Isothermal Titration Calorimetric Studies of Saccharomyces cerevisiae Myristoyl-CoA:Protein N-Myristoyltransferase

DETERMINANTS OF BINDING ENERGY AND CATALYTIC DISCRIMINATION AMONG ACYL-CoA AND PEPTIDE LIGANDS*

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Saccharomyces cerevisiae myristoyl-CoA-protein N-myristoyltransferase (Nmt1p) is an essential, monomeric enzyme that catalyzes the transfer of myristate from CoA to the amino-terminal Gly residue of cellular proteins. Product inhibition studies indicate that Nmt1p has an ordered Bi Bi reaction mechanism with myristoyl-CoA binding to the apo-enzyme to form a high affinity binary complex followed by binding of peptide with subsequent release of CoA and then the myristoylpeptide product. We have used isothermal titration calorimetry to quantify the effects of varying acyl chain length and removing the 3'-phosphate group of CoA on the energetics of interaction between Nmt1p and acyl-CoA ligands. Myristoyl-CoA binds to apo-Nmt1p with an affinity of 15 nm, corresponding to a binding free energy of ~10.9 kcal/mol. This free energy is composed of a large favorable enthalpy of ~24 kcal/mol and a large unfavorable entropic term. This large negative ΔH° is consistent with a conformational change in the enzyme upon ligand, allowing synthesis of a functional peptide binding site. Binding of palmitoyl-CoA and lauroyl-CoA is driven by an exothermic enthalpy change which is much smaller than the corresponding parameter for myristoyl-CoA binding. The large differences in binding enthalpy and entropy (ΔΔH° and TΔΔS° = 8–9 kcal/mol) demonstrate that the "off-length" acyl-CoA binds to Nmt1p in a significantly different energetic fashion from myristoyl-CoA, even though the enzyme does not have a great deal of specificity among these different ligands in terms of binding free energy (ΔΔG° ≤ 1 kcal/mol). The effect of removing the CoA 3'-phosphate group from myristoyl-CoA is similar to the effect of a two-carbon change in acyl chain length: i.e. an enthalpy dominated reduction in binding affinity. However, kinetic studies reveal that removing the 3'-phosphate from myristoyl-CoA has little effect on Nmt1p's catalytic efficiency, indicating that the 3'-phosphate group contributes binding free energy but little catalytic destabilization. The greater ΔΔG°, with smaller ΔΔH° and ΔΔS° components, produced by removing the 3'-phosphate compared to increasing chain length suggests that it is not primarily the interactions of the 3'-phosphate which are disrupted when palmitoyl-CoA is substituted for myristoyl-CoA.

No detectable interactions were noted between apo-Nmt1p and the substrate peptide, GAAPSKIV-NH₂, providing additional support for the preferred ordered reaction mechanism. In contrast, GAAPSKIV-NH₂ is able to bind with high affinity to Nmt1p saturated with either nonhydrolyzable myristoyl-CoA or palmitoyl-CoA analogs. The 3.6 μM dissociation constant for binding of GAAPSKIV-NH₂ to Nmt1p-S-(2-oxo)pentadecyl-CoA is driven by favorable enthalpy (~6.5 kcal/mol) and entropy (0.9 kcal/mol at 300 K). The fact that a high affinity peptide binding site is also formed with a nonhydrolyzable palmitoyl-CoA analog, S-(2-oxo)heptadecyl-CoA, indicates that the energy of binding of the off-length acyl-CoA is sufficient to induce the cooperative transition which allows peptide binding but not to generate a highly efficient active site. The similar affinities of Nmt1p for myristoyl-CoA, palmitoyl-CoA, and lauroyl-CoA provide little specificity for binding one acyl-CoA rather than another, suggesting that for this myristoyltransferase to avoid catalyzing the transfer of other acyl chains or being competitively inhibited by them, there must be some form of functional segregation of cellular acyl-CoA pools.

