Titration Calorimetric Analysis of AcylCoA Recognition by MyristoylCoA:Protein *N*-Myristoyltransferase†

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ABSTRACT: *Saccharomyces cerevisiae* myristoylCoA:protein *N*-myristoyltransferase (Nmt1p) is an essential enzyme that catalyzes the transfer of myristic acid (C14:0) from myristoylCoA to the N-terminus of cellular proteins with a variety of functions. Nmts from an assortment of species display remarkable *in vivo* specificity for this rare acyl chain. To better understand the mechanisms underlying this specificity, we have used isothermal titration calorimetry as well as kinetic measurements to study the interactions of Nmt1p with acylCoA analogs having variations in chain length and/or conformation, analogs with alterations in the thioester bond, and analogs with or without a 3′-phosphate in their CoA moiety. MyristoylCoA binds to Nmt1p with a K_d of 15 nM and a large exothermic ΔH (−25 kcal/mol). CoA derivatives of C12:0−C16:0 fatty acids bind to Nmt1p with similar affinity, but with much smaller ΔH and a correspondingly less negative $T\Delta S$ than myristoylCoA. Replacing the thioester carbonyl group with a methylene or removing the 3′-phosphate of CoA is each sufficient to prevent the low enthalpy binding observed with myristoylCoA. The carbonyl and the 3′-phosphate have distinct and important roles in chain length recognition over the range C12−C16. Acyltransferase activity parallels binding enthalpy. The naturally occurring *cis*-5-tetradecenoylCoA and *cis*-5,8-tetradecadienoylCoA are used as alternative Nmt substrates in retinal photoreceptor cells, even though they do not exhibit *in vitro* kinetic or thermodynamic properties that are superior to those of myristoylCoA. The binding of an acylCoA is the first step in the enzyme's ordered reaction mechanism. Our findings suggest that within cells, limitation of Nmt substrate usage occurs through control of acylCoA availability. This indicates that full understanding of how protein acylation is controlled not only requires consideration of the acyltransferase and its peptide substrates but also consideration of the synthesis and/or presentation of its lipid substrates.

MyristoylCoA:protein *N*-myristoyltransferase (Nmt,[1] EC 2.3.1.97) catalyzes the transfer of myristate (C14:0) from CoA to the N-terminal glycine of a variety of eukaryotic and viral proteins. This cotranslational modification occurs via formation of an amide bond and appears to be irreversible. Known Nmt substrates include proteins involved in signal transduction pathways (serine/threonine and tyrosine kinases, kinase substrates, phosphatases, the α subunits of heterotrimeric G proteins), proteins involved in vesicular trafficking (Arfs), and viral proteins with structural and nonstructural functions (*1*, *2*).

The role of the covalently attached myristoyl group is different for different N-myristoylproteins. Several proteins require the acyl chain for membrane association (*3*). Other proteins use C14:0 to promote protein−protein interactions. For example, N-myristoylation of G_α subunits is required for their association with $\beta\gamma$ subunits (*4*). Poliovirus VP4 forms a homopentameric capsid component, with the acyl chains stabilizing interactions among VP4 monomers and with other viral surface proteins (*5*).

The affinity of an acylpeptide for model phospholipid bilayers is highly dependent upon acyl chain length (*6*, *7*), as would be expected from the hydrophobic effect. Recent studies have indicated that myristate provides a means for generating a *weak* affinity for cellular membranes (*3*, *6*). This weak attraction may be modified by other features in a protein, such as a reversible post-translational modification (protein S-palmitoylation or phosphorylation; ref *8*), by the physical properties of other domains within the protein (polybasic regions, hydrophobic patches; ref *3*), or by alterations induced through noncovalent binding of ligands (calcium in the case of recoverin; ref *9*).

Myristate and myristoylCoA are rare cellular species compared to the corresponding abundant C16:0 compounds (*10*, *11*). Myristate is the only acyl chain utilized by Nmt in most cell lineages. Retinal photoreceptor cells represent the only known exception. The catalytic subunit of protein kinase A is acylated with C14:0 in cardiac myocytes and neurons but with laurate (C12:0), myristate, 5*Z*-tetradecenoate (C14:1$^{\Delta 5}$), and 5*Z*,8*Z*-tetradecadienoate (C14:2$^{\Delta 5,8}$) in rod outer segments (*12*). Heterogeneous acylation has

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[1] Abbreviations: Nmt1p, *Saccharomyces cerevisiae* myristoylCoA:protein *N*-myristoyltransferase; CoA, coenzyme A; Z, *cis*-double bond; MCAD, medium chain acylCoA dehydrogenase

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also been observed with other proteins in retinal cells from several species (e.g., *12*, *13*). The phenomenon of heterogeneous acylation of N-myristoylproteins raises a question about whether Nmt's selectivity for C14:0 is ascribable entirely to the molecular recognition properties of the enzyme itself or whether the specificity of acylation is due in large measure to the cellular acylCoAs available to the acyltransferase.

The 455 amino acid, monomeric *Saccharomyces cerevisiae* enzyme is the best characterized of the six orthologous Nmts whose primary structures have been defined. Nmt1p has no known cofactor requirement (*14*). Its reaction mechanism is ordered Bi−Bi: myristoylCoA binds before the acyltransferase is able to bind a peptide substrate; after transfer of the acyl chain, CoA is released prior to myristoylpeptide (*15*). The requirement for acylCoA binding before a high-affinity peptide recognition site is generated links variation in the structure of a bound acylCoA to the enzyme's peptide specificity (*16*). Nmt1p's acylCoA specificity has been analyzed *in vitro* with a panel of more than 300 acylCoA analogs. These kinetic studies have indicated that (i) Nmt1p is sensitive to changes in acyl chain length of ±2 methylenes; (ii) the acyl chain is bound in a bent conformation, with a principal bend occurring in the vicinity of C5−C6; (iii) the enzyme's acyl chain binding site is able to accommodate analogs with increased polarity; and (iv) the ω-terminus of the acyl chain must fit into a sterically restricted site (*16*−*18*).

To better understand the mechanisms that determine the specificity of protein N-myristoylation *in vivo*, we initiated a study of the thermodynamics of Nmt1p's interaction with acylCoA and peptide substrates (*19*). Isothermal titration calorimetry was used to determine standard free energies and enthalpies of Nmt1p binding to the CoA esters of myristate and palmitate. The enzyme has nearly identical affinity for the two acylCoAs. However, the binding of myristoylCoA is associated with a greater release of enthalpy ($\Delta\Delta H \approx 9$ kcal/mol). In addition, a high-affinity peptide binding site can be generated when nonhydrolyzable analogs of either myristoylCoA or palmitoylCoA are bound to Nmt1p (*19*).

These thermodynamic studies have now been extended. The effects of differing chain length, of introducing double and triple bonds in the polymethylene chain, of altering the acyl−CoA thioester bond, and of deleting the 3′-phosphate group from CoA have been explored. A recently described peptidomimetic inhibitor (*20*) has been used to probe the effects of variations in acylCoA structure on the enzyme's peptide binding site. The results not only provide insights about the structural features of acylCoAs that are important determinants of molecular recognition, but also emphasize how vulnerable the enzyme may be to variations in acylCoA availability.

