,10}$-*trans*) CoA is also a significantly better substrate than myristoleoyl (14:1, $\Delta^{9,10}$-*cis*) CoA. The fatty acyl group bound to NMT profoundly influences the rate of acylpeptide formation and the affinity of NMT for peptide substrates. However, the peptide substrate bound to NMT does not produce significant alterations in the enzyme's affinity for myristoyl CoA. *In vitro* characterization of these heteroatom substituted analogs suggests that they will be efficiently incorporated into proteins *in vivo* and may markedly alter acylprotein targeting and function.**

Numerous viral and cellular proteins are modified by the covalent attachment of fatty acyl groups (reviewed in refs. 1–4). Three distinct types of acylation have been described: Glycosylphosphatidylinositol-linked acylation, thioester- or ester-linked acylation, and N-myristoylation. Protein N-myristoylation involves the cotranslational attachment of a 14:0 fatty acid to N-terminal glycine residues via an amide linkage. This modification appears to be important for interactions with both membranes and other proteins.

The reason for selection of myristate (a relatively rare fatty acid) for covalent attachment to specific proteins remains poorly understood. N-myristoylated proteins are found in numerous cellular compartments including plasma membrane, endoplasmic reticulum, nucleoskeleton, and cytosol (reviewed in ref. 1). While N-myristoylation appears crucial for membrane association in some cases (5–8), it is not sufficient for targeting to particular cellular membranes. It has been suggested (9) that the myristoyl group allows reversible membrane association or that there may be *N*-myristoyl protein receptors (10, 11). The fatty acyl structural features required for proper targeting and function of *N*-myristoyl proteins have not been determined.

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Myristoyl-CoA:protein *N*-myristoyltransferase (NMT), the enzyme that transfers the myristoyl group from myristoyl CoA thioester to N-terminal glycine residues of nascent acylproteins, is remarkably specific for both peptide and fatty acyl CoA substrates (9, 12, 13). The substrate specificity of NMT has been highly conserved through evolution; yeast, wheat germ, and rat liver NMT activities are strikingly similar (13, 14). The high degree of evolutionary conservation of both peptide and fatty acyl CoA substrate specificity suggests that the myristoyl group may have unique structural characteristics essential for acylprotein function.

Now we have examined the fatty acyl specificity of yeast NMT using naturally occurring C_{10}–C_{16} acyl CoA esters and characterized a number of heteroatom-substituted fatty acyl analogs. Since some analogs differ dramatically in hydrophobicity from myristate while maintaining approximately the same chain length, we were able to assess the relative contributions of chain length and hydrophobicity to NMT-fatty acyl CoA interactions. Several analogs with markedly reduced hydrophobicity are excellent NMT substrates *in vitro*. If incorporated into *N*-myristoyl proteins, these analogs may radically alter the acylprotein's subsequent interactions with membranes or other hydrophobic proteins.

MATERIALS AND METHODS

Yeast NMT Purification. Yeast NMT was partially purified (150-fold; ref. 9) from *Saccharomyces cerevisiae* strain BJ405 (15).

Preparation of Gly-Asn-Ala-Ala-Ser-Tyr(^{125}I)-Arg-Arg (GNAAS[^{125}I]YRR). [^{125}I]Iodotyrosine ([^{125}I]Y-containing GNAAS[^{125}I]YRR with a specific activity of ≈3000 cpm/pmol was prepared and purified as described (14).

Determination of Peptide Kinetic Characteristics for GNAAS[^{125}I]YRR with Each Fatty Acid Analog. Acyl CoA thioesters were prepared (16) with *Pseudomonas* acyl-CoA ligase (0.3 unit/ml) in a reaction mixture containing 33 μM fatty acid. Triton X-100 (0.06%) was included to ensure fatty acid or analog solubility. Typically this acyl CoA mixture was diluted to 15 μM (total fatty acid concentration) in an acylpeptide-generating system that contained yeast NMT (0.5 μg; specific activity with saturating concentrations of GNAAS[^{125}I]YRR and myristoyl CoA = 3200 pmol·min^{-1}·mg^{-1}) and variable concentrations of GNAAS[^{125}I]YRR. The ^{125}I-labeled acylpeptide was purified by reverse-phase C_{18} HPLC (16). Kinetic characteristics of the peptide substrate were determined in parallel with each analog and myristoyl

Abbreviations: NMT, myristoyl-CoA:protein *N*-myristoyltransferase; [^{125}I]Y, [^{125}I]iodotyrosine or Tyr(^{125}I); GNAAS[^{125}I]YRR, Gly-Asn-Ala-Ala-Ser-Tyr(^{125}I)-Arg-Arg.