Myristoyl-CoA-protein N-myristoyltransferase (Nmt, EC 2.3.1.97) catalyzes the co-translational transfer of myristate (tetradecanoic acid; C14:0) from myristoyl-CoA to the amino-terminal glycine nitrogen of a variety of eukaryotic cellular and viral proteins. These proteins have diverse functions and subcellular locations (reviewed in Ref. 1). The myristoyl moiety is required for expression of the full biological function of proteins encoded by the genomes of enveloped and nonenveloped viruses and for the transforming functions of some tyrosine kinases (reviewed in Refs. 1 and 2), making human Nmt a potential target for treatment of viral infections and certain neoplasias. In addition, species-specific inhibitors of Candida albicans and Cryptococcus neoformans Nmts may be cidal to these leading causes of systemic fungal infections in immunocompromised humans (3–7).

Information about Nmt's kinetic mechanism and its substrate specificities is not only important for understanding how protein N-myristoylation is regulated in vivo but also for rational design of therapeutically useful alternative substrates and/or inhibitors. The 455-residue (3) monomeric (8) Saccharomyces cerevisiae enzyme (Nmt1p) is the best studied N-myristoyltransferase.

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§ The abbreviations used are: Nmt, myristoyl-CoA-protein N-myristoyltransferase; Nmt1p, S. cerevisiae myristoyl-CoA-protein N-myristoyltransferase; HPLC, high performance liquid chromatography; ACBP, acyl-CoA binding protein; E2p, catalytic subunit of dihydrolipoyl transacetylase.

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toytransferase. Product inhibition studies have indicated that its kinetic reaction mechanism is ordered Bi Bi (9). A binary complex of Nmtlp-myristoyl-CoA is formed prior to binding of a peptide substrate. After catalysis, CoA is released from the ternary complex, followed by myristoylpeptide. The binding of myristoyl-CoA to apo-Nmtlp is required for formation of a functionally competent peptide binding site (10). This heterotrophic cooperativity is also seen in the profound effect that variations in the chemical properties of the acyl-CoA presented to Nmtlp can have on the enzyme’s subsequent interactions with peptide ligands (10–13). Nmtlp is highly selective for myristoyl-CoA in vivo, despite the fact that the concentration of palmitoyl-CoA is at least 5-fold higher than that of myristoyl-CoA (14). The reaction mechanism and acyl-CoA specificity of human Nmt are indistinguishable from those of Nmtlp (15, 16).

The origins of Nmtlp’s specificity for myristoyl-CoA as well as the structural features of its acyl-CoA binding site have been explored by testing over 200 fatty acid analogs in an in vitro enzyme assay (11–19). These studies provided a functional view of conformational and electronic characteristics of acyl chains that make their CoA derivatives good substrates for Nmtlp. The results indicate that: (i) the enzyme is sensitive to changes of plus or minus two methylenes in the length of the acyl chain; (ii) the acyl chain is bound in a bent conformation with a principal bend occurring in the vicinity of C5–C6; (iii) the acyl-CoA binding site is able to accommodate increases in the polarity of the acyl chain; and (iv) the alpha terminus of the acyl chain must fit into a sterically restricted pocket which seems to be conical in shape. Likewise, the peptide specificity of Nmtlp has been explored with more than 100 synthetic peptides. The general features required for peptide recognition are known and can be used to predict whether newly defined proteins are likely substrates for the acyltransferase (8, 10, 20–23).

Our previous studies have probed the acyl-CoA and peptide binding sites of Nmtlp by observing kinetic parameters (K_m and V_max) or the overall activity of the peptide acylation reaction. Thus, the results reflect the combined effects of the binding affinity and the catalytic activity for the ligand in question. When the kinetic analysis of Nmtlp’s interactions with substrates, a description of the thermodynamics of ligand binding provides a more direct probe of the structural determinants of an enzyme’s molecular recognition. Recent advances in isothermal titration calorimetry have made this technique suitable for the study of the thermodynamics of biological reactions (24, 25). Rather than monitoring an optical signal or partitioning bound and free ligand as in most traditional methods of determining binding constants, isothermal titration calorimetry allows determination of the free energy, enthalpy, and entropy of a binding event in a single experiment by monitoring the heat of reaction. We have now used this method to study the thermodynamics of substrate interactions with Nmtlp. We have explored the effect of acyl chain length variation and the importance of the CoA 3'-phosphophate group on formation of binary enzyme:acyl-CoA complexes, and have studied the binding of peptide substrates to the apoenzyme and to binary complexes of Nmtlp and various acyl-CoAs.