MATERIALS AND METHODS

Synthesis of Acyl- and AlkylCoAs. Lauric, myristic, and palmitic acids were purchased from NuChek Prep (Elysian, MN). C14:1 and C16:1 fatty acids were synthesized using previously reported procedures (*17*). 5*Z*,8*Z*-tetradecadienoic acid was kindly supplied by Lubert Stryer and Tom Neubert (Stanford University). The CoA esters of C12:0−C17:0 were purchased from Sigma. AlkylCoAs were prepared from commercially available CoA (Sigma) and the alkyl tosylate. AcylCoAs were prepared from CoA or 3′-dephosphoCoA (Sigma) and the oxysuccinimide derivatives of fatty acids (*21*). All new compounds were characterized by IR, NMR, fast atom bombardment high resolution mass spectrometry (HRMS), and combustion analysis, as appropriate.

S-n-TetradecylCoA was prepared as a white powder in 82% yield. *Anal. calcd* for $C_{35}H_{64}N_7O_{16}P_3S$: C, 43.61; H, 6.69; N, 10.17%. *Found*: C, 43.29; H, 6.71; N, 9.88%. *S-n*-HexadecylCoA was prepared as a white powder in 60% yield. HRMS ($[M + H]^+$) calculated for $C_{37}H_{69}N_7O_{16}P_3S$: 992.3734. Found: 992.3710. *S*-[2-(±)-Hydroxytetradecanoyl]CoA was prepared in 63% yield [white powder, mp 146 °C (soften and decompose (dec)]. HRMS ($[M + H]^+$) calculated for $C_{35}H_{63}N_7O_{18}P_3S$: 994.3163. Found: 994.3157. *S-Z*5-TetradecenoylCoA was prepared in 91% yield [white powder, mp 143−145 °C (soften and dec)]. HRMS ($[M + H]^+$) calculated for $C_{35}H_{61}N_7O_{17}P_3S$: 976.3057. Found: 976.3044. *S-Z*5,*Z*8-TetradecadienoylCoA was prepared in 81% yield [white powder mp 105 °C (soften) ∼145 °C (dec)]. HRMS ($[M + H]^+$) calculated for $C_{35}H_{59}N_7O_{17}P_3S$: 974.2901. Found: 974.2906. *S*-6-TetradecynoylCoA was prepared as a white powder in 60% yield [mp 130−132 °C (dec)]. HRMS ($[M + H]^+$) calculated for $C_{35}H_{59}N_7O_{17}P_3S$: 974.2901. Found: 974.2877. *S*-Dodecanoyl 3′-dephosphoCoA was prepared as a white powder in 62% yield [mp 105 °C (soften), 165 °C (dec)]. HRMS ($[M + H]^+$) calculated for $C_{33}H_{58}N_7O_{14}P_2S$: 870.3237. Found: 870.3234. *S*-Tetradecanoyl 3′-dephosphoCoA was prepared in 87% yield [white powder, mp 110 °C (soften) 160 °C (dec)]. HRMS ($[M + H]^+$) calculated for $C_{35}H_{62}N_7O_{14}P_2S$: 898.3550. Found: 898.3554. *S*-Hexadecanoyl 3′-dephosphoCoA was prepared in 92% yield [white powder, mp 108 °C (soften) ∼158 °C (dec)]. HRMS ($[M + H]^+$) calculated for $C_{37}H_{66}N_7O_{14}P_2S$: 926.3863. Found: 926.3856. *S-Z*5-Tetradecenoyl 3′-dephosphoCoA was prepared in 68% yield [white powder, mp 149−152 °C (dec)]. HRMS ($[M + H]^+$) calculated for $C_{35}H_{60}N_7O_{14}P_2S$: 896.3394. Found: 896.3376. *S*-6-Tetradecynoyl 3′-dephosphoCoA was prepared as a white powder [mp ∼134 °C (soften) ∼150 °C (dec)]. HRMS ($[M + H]^+$) calculated for $C_{35}H_{58}N_7O_{14}P_2S$: 894.3237. Found: 894.3215.

Purification of Recombinant S. cerevisiae Nmt1p from Escherichia coli. Nmt1p was expressed in *E. coli* strain JM101 transformed with pBB131 (*22*). The enzyme was purified to apparent homogeneity, free of any associated thioesterase activity, as described (*19*).

Kinetic assays of Nmt1p activity with acylCoAs - An *in vitro* Nmt assay (*17*) was modified for the present study by using purified acylCoAs or acyl-3′-dephosphoCoAs, rather than the products of an initial *Pseudomonas* acylCoA synthetase reaction (*19*). Briefly, an unlabeled purified acylCoA (isolated according to ref *23* and used at a final concentration of 18 μM) was incubated with Nmt1p and a radiolabeled octapeptide substrate derived from the N-terminus of the human immunodeficiency virus $Pr55^{gag}$ protein [GAR[^{3}H]ASVLS-NH_2; specific activity = 1.2 Ci/mmol; final concentration = 25 μM; ref *17*)]. The tritiated acylpeptide product was then purified from the reaction mixture by C18 reverse phase HPLC (*17*) and quantitated with an inline scintillation counter. All enzyme assays were performed in triplicate on at least two occasions.

An additional series of experiments were performed in which Nmt1p and the tritiated octapeptide were incubated

with purified myristoylCoA alone, in combination with C12:0−CoA or C16:0−CoA or with both C12:0−CoA and C16:0−CoA (each at a final concentration of 1 μM). Acylpeptide products were resolved by C18 reverse phase HPLC (*17*) and quantitated as above.

Isothermal Titration Calorimetry. Purified Nmt1p was dialyzed against a buffer containing 100 mM HEPES, pH 7.4, 3 mM 2-mercaptoethanol, and 0.1 mM EDTA. Ligand solutions were prepared in the same buffer. The concentrations of Nmt1p and CoA-containing ligands were estimated spectrophotometrically, using extinction coefficients of 7.44 $\times$ 10^4 M^{-1} cm^{-1} at 280 nm and 1.54 $\times$ 10^4 M^{-1} cm^{-1} at 260 nm, respectively (*19*). In a few titrations, Nmt1p concentrations were defined by assuming a 1:1 binding stoichiometry and fitting to enzyme concentration instead of stoichiometry as a variable parameter. The concentration of the peptidomimetic inhibitor SC-58272 was determined by dry mass and purity (defined by HPLC and mass spectrometry, ref *20*).

Calorimetric titrations were performed using an Omega calorimeter (MicroCal, Northampton, MA) (*19*, *24*). Data from each titration were collected in MicroCal Origin software (*24*). Baseline thermal current subtraction and peak integration limits were adjusted within Origin. Titrations were carried out at 300.1 K. The concentration of Nmt1p in the reaction cell was typically 2−5 μM. The concentration of ligand in the syringe was typically 50−250 μM. Higher concentrations (Nmt1p up to 15 μM; ligand up to 2000 μM) were used for titrations involving the 3′-dephosphoCoA esters of dodecanoate, tetradecenoate, and tetradecynoate. The 2−3 order of magnitude dilution of ligand into the reaction cell was occasionally accompanied by a detectable heat. In many instances, this effect could be eliminated by reducing the concentration of the ligand within the syringe. In instances where this heat (typically <2 kcal/mol) could not be eliminated, data were corrected by subtracting a constant heat from each injection. The value of this correction was chosen so as to fit the upper plateau of the binding curve to 0 kcal/mol and was typically smaller than the heat measured in control titrations of the same solution of ligand into buffer alone.