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CoA (Table 1). All peptide V_{max} data were normalized to the V_{max} of the reaction with myristoyl CoA, which was measured concurrently. Kinetic data represent averages of at least duplicate experiments.

Fatty Acyl and Fatty Acyl Analog Kinetic Characterization. Acyl CoA thioesters for each fatty acid or analog were generated as described above except that 10-fold more *Pseudomonas* acyl-CoA ligase (3 units/ml) was used. GNAAS[^{125}I]YRR was used *at its* K_m *for the particular fatty acyl substrate or analog to be tested.* Acyl CoA concentration varied from reaction to reaction. Kinetic characteristics for myristoyl CoA and each analog were determined in parallel (Table 1). All V_{max} data were normalized to the V_{max} for myristoyl CoA. Experiments were performed in duplicate, and the data were averaged.

Determination of the Efficiency of Enzymatic Acyl CoA Thioester Synthesis. Acyl CoA synthesis was quantitated by a modification of the procedure of Hosaka *et al.* (17). Myristoyl, palmitoyl, and 11-oxymyristoyl CoA thioesters were generated enzymatically (16) in reaction mixtures containing 0.033 nmol of tritiated fatty acid, 3.3 nmol of unlabeled fatty acid, 0.06% Triton X-100, and either 0.3 unit/ml or 3 units/ml of *Pseudomonas* acyl-CoA ligase. Radioactivity remaining in the aqueous phase after acidification to pH 2 with HCl and extraction three times with heptane was used as a measure of acyl CoA generated. Each heptane extraction removed >90% of the free fatty acid.

2-Octanol/Water Partition Coefficients. Enzymatically generated acylpeptides were purified by C_{18} reverse-phase HPLC, dried under a stream of nitrogen, and resuspended in 2 ml of 1:1 (vol/vol) 2-octanol/water. Mixtures were vigorously mixed in three 30-sec spurts. Aliquots (800 μl) of the aqueous and organic phases were assayed in a γ counter.

Determination of Myristoyl CoA K_m as a Function of Peptide Substrate. Myristoyl CoA K_m was determined with three peptides: Gly-Ala-Gln-Leu-Ser-Thr-Leu-Gly (K_m = 3 μM), Gly-Asn-Ala-Ala-Ala-Ala-Arg-Arg (K_m = 60 μM), and Gly-Ala-Arg-Ala-Ser-Val-Ser-Gly (K_m = 430 μM). [^{3}H]Myristoyl CoA was generated with *Pseudomonas* acyl-CoA ligase (3 units/ml) and Triton X-100 (0.06%) so that the final total myristoyl concentration was 4.4 μM. Each peptide was used at its K_m as determined (9, 12).

Synthesis of Fatty Acid Analogs. Analogs were synthesized by combination of an appropriate ω-bromo acid with thiol or alcohol in the presence of base and generally were purified by extraction into ethyl acetate at pH 1 and silica dry column chromatography. The purified compounds were characterized by TLC, melting point, ^{1}H NMR, ^{13}C NMR, and MS. Synthesis of 11-(ethylthio)undecanoic acid (12-thiamyristic acid)¶ has been described (14). Information concerning the synthesis of other analogs is available from the authors upon request.

Synthesis of Tritiated 10-(Propoxy)decanoic Acid (11-Oxymyristic acid). Tritiated 11-oxymyristic acid was prepared by catalytic reduction of 10-(propynoxy)decanoic acid with tritium gas diluted with hydrogen. The product was purified by TLC, and radiochemical purity was assessed by HPLC. Product identity was verified by coelution with unlabeled 10-(propoxy)decanoic acid and by MS.