The heat of each injection, normalized to the amount of ligand injected, was fit to a differential form single set of binding sites model for stoichiometry, binding constant (K_b), and enthalpy (ΔH) with Scientist (MicroMath, Salt Lake City, UT). Data are presented in Table 1 in the form of a best fit value and 95% support plane confidence intervals for each parameter, derived from a simultaneous fit to multiple titrations of each ligand (e.g., Figure 1). With the exception of *Z*5-myristoyl-3′-dephosphoCoA (n = 1.3; 95% interval 1.0−1.5), fit stoichiometry did not vary from 1:1 by more than 5%. For lauroyl-3′-dephosphoCoA, the affinity was too low to collect data over the complete binding isotherm, making it difficult to fit to all 3 parameters (Figure 1; ref *24*), so the data were fit to K_b and ΔH with the stoichiometry fixed at 1:1.

RESULTS

Acyl Chain Length Discrimination. Table 1 lists thermodynamic parameters for the ligation of apo-Nmt1p with fully saturated acylCoAs having chain lengths between 12 and 17 carbons. Although enzymatic activity falls off as chain length is varied in either direction from C14:0, binding affinity does not change significantly until C12:0 and C17:0 are reached. Variations in chain length are accompanied by large changes in the enthalpic and entropic components of $\Delta G°$. As chain length is either increased or decreased from C14, the contribution of ΔH to affinity decreases, with compensating decreases in $T\Delta S$ ($\Delta\Delta H \approx T\Delta S \approx 6$ kcal/mol for a change of one methylene).

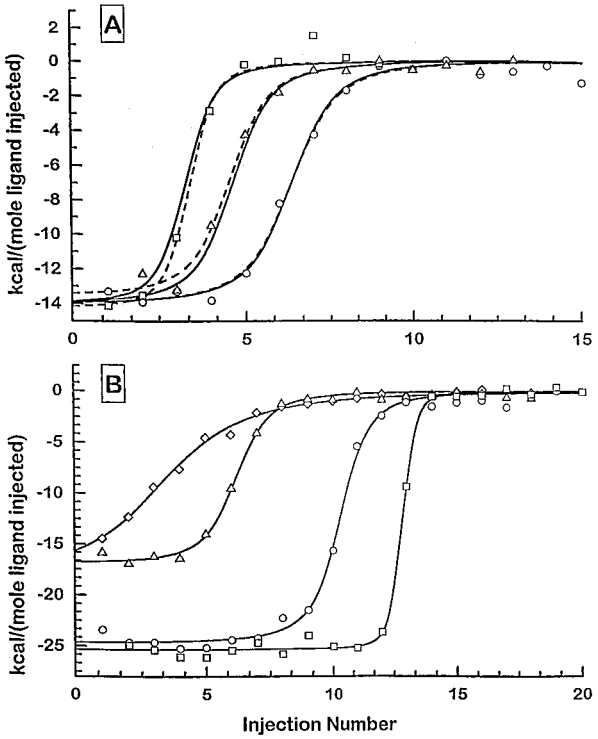


FIGURE 1: Calorimetric titrations. Panel A, titrations of SC-58272 to Nmt1p:myristoylCoA binary complexes, illustrating the fitting procedure. Three individual titrations are shown, with the best fit parameters for each individual titration plotted as a dashed line and the best single set of parameters describing the three titrations simultaneously plotted as solid lines. The derived parameters are squares, K_d = 19.6 nM, ΔH = −14.25 kcal/mol; triangles, K_d = 31.0 nM, ΔH = −13.58 kcal/mol; circles, K_d = 32.5 nM, ΔH = −14.07 kcal/mol; solid lines (global fit), K_d = 29.1 nM, ΔH = −14.02 kcal/mol. Panel B, data and best fit curves for Nmt1p binding to *S*-(2-oxo)pentadecylCoA (squares), myristoylCoA (circles), myristoyl-3′-dephosphoCoA (triangles), and 6-tetradecynoyl-3′-dephosphoCoA (diamonds). The shape of the curve is determined by the product of the binding constant and the concentration of enzyme. At high values of this product, the curve approaches a step function and becomes increasingly insensitive to changes in the binding constant. At low values, the curve becomes flat and it becomes difficult to titrate over the full width of the curve. The stoichiometry and enthalpy are less well determined. The parameters describing these titrations are *S*-(2-oxo)pentadecylCoA, K_b = 3.1 $\times$ 10^8 M^{-1}, ΔH = −25.3 kcal/mol; myristoylCoA, K_b = 7.1 $\times$ 10^7 M^{-1}, ΔH = −24.7 kcal/mol; myristoyl-3′-dephosphoCoA, K_b = 1.2 $\times$ 10^7 M^{-1}, ΔH = −16.9 kcal/mol; 6-tetradecynoyl-3′-dephosphoCoA, K_b = 1.3 $\times$ 10^6 M^{-1}, ΔH = −18.7 kcal/mol.

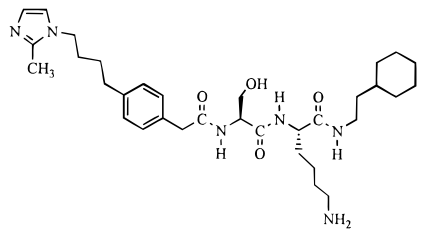
Acyltransferase activity parallels binding enthalpy for these ligands: both decline in magnitude as acyl chain length diverges from C14 (Figure 2). Thus, Nmt1p is able to sense changes as small as one carbon in chain length, as reflected in the enthalpy and entropy of binding, but is unable to discriminate among acyl chain lengths at the level of binding affinity.

An alternative view of this acyl chain length discrimination was obtained by presenting the enzyme with 1 μM myristoylCoA in the presence of equimolar lauroylCoA, or

Table 1: Thermodynamic Parameters of the Binding of Ligands to Nmt1p

	number of experiments	K_d (nM)	ΔG° (kcal/mol)	ΔH (kcal/mol)	$T\Delta S$ (kcal/mol)	acyltransferase activity[a]
			acylCoA → apo-Nmt1p[b]			
varying acyl chain length						
lauroylCoA	4	62 (41 to 91)[c]	−9.9 (−9.7 to −10.1)	−15.5 (−15.0 to −16.0)	−5.6 (−4.9 to −6.3)	44 ± 0.4
tridecanoylCoA	3	14 (5 to 31)	−10.8 (−10.3 to −11.4)	−18.3 (−17.4 to −19.2)	−7.5 (−6.0 to −8.9)	84 ± 7
myristoylCoA	9	15 (8 to 24)	−10.8 (−10.5 to −11.1)	−24.8 (−24.1 to −25.5)	−14.1 (−13.1 to −15.1)	100[c]
pentadecanoylCoA	4	13 (7 to 22)	−10.8 (−10.5 to −11.2)	−18.8 (−18.2 to −19.4)	−8.0 (−7.0 to −8.9)	12 ± 2
palmitoylCoA	5	26 (13 to 49)	−10.4 (−10.0 to −10.8)	−15.1 (−14.4 to −15.8)	−4.6 (−3.5 to −5.7)	1.6 ± 0.3
heptadecanoylCoA	2	79 (37 to 147)	−9.7 (9.4 to −10.2)	−10.3 (−9.6 to −11.2)	−0.6 (0.6 to −1.8)	0.6 ± 0.1
role of thioester carbonyl						
S-(2-oxo)pentadecylCoA	3	3 (1 to 6)	−11.7 (−11.3 to −12.3)	−25.8 (−25.3 to −26.3)	−14.1 (−13.0 to −15.0)	
S-(2-oxo)heptadecylCoA	3	33 (14 to 64)	−10.3 (−9.9 to −10.8)	−13.7 (−13.2 to −14.3)	−3.5 (−2.5 to −4.4)	
tetradecylCoA	3	11 (5 to 20)	−10.9 (−10.6 to −11.4)	−17.5 (−16.9 to −18.2)	−6.6 (−5.5 to −7.6)	
hexadecylCoA	4	40 (24 to 63)	−10.2 (−9.9 to −10.5)	−18.7 (−17.9 to −19.6)	−8.5 (−7.4 to −9.7)	
2-hydroxymyristoylCoA	3	33 (19 to 54)	−10.3 (−10.0 to −10.6)	−23.6 (−22.6 to −24.7)	−13.3 (−12.0 to −14.7)	
role of 3′-PO_4 of CoA						
lauroyl-3′-dephosphoCoA[d]	3	8380 (6870 to 10200)	−7.0 (−6.9 to −7.1)	−12.7 (−11.9 to −13.6)	−5.8 (−4.8 to −6.8)	21 ± 2
myristoyl-3′-dephosphoCoA	4	124 (81 to 182)	−9.5 (−9.2 to −9.7)	−17.5 (−16.8 to −18.1)	−8.0 (−7.1 to −8.9)	73 ± 5
palmitoyl-3′-dephosphoCoA	3	232 (101 to 504)	−9.1 (−8.6 to −9.6)	−11.6 (−10.2 to −13.8)	−2.5 (−0.6 to −5.2)	4 ± 1
conformational restrictions						
5*Z*-tetradecenoylCoA	2	19 (12 to 28)	−10.6 (−10.4 to −10.9)	−16.4 (−15.9 to −16.8)	−5.7 (−5.0 to −6.4)	106 ± 29
6-tetradecynoylCoA	2	17 (0.6 to 37)	−10.7 (−10.2 to −12.7)	−20.7 (−19.5 to −22.0)	−10.0 (−6.8 to −11.8)	155 ± 23
5*Z*,8*Z*-tetradecadienoylCoA	3	75 (38 to 137)	−9.8 (−9.4 to −10.2)	−15.0 (−14.0 to −16.1)	−5.2 (−3.8 to −6.7)	5.3 ± 3.7
5*Z*-tetradecenoyl-3′-dephosphoCoA	2	1100 (500 to 2100)	−8.2 (−7.8 to −8.6)	−12.3 (−9.9 to −16.8)	−4.1 (−1.2 to −9.0)	219 ± 14
6-tetradecynoyl-3′-dephosphoCoA	3	969 (713 to 1310)	−8.3 (−8.1 to −8.4)	−20.8 (−18.4 to −24.2)	−12.5 (−10.0 to −16.1)	155 ± 21
SC-58272 → Nmt1p:AcylCoA[e]						
lauroylCoA	2	37 (14 to 80)	−10.2 (−9.7 to −10.8)	−14.4 (−13.2 to −15.6)	−4.2 (−2.5 to −5.9)	
myristoylCoA	3	29 (15 to 51)	−10.3 (−10.0 to −10.7)	−14.0 (−13.2 to −14.9)	−3.7 (−2.5 to −4.8)	
palmitoylCoA	3	23 (13 to 37)	−10.5 (−10.2 to −10.8)	−16.3 (−15.6 to −17.1)	−5.8 (−4.7 to −6.9)	

[a] Mean values ± 1 SD. Data are expressed as a percentage of the activity observed with myristoylCoA and GARASVLS-NH_2; 100% = 4.51 ± 0.52 × 10^5 pmol myristoylpeptide/min/mg Nmt1p. [b] Thermodynamic parameters for binding of the indicated acylCoA to apo-Nmt1p. Note that less rigorously defined parameters for binding of C12:0−CoA, C14:0−CoA, C16:0−CoA, myristoyl-3′-dephosphoCoA, and the oxo-alkylCoAs were reported earlier (*19*). [c] Numbers in parentheses represent 95% confidence intervals, determined as described in Materials and Methods. [d] Thermodynamic parameters and confidence intervals for lauroyl-3′-dephosphoCoA are calculated with the binding soitchiometry fixed at 1:1, as described in Materials and Methods. [e] Thermodynamics of binding of the peptidomimetic inhibitor SC-58272 to binary complexes of Nmt1p and the indicated acylCoA. The peptidomimetic does not bind to apo-Nmt1p in the concentration range examined (up to 30 μM enzyme), consistent with the yeast enzyme's ordered Bi Bi reaction mechanism. SC-58272 =



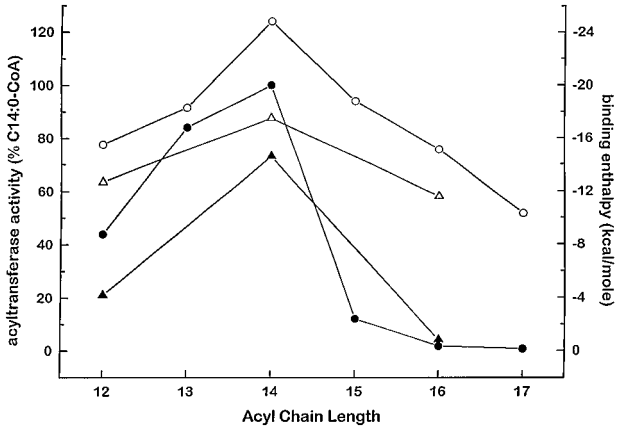


FIGURE 2: Binding enthalpy and acyltransferase activity vary as a function of chain length. Enthalpy of binding to Nmt1p (open symbols) and acyltransferase activity (filled symbols) are shown for CoA (circles) and 3′-dephospho-CoA (triangles) esters of fatty acids of varying chain length. For both series of compounds, activity and binding enthalpy are maximal for the 14-carbon chain length. Note that activity falls off more steeply when chain length is increased from C14 than when chain length is decreased.

palmitoylCoA, or both lauroylCoA and palmitoylCoA. Acyl-GAR[^{3}H]ASVLS-NH_2 products were resolved by C18 reverse phase HPLC. When Nmt1p was simultaneously presented with palmitoylCoA and myristoylCoA, myristoylpeptide production was reduced by 39 ±7.5% (reference control = myristoylCoA alone). The palmitoylated peptide represented 0.6% of total acylpeptide. When the enzyme was exposed to equimolar lauroylCoA and myristoylCoA, myristoylpeptide production decreased by 19 ± 9% and the lauroylated peptide comprised 1.3% of the acylpeptide species. In the presence of all three acylCoAs, myristoylpeptide production was reduced by 43 ± 16% with lauroyl- and palmitoylpeptide representing 1.3 and 0.7%, respectively, of the total products.

Studies using synthetic peptides and peptidomimetics indicate that Nmts from a variety of species require three critical elements for recognition of nascent protein substrates: an N-terminal amine, a Ser^5 hydroxyl and a Lys^6 ϵ-amino group (25). SC-58272 is a peptidomimetic inhibitor of Nmt1p (20). It was derived from the N-terminal sequence of a known *S. cerevisiae* N-myristoylprotein, Arf2p (GLYASKLS-NH_2). Gly^1 was replaced with a 2-methylimidazole. Leu^2-Tyr^3-Ala^4 were replaced with a (4-butyl)-phenacetyl group that was N-linked to the imidazole. Ser^5 and Lys^6 were retained while the C-terminal Leu^7-Ser^8 dipeptide was replaced with a N-cyclohexylethyl carboxamide. The structure of SC-58272 is shown in Table 1. Kinetic studies have shown that the inhibitor is competitive for peptide and noncompetitive for myristoylCoA (*20*).

SC-58272 allowed us to survey the peptide binding site created when Nmt1p binds hydrolyzable acylCoA ligands. No significant difference was noted in the affinity ($\Delta G°$), ΔH, or $T\Delta S$ of binding of SC-58272 to Nmt1p complexed with lauroylCoA, myristoylCoA, or palmitoylCoA (Table 1). Thus, acquisition of acylCoAs of differing chain length does not result in apparent changes in recognition of this substrate analog.