RESULTS

Synthesis of Fatty Acid Analogs. The peptide substrate specificity of NMT has been characterized in detail by using >90 synthetic peptides (9, 12, 13, 16). The structural requirements for the fatty acyl CoA substrates of NMT have not been as carefully defined. In this study, we have synthesized a number of oxygen- and sulfur-containing fatty acid analogs to examine the details of NMT–fatty acyl CoA interaction. These analogs allow us to determine the kinetic effects of

¶We have adopted the nomenclature used by Pascal *et al.* (18) in which analogs are identified by type and position of heteroatom substitution as well as by chain length. Thus, 11-(ethylthio)undecanoic acid ($CH_3CH_2S(CH_2)_{10}COOH$) is referred to as 12-thiamyristic acid.

Table 1. Kinetic characterization of CoA thioester substrates formed with fatty acids and fatty acid analogs

Fatty acid formula	Acyl-peptide elution time, min	Peptide		Acyl CoA		Fatty acid name
		K_m, μM	V_{max},* relative %	K_m, μM	V_{max},* relative %	
Unsubstituted fatty acids						
$CH_3(CH_2)_{12}COOH$	26	10	100	0.6	100	Myristic acid
$CH_3(CH_2)_8COOH$	14	95	80	5.5	230	Decanoic acid
$CH_3(CH_2)_{10}COOH$	21	50	140	1.4	280	Dodecanoic acid
$CH_3(CH_2)_{14}COOH$	32	35	14	1	28	Palmitic acid
$CH_3(CH_2)_3CH{=}CH(CH_2)_7COOH$						
$\Delta^{9,10}$-*cis*	22	11	55	2	30	Myristoleic acid
$\Delta^{9,10}$-*trans*	23	8	150	0.5	60	Myristelaidic acid
Position 6, 12, or 13 heteroatom-substituted analogs						
$CH_3CH_2S(CH_2)_{10}COOH$	18	19	98	1.4	130	12-Thiamyristic acid
$CH_3(CH_2)_7S(CH_2)_4COOH$	21	11	213	1.5	160	6-Thiamyristic acid
$CH_3O(CH_2)_{11}COOH$	12–13	19	177	1.2	70	13-Oxymyristic acid
$CH_3CH_2O(CH_2)_{10}COOH$	12	15	62	1.8	64	12-Oxymyristic acid
$CH_3(CH_2)_7O(CH_2)_4COOH$	16	31	335	1.6	675	6-Oxymyristic acid
Position 11 heteroatom-substituted analogs						
$CH_3(CH_2)_2S(CH_2)_9COOH$	18	47	245	1.5	250	11-Thiamyristic acid
$CH_3(CH_2)_2O(CH_2)_9COOH$	11–12	34	150	6.1	270	11-Oxymyristic acid
$HC{\equiv}CCH_2O(CH_2)_9COOH$	9	28	200	6.5	320	11-Oxy-13-yne-myristic acid
Analogs with altered chain length and heteroatom substitution						
$CH_3O(CH_2)_{10}COOH$	12–13	47	232	6.9	163	12-Oxytridecanoic acid
$CH_3(CH_2)_3O(CH_2)_{10}COOH$	19	42	25	1	80	12-Oxypalmitic acid

*Since fatty acyl concentration was 15 μM for determining peptide V_{max}, the values reported for peptide V_{max} with decanoyl, 11-oxymyristoyl, 11-oxy-13-yne-myristoyl, and 12-oxytridecanoyl CoA (i.e., the compounds with high fatty acyl CoA K_m) may be slightly lower than the actual peptide V_{max}. In all these cases, the compounds were used at >2.1 times their K_m, so actual peptide V_{max} values with these fatty acids should not be more than 50% greater than the value reported.

hydrophobicity and chain length as independent parameters. The majority of these compounds are analogs of myristic acid, a 14:0 fatty acid. They differ from myristic acid by substitution of oxygen or sulfur for a methylene group.¶

Characterization of Acylpeptides. Fatty acids and fatty acid analogs were tested *in vitro* as NMT substrates after enzymatic conversion to their CoA thioesters with *Pseudomonas* acyl-CoA ligase. This ligase is largely nonspecific for fatty acid substrates (19). Enzymatically generated acyl CoA thioesters were used for synthesis of acylpeptides by adding the iodinated peptide substrate GNAAS[^{125}I]YRR and yeast NMT. Acylpeptides were purified by C_{18} reverse-phase HPLC using a linear gradient of acetonitrile in water.