Role of the Thioester Carbonyl in Acyl Chain Length Recognition. Additional compounds were synthesized to identify functional groups in acylCoA ligands that serve as important landmarks for the enzyme's recognition of acyl chain length. The nonhydrolyzable myristoylCoA and palmitoylCoA analogs *S*-(2-oxo)pentadecylCoA and S-(2-oxo)heptadecylCoA are Nmt inhibitors (*15*, *21*). These compounds represent the insertion of a single methylene group between the CoA sulfur and the fatty acid carbonyl carbon, resulting in a thioether bond that is more stable than the natural thioester. The similarities in ΔH and $T\Delta S$ observed with the oxoalkylCoAs and the corresponding acylCoAs (compared in Table 2) suggest that apo-Nmt1p perceives *S*-(2-oxo)pentadecylCoA and *S*-(2-oxo)heptadecylCoA as close approximations of myristoylCoA and palmitoylCoA, respectively. However, the length of the analogs' acyl chains is 1 methylene greater than the acylCoAs they model. The enthalpy of binding of S-(2-oxo)pentadecylCoA (−25.8 kcal/mol) is much closer to that of myristoylCoA (−24.8 kcal/mol) than to pentadecanoylCoA (−18.8 kcal/mol). A similar conclusion can be reached by comparing ΔH among S-(2-oxo)heptadecylCoA (−13.7 kcal/mol), palmitoylCoA (−15.1 kcal/mol), and heptadecanoylCoA (−10.3 kcal/mol). Thus, whatever mechanism the enzyme uses to assess acyl chain length, it is sensitive to the distance from carbonyl to ω-terminus and relatively insensitive to the distance from the sulfur (or other portions of CoA) to the fatty acid's terminal methyl.

TetradecylCoA and hexadecylCoA are equivalent to myristoylCoA and palmitoylCoA with the carbonyl group replaced by a methylene. The K_d of tetradecylCoA is not significantly different from that of myristoylCoA. Likewise, removal of the carbonyl oxygen from palmitoylCoA does not affect binding affinity (Table 1). The enthalpy of binding of the two alkylCoAs is quite similar (−17.5 versus −18.7 kcal/mol) in contrast to the differences observed between myristoyl- and palmitoylCoA (−24.8 versus −15.1 kcal/mol). Nmt1p's diminished enthalpic discrimination between the alkylCoAs emphasizes the importance of the carbonyl moiety in acyl chain length recognition.

2-HydroxymyristoylCoA is an inhibitor of Nmts (e.g., ref *26*). The presence of the hydroxyl group vicinal to the carbonyl suggests that this compound may inhibit Nmt by interacting with electrophilic functional groups in the enzyme which normally associate with the carbonyl oxygen of myristoylCoA. Introduction of the hydroxyl has no significant effect on the thermodynamics of binary complex formation (Table 1). Moreover, there are no significant differences in the K_d of SC-58272 for myristoylCoA:Nmt1p, tetradecylCoA:Nmt1p, hexadecylCoA:Nmt1p, and 2-hydroxymyristoylCoA:Nmt1p binary complexes (data not shown). This latter finding indicates that neither the lack of the carbonyl, nor the presence of the neighboring hydroxyl group, prevents binding of an acylCoA analog and induction of a high-affinity recognition site for the peptide substrate analog.

Role of the 3′-Phosphate Group of CoA in Molecular Recognition. Analysis of the interactions between apo-Nmt1p and lauroyl-3′-dephosphoCoA, myristoyl-3′-dephosphoCoA, and palmitoyl-3′-dephosphoCoA disclosed that the thermodynamic role of this group is linked to the length of the acyl chain (Tables 1 and 2). Myristoyl-3′-dephosphoCoA binds to apo-Nmt1p with 9-fold lower affinity than myristoylCoA. The enthalpy is reduced by 7.3 kcal/mol. Comparing the thermodynamics of binding of palmitoyl-3′-dephosphoCoA with palmitoylCoA reveals a similar reduction in affinity, but a much smaller reduction in ΔH ($\Delta\Delta H$ =

Table 2: Summary of the Effects of Altering AcylCoA Structure on Recognition by Nmt1p[a]

alteration	reference compound	$\Delta\Delta G°$ (kcal/mol)	$\Delta\Delta H$ (kcal/mol)	$T\Delta\Delta S$ (kcal/mol)	acyltransferase activity
2-carbon chain length increase	myristoylCoA	0.4	9.7	9.3	98% loss
	myristoyl-3′-dephosphoCoA	0.4	5.9	5.5	94% loss
	tetradecylCoA	0.7	−1.2	−1.9	
	S-2(oxo)pentadecylCoA	1.4	12.1	10.7	
loss of 3′-phosphate	myristoylCoA	1.3	7.3	6.0	27% loss
	palmitoylCoA	1.3	3.5	2.2	162% increase
	lauroylCoA	2.9	2.8	−0.1	52% loss
	5*Z*-tetradecenoylCoA	2.4	4.1	1.7	107% increase
	6-tetradecynoylCoA	2.4	−0.1	−2.5	no change
replacement of C═O with CH_2	myristoylCoA	−0.1	7.3	7.4	
	palmitoylCoA	0.2	−3.6	−3.8	
insertion of methylene between carbonyl and sulfur	myristoylCoA	−0.9	−1.0	−0.1	
	palmitoylCoA	0.1	1.4	1.3	
cis-double bond between C5−C6	myristoylCoA	0.2	8.4	8.2	6% increase
	myristoyl-3′-dephosphoCoA	1.3	5.2	3.9	200% increase
Triple bond between C6−C7	myristoylCoA	0.1	4.1	4.0	55% increase
	myristoyl-3′-dephosphoCoA	1.2	−3.3	−4.5	112% increase

[a] Values presented represent the *change* in each parameter when a given structural alteration (first column) is made in the context of the indicated acylCoA or analog (second column). For example, the second row presents the differences in thermodynamic and kinetic parameters between palmitoyl-3′-dephosphoCoA and myristoyl-3′-dephosphoCoA. $\Delta\Delta G°$ and $\Delta\Delta H$ are taken from Table 1. $T\Delta\Delta S$ is calculated as $\Delta\Delta H - \Delta\Delta G°$ to minimize rounding errors.

3.5 kcal/mol). The role of the 3′-phosphate in lauroylCoA binding to apo-Nmt1p appears to be different from both the myristoylCoA and palmitoylCoA cases. Removal of the 3′-phosphate from lauroylCoA produces an unfavorable 2.8 kcal/mol change in binding enthalpy without a compensating reduction in entropy, resulting in a large reduction in affinity (K_d for lauroylCoA = 62 nM; for lauroyl-3′-dephosphoCoA = 8400 nM).

The effect of removing the 3′-phosphate of CoA on acyltransferase activity also varies with acyl chain length (Table 1). A modest (27%) reduction in activity is observed when the 3′-phosphate is removed from myristoylCoA. The effect on lauroylCoA is greater (50% loss of activity). However, loss of the 3′-phosphate from palmitoylCoA is associated with a 2.5-fold *increase* in activity. Loss of the 3′-phosphate from lauroylCoA, myristoylCoA, and palmitoylCoA does not produce significant changes in the enzyme's affinity for the peptidomimetic inhibitor (data not shown).