The C_{18} reverse-phase HPLC elution time for acylpeptides is strongly dependent on fatty acid structure and chain length (see Table 1 or Fig. 1). Myristoyl-GNAAS[^{125}I]YRR was eluted at 26 min, while decanoyl, dodecanoyl, and palmitoyl acylpeptides were eluted at 14, 21, and 32 min, respectively. Thus, for every two-methylene-group increase in chain length, reverse-phase HPLC acylpeptide elution time increased by ≈6 min. Both cis and trans double bonds decreased elution time by 3–4 min. Substitution of sulfur or oxygen for a methylene group dramatically altered the elution time. A single sulfur in place of a methylene group decreased acylpeptide elution time by 5–8 min. This gave an elution time comparable to that expected for the acylpeptide of an 11:0 or 12:0 fatty acid. Oxygen-for-methylene substitutions decreased HPLC elution time by 10–15 min. Similar acylpeptide elution times are expected for 9:0 to 11:0 fatty acids. Unsaturation and oxygen substitution had an additive effect on elution time. An oxygen-substituted myristic acid analog with a triple bond was eluted as an acylpeptide at 9 min on reverse-phase HPLC as might be expected for acylpeptides of an 8:0 or 9:0 fatty acid (see 11-oxy-13-yne-myristic acid in Table 1).

Partitioning between 2-octanol and water is a standard biochemical assay for characterizing the hydrophobicity of organic molecules (20, 21). There is a strong correlation between acylpeptide C_{18} reverse-phase HPLC elution times and octanol/water partition coefficients (Fig. 2). Therefore,

06	6-oxymyristic acid	S6	6-thiamyristic acid	C10	decanoic acid
011	11-oxymyristic acid	S11	11-thiamyristic acid	C12	dodecanoic acid
011 yne	11-oxy-13-yne-myristic acid	S12	12-thiamyristic acid	C14	myristic acid
012	12-oxymyristic acid			C16	palmitic acid
012, 13 atom	12-oxytridecanoic acid			$\Delta^{9,10}{}_{cis}$	myristoleic acid
012, 16 atom	12-oxypalmitic acid			$\Delta^{9,10}{}_{trans}$	myristelaidic acid
013	13-oxymyristic acid				

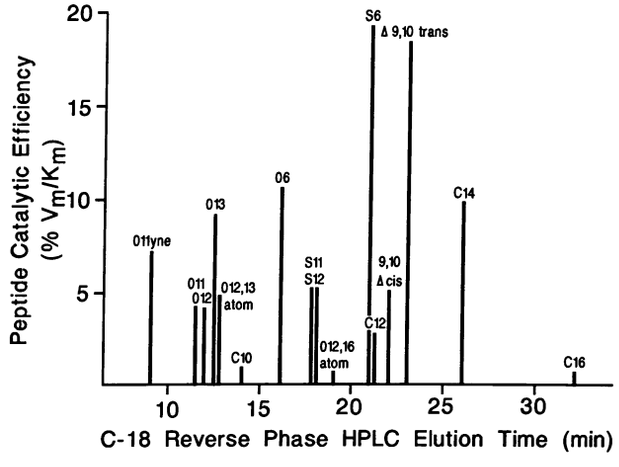

FIG. 1. Catalytic efficiency (V_{max}/K_m) for the peptide substrate as a function of C_{18} reverse-phase HPLC acylpeptide elution time. Peptide catalytic efficiencies were calculated from the data in Table 1 and plotted versus C_{18} reverse-phase HPLC elution time for the acylpeptide generated.