The acyltransferase activities observed with the 3′-dephosphoCoA esters parallel their binding enthalpies, in a fashion similar to that observed with the corresponding CoA esters (Figure 2). A linear correlation exists between binding enthalpy and acyltransferase activities for both the 3′-dephosphoCoA and CoA esters (Figure 3). The CoA esters of C15:0 and C16:0 represent the only exceptions: their activities fall below what would be predicted from their enthalpies. Together, these findings suggest that interactions between Nmt1p and the 3′-phosphate group may be part of the enzyme's mechanism of *in vitro* acyltransferase specificity (see *Discussion*).

Effects of Introducing Conformational Restrictions in the Acyl Chain. Restricting the conformational space available to a C14 acyl group by introducing double or triple bonds can have marked effects on Nmt1p's acyltransferase activity. Placement of a *cis* (*Z*) double bond at C5 or a triple (Y) bond at C4 or C6 increases activity, while a *trans* (*E*) double bond or a triple bond at C5 diminish activity (*17*). These findings suggest that myristoylCoA binds to Nmt1p with a bend in the vicinity of C5−C6. Introducing or preventing a bend in the vicinity of C8 has similar, but less dramatic effects (*27*). This conformation-dependent alteration has been noted in orthologous Nmts (*27*).

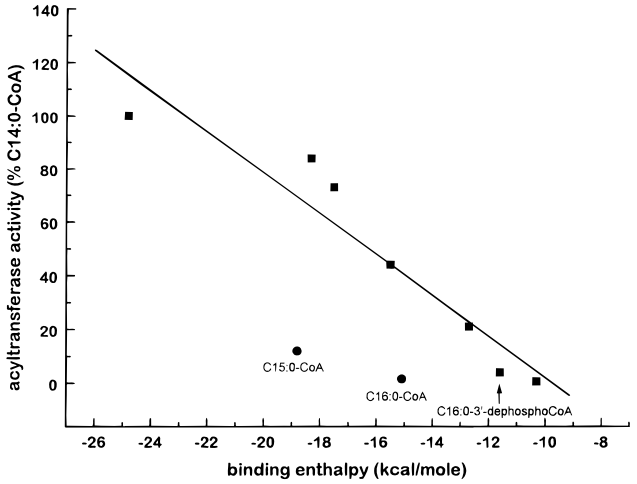


FIGURE 3: Correlation between acyltransferase activity and binding enthalpy for saturated acylCoAs and acyl-3′-dephosphoCoAs. Acyltransferase activity is plotted as a function of enthalpy of binding to Nmt1p for all saturated thioester compounds used in this study (see Table 1). The line indicates the linear correlation (r = 0.95) between activity and enthalpy for these compounds, excluding the CoA esters of C15:0 and C16:0. Note that palmitoyl-3′-dephosphoCoA has greater acyltransferase activity than palmitoylCoA and falls on this line.

The phenomenon of heterogeneous acylation of N-myristoylproteins led us to define the nature of the interactions between Nmt1p and 5*Z*-tetradecenoylCoA, 6-tetradecynoylCoA, and 5*Z*,8*Z*-tetradecadienoylCoA. The C14:1 derivatives do not have significantly different affinities for Nmt1p compared to myristoylCoA (Table 1). However, as with other changes in the structure of myristoylCoA, the entropic and enthalpic contributions are reduced. 5*Z*-tetradecenoylCoA and 6-tetradecynoylCoA bind with enthalpies that are 8.4 and 4.1 kcal/mol less exothermic than myristoylCoA binding, respectively. 5*Z*,8*Z*-tetradecadienoylCoA binds with slightly lower affinity than myristoylCoA and with an enthalpy that is 9.8 kcal/mol less exothermic (Table 1).

Table 3: Comparison of the Thermodynamic and Kinetic Properties of AcylCoA Substrates Known to be Used by Nmt *in Vivo* with the Properties of MyristoylCoA

	K_d (nM)	ΔH (kcal/mol)	$T\Delta S$ (kcal/mol)	acyltransferase activity
acylCoA → apo-Nmt				
lauroylCoA (C12:0−CoA)	62[a]	−15.5	−5.6	44[b]
myristoylCoA (C14:0−CoA)	15	−24.8	−14.1	100
5*Z*-tetradecenoylCoA (C14:1$^{\Delta 5}$−CoA)	19	−16.4	−5.7	106
5*Z*,8*Z*-tetradecadienoylCoA (C14:1$^{\Delta 5,8}$−CoA)	75	−15.0	−5.2	5
palmitoylCoA (C16:0−CoA)	26	−15.1	−4.6	2

[a] Best fit values are taken from Table 1. [b] Activity is expressed as a percentage of the activity observed with myristoylCoA and GARASVLS-NH_2 (see Table 1).

The *in vitro* acyltransferase activity of Nmt1p is identical whether the enzyme is presented with myristoylCoA or 5*Z*-tetradecenoylCoA. However, the other two acylCoAs used by Nmt in photoreceptor cells, 5*Z*,8*Z*-tetradecadienoylCoA and dodecanoylCoA, are inferior to myristoylCoA as substrates (5 and 44% of C14:0−CoA; Table 1). These kinetic and thermodynamic observations are summarized in Table 3 and support the notion that heterogeneous acylation occurs as a consequence of making the various acylCoAs available to Nmt rather than as a consequence of their being superior substrates to myristoylCoA.

DISCUSSION

Molecular Recognition. Table 2 summarizes the effects of structural changes in myristoylCoA on the interactions of Nmt1p with acylCoA ligands. MyristoylCoA produces an exothermic ΔH of −24.8 kcal/mol when binding to apo-Nmt1p. There is a large reduction in the magnitude of this parameter when any of several changes are made in acylCoA structure, without a coincident reduction in affinity. The enthalpy and entropy of binding acylCoAs fall off as the chain is either extended or shortened (Figure 2), suggesting that acyl chain length recognition is not caused by a single physical phenomenon such as the hydrophobic effect, which would be expected to vary monotonically with chain length. The large ΔH for myristoylCoA binding may represent formation of a unique bound state that is inaccessible to most of the other compounds tested. The only modifications of myristoylCoA that allow binding with a similar enthalpy, addition of the 2-hydroxyl group or insertion of a methylene between the carbonyl carbon and CoA sulfur, indicate tolerance to minor structural changes in the immediate vicinity of the thioester bond.

Studies of acyl chain length recognition by medium chain acylCoA dehydrogenase (MCAD) using alkyCoA analogs of acylCoA substrates revealed a segregation of catalytic and binding specificity. AlkylCoAs of increasing chain length are bound with monotonically increasing affinity beyond the range of dehydrogenase activity (*28*). More recent analysis of this enzyme's interactions with nonsubstrate acylCoA analogs disclosed that ligands containing a thioester carbonyl bind to MCAD with a chain length-dependent affinity that corresponds to its activity profile (*29*). These findings indicate that the thioester carbonyl is essential for MCAD's recognition of acyl chain length. The data also demonstrated that polarization of the carbonyl, a prerequisite to dehydrogenation, varies with chain length in a pattern corresponding to both activity and affinity (*29*).

Our results also reveal a critical role for the thioester carbonyl in chain length recognition by Nmt1p. The similarity in Nmt1p's binding enthalpy for alkylCoAs (tetradecylCoA and hexadecylCoA) contrasts with the chain length-dependent variation of binding enthalpy for acylCoAs (and acyl-3′-dephosphoCoAs) (Figure 2) and is analogous to the results obtained with MCAD. Furthermore, data obtained with *S*-(2-oxo)alkylCoA ligands allows us to conclude that the carbonyl and ω-terminal methyl groups are important landmarks for acyl chain length measurement.