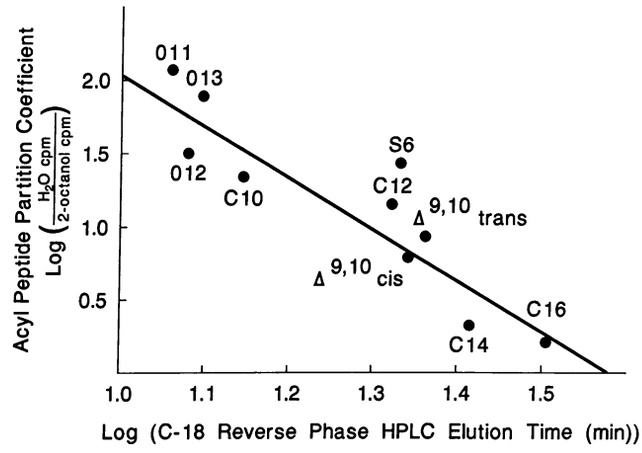

FIG. 2. Correlation of C_{18} reverse-phase HPLC elution time with 2-octanol/water partition coefficients. Enzymatically generated acylpeptides were purified by C_{18} reverse-phase HPLC. The partitioning of purified acylpeptides between water and 2-octanol was performed as described. A line was fit to the data by using least-squares analysis (correlation coefficient $R = -0.907$). Abbreviations are as in Fig. 1.

we believe that C_{18} reverse-phase HPLC elution time is a good indicator of the relative hydrophobicity of naturally occurring and heteroatom-containing fatty acids.

Peptide Substrate Kinetic Characteristics Are Dramatically Affected by Fatty Acid Choice. The kinetic characteristics for GNAAS[^{125}I]YRR were tested for each analog or naturally occurring fatty acid in parallel with enzymatically generated myristoyl CoA. The fatty acyl CoA bound to NMT significantly affects NMT's affinity for peptide substrates (Table 1). With myristoyl CoA, GNAAS[^{125}I]YRR was bound by NMT with high affinity (K_m = 10 μM), and acylpeptide was generated efficiently. Both increases and decreases in fatty acyl chain length adversely affect peptide K_m. Peptide K_m increased to 35 μM with palmitoyl, 50 μM with dodecanoyl, and 95 μM with decanoyl CoA thioesters. These changes in peptide K_m as a function of fatty acyl chain length were also seen with oxygen-substituted fatty acid analogs. Enzymatically generated 12-oxytridecanoyl CoA and 12-oxypalmitoyl CoA gave peptide K_m values of 47 μM and 42 μM, respectively. Peptide V_{max} was also dramatically decreased for both palmitoyl CoA (14% of myristoyl CoA) and 12-oxypalmitoyl CoA (25% of myristoyl CoA). Thus, fatty acyl moieties two atoms longer than myristate are selected against at the level of both peptide binding and acylpeptide formation.

In contrast to the marked changes in peptide kinetic characteristics observed as a function of fatty acyl chain length, relatively minor alterations resulted from heteroatom substitution. Although 12-thiamyristoyl and 6-thiamyristoyl peptides migrated like acylpeptides of 11:0 or 12:0 fatty acids on reverse-phase HPLC, GNAAS[^{125}I]YRR was still bound tightly by NMT (K_m = 19 μM and 11 μM, respectively) and acylpeptides were efficiently generated when these analogs were used as substrates (V_{max} = 98% and 213% of myristoyl CoA V_{max}, respectively). Oxygen-substituted 12-oxymyristoyl and 13-oxymyristoyl groups also allowed tight peptide binding (K_m = 15 μM and 19 μM, respectively) and were efficiently transferred to synthetic peptides (62% and 177% of myristoyl CoA V_{max}, respectively). These analogs were significantly better substrates than decanoyl CoA even though their apparent hydrophobicity is comparable.

Some heteroatom substitutions are less well accepted by NMT. 6-Oxymyristoyl CoA, for example, caused an increase in peptide K_m to 31 μM. Peptide V_{max}, however, increased to 335% of the V_{max} with myristoyl CoA, so that catalytic efficiency for the peptide substrate was virtually identical

with myristoyl CoA and 6-oxymyristoyl CoA (V_{max}/K_m = 10 and 10.8, respectively). Substitution at fatty acid position 11 by either sulfur or oxygen led to 3- to 5-fold increases in peptide K_m. Nonetheless, peptide V_{max} was also increased, suggesting that the CoA thioesters of these fatty acid analogs may function as substrates for NMT *in vivo*.

The effect of fatty acid or analog choice on peptide substrate catalytic efficiency (V_{max}/K_m) is summarized in Fig. 1. The CoA thioester of myristic acid (acylpeptide elution time, 26 min) gave the best peptide catalytic efficiency of the saturated, naturally occurring fatty acids tested. The peptide catalytic efficiency with dodecanoyl CoA (acylpeptide elution time, 21 min) is about one-third of that with myristoyl CoA. Decanoyl CoA (acylpeptide elution time, 14 min) and palmitoyl CoA (acylpeptide elution time, 32 min) caused significant decreases in GNAAS[^{125}I]YRR catalytic efficiency. Clearly, several fatty acyl CoA analogs with dramatically different C_{18} acylpeptide elution times from that of *N*-myristoyl peptide serve as good NMT substrates *in vitro*. 6-Oxymyristoyl CoA and 13-oxymyristoyl CoA, for example, gave peptide substrate catalytic efficiencies almost identical to that for myristoyl CoA (Fig. 1).

Kinetic Characteristics of Myristoyl CoA Are Not Affected by Peptide Substrate Choice. The average K_m for myristoyl CoA with GNAAS[^{125}I]YRR at a half-saturating concentration was 0.6 μM (Table 2). To test the effect of peptide substrate choice on fatty acyl CoA K_m, the kinetic characteristics of myristoyl CoA were determined with peptide substrates that have a >100-fold range in their affinity for yeast NMT (K_m = 3–430 μM). Myristoyl CoA K_m was the same with all peptide substrates tested (Table 2).

Acyl CoA Substrate Kinetic Characteristics Are Affected by Both Chain Length and the Position of Heteroatom Substitution. The K_m for CoA thioesters of saturated fatty acids two carbons longer and two carbons shorter than myristic acid as well as a large number of heteroatom-substituted analogs varied between 1 μM and 1.8 μM (Table 1). However, when fatty acid chain length decreased by four methylene groups, fatty acyl CoA K_m increased severalfold to 5.5 μM. Substitution of an oxygen at position 12 and decrease in chain length by one methylene group (12-oxytridecanoic acid) also significantly increased fatty acyl CoA K_m (to 6.9 μM). Comparable increases in K_m were observed when an oxygen atom was substituted in position 11 (K_m = 6.1–6.5 μM). Surprisingly, substitution of a sulfur in position 11 had only a modest effect on fatty acyl CoA K_m.

The acyl moiety of most fatty acyl and fatty acyl analog CoA thioesters tested was efficiently transferred to the iodinated peptide substrate. Palmitoyl CoA gave the lowest V_{max} for any of the enzymatically generated saturated fatty acyl and analog CoA thioesters tested (28% of myristoyl CoA). The acyl moiety of all other saturated analogs (including 12-oxypalmitoyl CoA) were transferred at least 65% as well as the myristoyl group. Decanoyl CoA and dodecanoyl CoA had a fatty acid V_{max} that was 230% and 280% of that of myristoyl CoA, respectively. Position 11-substituted analogs also had high V_{max} (varying between 250% and 320% of myristoyl CoA V_{max}) even though both peptide and fatty acid tended to be poorly bound by NMT with these analogs. Strikingly, 6-oxymyristoyl CoA had a V_{max} that was 675% of that of myristoyl CoA and a fatty acid K_m of only 1.6 μM.

Table 2. K_m for myristoyl CoA with four different peptides

Peptide	Peptide K_m, μM	Myristoyl CoA K_m with indicated peptide, μM
GNAAS[^{125}I]YRR	10	0.6
GAQLSTLG	3	0.4
GNAAAARR	60	0.4
GARASVSG	430	0.5

K_m for myristoyl CoA was determined with unlabeled myristoyl CoA and the iodinated peptide GNAAS[^{125}I]YRR or with [^{3}H]myristoyl CoA and the unlabeled peptides indicated.