Trievel et al. (*29*) proposed that acyl chain length determines the residence time of the thioester bond in a conformation that optimizes hydrogen bonding with MCAD and consequent polarization of the carbonyl. A similar model may apply to Nmt1p where the enthalpy of acylCoA binding (and the corresponding entropy) reflects residence time in a conformation in which the thioester bond is sterically and electronically optimized for acyl transfer either to an enzyme nucleophile or directly to the peptide substrate's terminal nitrogen. A greater sensitivity of residence time to chain length could explain the more rapid falloff in activity and binding enthalpy observed with Nmt1p compared to MCAD.

Removing the 3′-phosphate from myristoylCoA results in binding with a less favorable enthalpy than the parent compound. The dependence of the 3′-phosphate's interactions with the enzyme on chain length (Table 2) indicates that chain length-dependent changes in the binding of the acylCoA are propagated as far as the 3′-phosphate. The greater dependence of C12:0−CoA than C14:0−CoA on this phosphate group for binding energy may reflect a difference in the way the two acylCoAs are recognized and bound. The shorter acyl chain may allow greater flexibility in the bound conformation of the ligand, allowing for improved interaction of the charged region of CoA with Nmt1p. Conversely, the smaller hydrophobic contribution to binding from the dodecanoyl chain may require stronger ionic interactions between the CoA and Nmt1p to generate high-affinity. Like C12:0−CoA, the CoA esters of C14 fatty acids with hydrophobicities less than that of myristate are very dependent upon the 3′-phosphate for high-affinity binding to apo-Nmt1p (e.g., see the effects of removing the 3′-phosphate from 5*Z*-tetradecenoylCoA and 6-tetradecynoylCoA in Table 2).

Among the fully saturated thioester-containing compounds examined, only pentadecanoylCoA and palmitoylCoA do not fit the correlation between acyltransferase activity and binding enthalpy (Figure 3). PalmitoylCoA may present the greatest challenge for this *N*-myristoyltransferase: it is a very abundant acylCoA in *S. cerevisiae* (*11*) and its incorporation into Nmt substrates would be expected to have marked effects on membrane−protein and protein−protein interactions (*1*, *3*). Our results suggest that interactions between the 3′-phosphate of palmitoylCoA and Nmt1p could underlie a mechanism that imparts selectivity against palmitoyltransferase activity, i.e., removal of the 3′-phosphate results in

an increase in palmitoyltransferase activity such that palmitoyl 3′-dephosphoCoA fits the activity−enthalpy correlation observed for the other saturated acylCoAs. This proposed 3′-phosphate-mediated inhibition of palmitoyltransferase activity may also apply to C15:0−CoA (Figure 3).

The Challenge of Being Nmt1p. Nmt1p does not possess an intrinsic, peptide-independent acylCoA thioesterase activity (*19*). In principle, such an activity could serve a useful editing function, allowing Nmt1p to rid itself of tightly bound nonsubstrate acylCoAs. Without such an editing function, and because there is little chain length specificity at the level of acylCoA binding affinity, Nmt1p is in a precarious position: faced with a pool of cellular acylCoAs of which myristoylCoA is only a minor component, the apo-enzyme would appear to be at continual risk of being ligated with the wrong acylCoA. This can lead to either inhibition of protein N-myristoylation or acylation of proteins with the wrong fatty acid, as demonstrated by the results obtained when purified Nmt1p was presented with equimolar mixtures of C12:0−CoA, C14:0−CoA, and C16:0−CoAs.

The phenomenon of heterogeneous protein N-acylation in retinal photoreceptor cells suggests that factors other than Nmt participate in regulating its acyl chain specificity *in vivo*. Nmt is encoded by a single gene in all species that have been examined (*30*), and there is no evidence for the existence of cell lineage-specific isoforms with varying substrate specificities. The alternate substrate acylCoAs used by Nmt in photoreceptor cells have binding affinities for the enzyme that are equal to myristoylCoA (C14:1$^{\Delta 5}$−CoA) or inferior to myristoylCoA (C12:0−CoA and C14:2$^{\Delta 5,8}$−CoA). None have superior kinetic properties (see Table 3). Together, these observations indicate that the lipid specificity of protein N-myristoylation in photoreceptor cells is defined, at least in part, by acylCoA availability. The mechanisms that regulate Nmt's access to acylCoAs are unknown. AcylCoA binding protein could contribute to the functional segregation of acylCoAs within cells: the affinity of this ubiquitous cytoplasmic protein for acylCoAs increases with chain length (*31*). Another potential mechanism may be thermodynamic selection of acylCoAs by cellular membranes. The affinity of acylCoAs for model membranes increases by an order of magnitude for each two-carbon increase in acyl chain length (*6*). Thus, one can speculate that the presence of cellular membranes may deplete the cytoplasm of more hydrophobic acylCoAs (e.g., C16:0−CoA). The result would be relative enrichment of less hydrophobic acylCoAs (C12:0−CoA, C14:2$^{\Delta 5,8}$-CoA; C14:1$^{\Delta 5}$-CoA, and C14:0−CoA) in the cytoplasm where they would be accessible to cytoplasmic Nmt. Cell lineage-specific differences in acylCoA metabolism would define the subset of less hydrophobic acylCoAs that are produced.

Implications for Protein S-Palmitoylation. Enzymatic activities responsible for post-translational addition of palmitate to Cys residues are associated with cellular membranes and variously termed palmitoylacyltransferase (PAT; ref *32*, *33*) or protein palmitoyltransferase (PPT; ref *34*). Heterogenous S-acylation of palmitoylproteins with C16, C18, and C20 species has been documented in a variety of tissues (*35*, *36*). Cellular membranes may help define both the lipid and protein substrate specificities of PAT/PPT. The partitioning of longer chain acylCoAs into membranes could be used to advantage by membrane-associated S-acyltransferases to aid in discrimination against shorter chain acylCoAs. Palmitoylation requires that protein substrates be targeted to the membrane-bound PAT/PPT. For example, this is accomplished by initial cotranslational N-myristoylation in the case of some src family tyrosine kinases (*37*) or by association with other membrane-bound proteins in the case of the of some N-myristoylated G-protein α-subunits (*38*).

The acyl chain specificity of protein S-acylation may not be as important as the acyl chain specificity of protein N-myristoylation. An apparent function of acyl chains in many N-myristoyl- and S-palmitoylproteins is to promote switchable membrane association (reviewed in ref *1*). For palmitoylproteins, switching is accomplished by acylation/deacylation. This switching mechanism imposes a lower, but not upper, limit for acyl chain hydrophobicity. The thioester-linked lipid must only confer sufficient membrane affinity to anchor the protein.[2] A more hydrophobic acyl chain (>C16) can be tolerated because the switch involves acyl chain removal. In contrast, cotranslational N-myristoylation appears to be irreversible. In the case of N-myristoylproteins, the switch depends upon the marginal membrane affinity conferred by their covalently bound acyl chains and the influences of reversible modifications (e.g., altering the protein's electrostatic attraction for membranes by adding or substrate phosphate groups; ligand-induced protein conformational changes that affect the exposure of the N-terminal acyl chain). Utilization of C12:0, C14:1$^{\Delta 5}$, or C14:2$^{\Delta 5,8}$ may yield acylated isoforms of N-myristoylproteins that can more readily partition to the aqueous phase in the membrane-rich microenvironments of photoreceptor cells, thereby permitting proteins such as recoverin and transducin to fulfill their roles in rapidly executed visual signal transduction cascades (*39*).