Unsaturation at Position 9,10 Markedly Affects Both Fatty Acyl CoA and Peptide Kinetic Parameters. Comparison of myristoleic acid ($\Delta^{9,10}$-*cis*) to myristelaidic acid ($\Delta^{9,10}$-*trans*) revealed a strong preference for the trans double bond. K_m for enzymatically generated myristelaidoyl CoA (0.5 μM) was similar to that for myristoyl CoA (0.6 μM) (Table 1). In contrast, K_m for myristoleoyl CoA was 4-fold higher. V_{max} for myristelaidoyl CoA was 2-fold higher than that for myristoleoyl CoA, and peptide V_{max} was 3-fold higher with the trans than with the cis unsaturated fatty acyl analog. Thus, the trans unsaturated fatty acyl group is both bound and transferred more efficiently to peptide than the cis unsaturated fatty acyl group.

Efficiency of Enzymatic Generation of Acyl CoA Thioesters. One potential problem with interpreting data about the substrate characteristics of the fatty acid analogs is that there may be variability in the efficiency of their enzymatic conversion to CoA esters by the reportedly "nonspecific" *Pseudomonas* CoA ligase. The major concern applies to that class of fatty acid analogs with high K_m values because the high K_m could reflect either poor binding to NMT or inefficient conversion to CoA esters. To test this, we compared the efficiency of acyl CoA ester generation from 11-oxymyristic acid (a heteroatom containing fatty acid which appears to be poorly bound by NMT) to that of myristic and palmitic acids. The conditions used to generate acyl CoA esters were the same as those used for all of the compounds listed in this report. We found that 43–48% of myristic acid, 39–42% of palmitic acid, and 51–68% of the 11-oxymyristic acid were converted to their respective CoA esters. These differences in the efficiency of acyl CoA formation should not significantly affect the calculated peptide K_m or V_{max} data and will alter the relative fatty acyl CoA K_m values by <2-fold. The results suggest that the high K_m for 11-oxymyristic acid reflects low-affinity binding to NMT rather than inefficient conversion to its CoA thioester.

DISCUSSION

Studies of *N*-myristoyl proteins by GC/MS or by isotopic labeling and fatty acid analysis show these proteins to be blocked at their N terminus almost exclusively by myristic acid (reviewed in ref. 1). Previous analysis of NMT *in vitro* also showed a strong preference for myristoyl CoA over other saturated long chain acyl CoA thioesters (9, 14). To investigate the interactions of NMT with fatty acyl CoA substrates in greater detail, we have synthesized a number of heteroatom-substituted fatty acid analogs. Our studies show that the acyl CoA bound to NMT profoundly influences both the affinity of NMT for peptides and the rate of acylpeptide formation. Kinetic characterization of the heteroatom-substituted fatty acid analogs suggests that fatty acid chain length is much more important than hydrophobicity in fatty acyl CoA substrate selection. Comparison of cis and trans unsaturated myristoyl CoA thioesters gives insight about the conformation of the fatty acyl moiety bound to NMT.

Much of yeast NMT's apparent fatty acid specificity is produced by changes in peptide K_m as a function of the bound fatty acyl CoA (Table 1). This suggests that NMT binds its fatty acyl CoA substrate before interacting with the nascent protein. The fatty acyl CoA bound to NMT then influences the ability of NMT to recognize particular peptide substrates. The lack of an effect of peptide substrate choice on the apparent K_m for myristoyl CoA (see Table 2) supports this ordered binding mechanism. Note in particular that palmitoyl CoA, one of the more abundant fatty acyl CoA esters in cells, is the worst substrate for NMT; its peptide catalytic effi-

ciency is only 4% of that of myristoyl CoA. This difference presumably accounts to a large extent for the failure of palmitoyl CoA to serve as an NMT substrate *in vivo*.