In summary, our results reveal that Nmt1p's intrinsic molecular recognition is insufficient to explain the observed *in vivo* specificity of N-myristoylation. These findings suggest that within the cell, limitation of Nmt substrate usage occurs through control of acylCoA availability. This notion adjusts the focus for control of protein lipidation from the acyltransferase and its peptide substrates to include the synthesis or presentation of the lipid. Such a perspective further suggests that lipid availability may play an important role in the numerous signaling pathways in which lipidated proteins function.

[2] The lower limit of acyl chain hydrophobicity could be defined by selecting from a pool of acylCoAs already affiliated with membranes.

REFERENCES

1. Bhatnagar, R. S., and Gordon, J. I. (1997) *Trends Cell Biol. 7*, 14−20.
2. Chow, M., and Moscufo, N. (1995) *Methods Enzymol. 250*, 495−509.
3. McLaughlin, S., and Aderem, A. (1995) *Trends. Biochem. Sci. 20*, 272−276.
4. Wedegaertner, P. B., Wilson, P. T., and Bourne, H. R. (1995) *J. Biol. Chem. 270*, 503−506.
5. Chow, M., Newman, J. F. E., Filman, D., Hogle, J. M., Rowlands, D. J., and Brown, F. (1987) *Nature 327*, 482−486.
6. Peitzsch, R. M., and McLaughlin, S. (1993) *Biochemistry 32*, 10436−10443.
7. Silvius, J. R., and l'Heureux, F. (1994) *Biochemistry 33*, 3014−3022.
8. Seykora, J. T., Myat, M. M., Allen, L.-A. H., Ravetch, J. V., and Aderem, A. (1996) *J. Biol. Chem. 271*, 18797−18802.

9. Ames, J. B., Tanaka, T., Ikura, M., and Stryer, L. (1995) *J. Biol. Chem. 270*, 30909−30913.
10. Hori, T., Nakamura, N., and Okuyama, H. (1987) *J. Biochem. (Tokyo) 101*, 949−956.
11. Schjerling, C. K., Hummel, R., Hansen, J. K., Borsting, C., Mikkelsen, J. M., Kristiansen, K., and Knudsen, J. (1996) *J. Biol. Chem.* 271, 22514−22521.
12. Johnson, R. S., Ohguro, H., Palczewski, K., Hurley, J. B., Walsh, K. A., and Neubert, T. A. (1994) *J. Biol. Chem. 269*, 21067−21071.
13. Dizhoor, A. M., Ericsson, L. H., Johnson, R. S., Kumar, S., Olshevskaya, E., Zozulya, S., Neubert, T. A., Stryer, L., Hurley, J. B., and Walsh, K. A. (1992) *J. Biol. Chem. 267*, 16033−16036.
14. Rudnick, D. A., McWherter, C. A., Gokel, G. W., and Gordon, J. I. (1993) *Adv. Enzymol. 67*, 375−430.
15. Rudnick, D. A., McWherter, C. A., Rocque, W. J., Lennon, P. J., Getman, D. P., and Gordon, J. I. (1991) *J. Biol. Chem. 266*, 9732−9739.
16. Rudnick, D. A., Lu, T., Jackson-Machelski, E., Hernandez, J. C., Li, Q., Gokel, G. W., and Gordon, J. I. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 10507−10511.
17. Kishore, N. S., Lu, T., Knoll, L. K., Katoh, A., Rudnick, D. A., Mehta, P. P., Devadas, B., Huhn. M., Atwood, J. L., Adams, S. P., Gokel, G. W., and Gordon, J. I. (1991) *J. Biol. Chem. 266*, 8835−8855.
18. Lu, T., Li, Q., Katoh, A., Hernandez, J., Duffin, K., Jackson-Machelski, E., Knoll, L. J., Gokel, G. W., and Gordon, J. I. (1994) *J. Biol. Chem. 269*, 5346−5357
19. Bhatnagar, R. S., Jackson-Machelski, E., McWherter, C. A., and Gordon, J. I. (1994) *J. Biol. Chem. 269*, 11045−11053.
20. Devadas, B., Zupec, M. E., Freeman, S. K., Brown, D. L., Nagarajan, S., Sikorski, J. A., McWherter, C. A., Getman, D. P., and Gordon, J. I. (1995) *J. Med. Chem. 38*, 1837−1840.
21. Paige, L. A., Zheng, G., DeFrees, S. A., Cassady, J. M., and Geahlen, R. L. (1989) *J. Med. Chem. 32*, 1667−1673.
22. Duronio, R. J., Jackson-Machelski, E., Heuckeroth, R. O., Olins, P. O., Devine, C. S., Yonemoto, W., Slice, L. W., Taylor, S. S., and Gordon, J. I. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 1506−1510.
23. Bhatnagar, R. S., and Gordon, J. I. (1995) *Methods Enzymol. 250*, 467−486
24. Wiseman, T., Williston, S., Brandts, J. F., and Lin, L. N. (1989) *Anal. Biochem. 179*, 131−137.
25. McWherter, C. A., Rocque, W. J., Zupec, M. E., Freeman, S. K., Brown, D. L., Devadas, B., Getman, D. P., Sikorski, J. A., and Gordon, J. I. (1997) *J. Biol. Chem. 272*, 11874−11880.
26. Zheng, G. Q., Hu, X., Cassady, J. M., Paige, L. A., and Geahlen, R. L. (1994) *J. Pharm. Sci. 83*, 233−238.
27. Kishore, N. S., Wood, D. C., Mehta, P. P., Wade, A. C., Lu, T., Gokel, G. W., and Gordon, J. I. (1993) *J. Biol. Chem. 268*, 4889−4902.
28. Powell, P. J., Lau, S-M., Killian, D., and Thorpe, C. (1987) *Biochemistry 26*, 3704−3710.
29. Trievel, R. C., Wang, R., Anderson, V. E., and Thorpe, C. (1995) *Biochemistry 34*, 8597−8605.
30. Lodge, J. K., Johnson, R. L., Weinberg, R. A., and Gordon, J. I. (1994) *J. Biol. Chem. 269*, 2996−3009.
31. Knudsen, J., Faergeman, N. J., Skott, H., Hummel, R., Borsting, C., Rose, T. M., Anderson, J. S., Hojrup, P., Roepstorff, P., and Kristiansen, K. (1994) *Biochem. J. 302*, 479−485.
32. Berthiaume, L., and Resh, M. D. (1995) *J. Biol. Chem. 270*, 22399−22405.
33. Dunphy, J. T., Greentree, W. K., Manahan, C. L., and Linder, M. E. (1996) *J. Biol. Chem. 271*, 7154−7159.
34. Liu, L., Dudler, T., and Gelb, M. H. (1996) *J. Biol. Chem. 271*, 23269−23276.
35. Hallak, H., Muszbek, L., Laposata, M., Belmonte, E., Brass, L. F., and Manning, D. R. (1994) *J. Biol. Chem. 269*, 4713−4716.
36. Schroeder, H., Leventis, R., Shahinian, S., Walton, P. A., and Silvius, J. R. (1996) *J. Cell Biol. 134*, 647−660.
37. Shenoy-Scaria, A. M., Dietzen, D. J., Kwong, J., Link, D. C., and Lublin, D. M. (1994) *J. Cell. Biol. 126*, 353−363.
38. Degtyarev, M. Y., Spiegel, A. M., and Jones, T. L. Z. (1994) *J. Biol. Chem. 269*, 30898−30903.
39. Hughes, R. E., Brzovic, P. S., Klevit, R. E., and Hurley, J. B. (1995) *Biochemistry 34*, 11410−11416.

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