A double bond in the backbone of the fatty acid restricts the stereochemistry at the position of unsaturation. The increased catalytic efficiency for peptide observed with the trans unsaturated fatty acyl CoA (compared to myristoyl) suggests that when the acyl CoA is bound to NMT, rotation is restricted around the 9,10 single bond and that a trans conformation is preferred. The entropic barrier for acylpeptide formation is somewhat reduced because the trans acyl group already has restricted rotation at the 9,10 position. The cis unsaturated fatty acyl group is both bound less tightly and transferred less efficiently than the trans unsaturated fatty acyl group. These data suggest that NMT binds myristoyl CoA in an "extended" rather than a "bent" conformation at the 9,10 position of the fatty acid chain. Examination of unsaturated analogs which differ in double-bond position and conformation should yield additional information about NMT–fatty acyl CoA interactions.

The substitution of sulfur or oxygen for a methylene group in the backbone of the fatty acid has dramatic effects on hydrophobicity. Both the octanol/water partition coefficients and C_{18} reverse-phase HPLC elution behavior of the acylpeptide derivatives of these heteroatom-substituted analogs indicate that they are significantly less hydrophobic than naturally occurring saturated fatty acids of similar chain length. Oxygen substitution causes a larger change in hydrophobicity than sulfur. This agrees with previously observed effects of oxygen- and sulfur-containing substituents on octanol/water partitioning ratios (22).

In contrast to their effects on hydrophobicity, sulfur and oxygen substitutions for $-CH_2-$ have only minor effects on bond lengths and bond angles (23). An oxygen-substituted myristic acid analog is expected to be about 1% shorter than myristic acid, while a sulfur-substituted analog is about 3% longer than myristic acid. For comparison, palmitic acid is about 15% longer than myristate. For straight chain hydrocarbons, the C—C—C angle is about 112.5° (24), whereas the C—O—C and C—S—C bond angles are 110.0 ± 3° and 105 ± 3°, respectively (23).

Fatty acids whose acylpeptides differ dramatically from *N*-myristoyl peptide in hydrophobicity (defined by HPLC elution time and 2-octanol/water partition coefficients) form significantly better substrates (as acyl CoA thioesters) than do comparably migrating naturally occurring saturated fatty acids. In fact, 6-oxymyristic acid, whose acylpeptide migrates like that of a 10:0 or 11:0 fatty acid, forms a CoA thioester giving a 2-fold higher catalytic efficiency than myristoyl CoA. Palmitoyl CoA and 12-oxypalmitoyl CoA both have similar effects on substrate kinetic parameters. However, analogs with heteroatom substitutions at different positions along their hydrocarbon backbone interact differently with NMT, even though their corresponding acylpeptides have comparable HPLC elution profiles. 11-Oxymyristoyl CoA, for example, is a significantly worse NMT substrate than is 13-oxymyristoyl CoA. These data suggest that particular residues of NMT react with specific parts of the fatty acid backbone and that the fatty acyl CoA may be rigidly bound by NMT. The introduction of sulfur or oxygen atoms into the fatty acid backbone may allow for hydrogen bonding between the amino acid side chains or amino groups of NMT and the heteroatom in the fatty acid.

The analogs synthesized and tested suggest novel approaches to study the role of fatty acylation in protein function. Several oxygen-substituted fatty acyl analogs are efficiently transferred to peptide substrates by NMT *in vitro*, yet their acylpeptides migrate on reverse-phase HPLC like those of 9:0 to 11:0 fatty acids. Studies by Franks and Lieb (25) on the partitioning of long-chain fatty alcohols into membrane bilayers demonstrated a 20-fold decrease in membrane association for every two-methylene-group decrease in chain length. Our acylpeptide 2-octanol/water partition coefficients changed up to 60-fold upon substitution of oxygen for a methylene group. Incorporation of these analogs into proteins *in vivo* may be expected to radically affect acylprotein subcellular localization, protein–protein interaction, or acylprotein function. Incorporation should be fairly specific since metabolism to either increase or decrease analog chain length should reduce utilization of the altered analog by NMT. The synthesis of analogs with multiple heteroatom substitutions should generate NMT substrates with even more dramatically reduced hydrophobicity. The utility of these compounds as antiviral and chemotherapeutic agents remains to be tested.

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