**EXPERIMENTAL PROCEDURES**

**Purification of S. cerevisiae Nmtlp from Escherichia coli.** S. cerevisiae Nmtlp was expressed in E. coli strain JM101 containing pBB131 (26, 27) and purified to apparent homogeneity as described previously (28). The enzyme was further purified to remove residual thioesterase activity by dialyzing it against buffer A (20 mM potassium phosphate, pH 7.4, 1 mM EDTA, 1 mM diethiothreitol) then passing it over a column of SP Sepharose fast flow (Pharmacia) equilibrated with the same buffer (1 ml of resin/mg of protein). A gradient from 0 to 500 mM NaCl in buffer A was used to elute material in a total of 10 column volumes.

Three-mI fractions were collected with absorbance monitored at 280 nm. The major absorbance peak contained Nmtlp without contaminating thioesterase activity as determined by the assay described below.

**Assay of Thioesterase Activity.** Tritiated myristoyl-CoA and palmitoyl-CoA were generated as described previously (15). To assay Nmtlp preparations for thioesterase activity, an aliquot of enzyme (1 μg of protein) was incubated for 30 min at 27 °C with 1 μCi of [3H]myristoyl-CoA or [3H]palmitoyl-CoA (specific activity = 10 Ci/mmol) in a reaction mixture (total volume = 110 μl) containing 100 mM HEPES, pH 7.4, 3 mM 2-mercaptoethanol, 0.1 mM EDTA, and 0.05% Triton X-100. The reaction was quenched by adding 100 μl of ice-cold MeOH, and the products analyzed by C4 reverse phase HPLC (16) using a gradient from 35 to 56% acetonitrile in 50 mM ammonium acetate, pH 5.4, with 5 mM tetrabutylammonium phosphate, followed by an 85% acetonitrile bump. This system is sufficiently sensitive to detect hydrolysis of less than 0.5% of the [3H]acyl-CoA (data not shown).

**Kinetic Assays of Nmtlp Activity.** The in vitro Nmt assay has been described in detail elsewhere (28). Briefly, [3H]acyl-CoAs were generated from the radiolabeled fatty acid and CoA (or 3'-dephospho-CoA) using the relatively nonspecific Pseudomonas acyl-CoA synthetase (12, 19). The [3H]acyl-CoA product was then incubated with purified thioesterase-free Nmtlp and a peptide substrate. The initial reaction velocity of myristoyl-CoA acylation was determined by measuring the relative amount of unreacted acyl-CoA product using C4 or C18 reverse phase HPLC (28) and an in-line scintillation counter. The apparent kinetic parameters K_m and V_max were determined by varying the concentration of acyl-CoA or peptide substrate and analyzing the data by Lineweaver-Burk plots. Kinetic parameters for peptide substrates were determined in the presence of several acyl-CoAs with each acyl-CoA at saturating concentrations.

The kinetic parameters for each acyl-CoA were determined with the peptide concentration equal to its K_m. Experiments were performed using the high affinity substrate GAAPSKIV-NH_2 (representing residues 2–9 of the primary translation product of Cnbl mRNA; cf. Ref. 29) and its derivatives as well as the previously well characterized octapeptide substrate GNAARRK-NH_2 (derived from the amino-terminal sequence of the catalytic subunit of cAMP-dependent protein kinase; cf. Refs. 8 and 20). Each assay was performed in duplicate on at least two separate occasions.

**Determination of Reagent and Buffer Costs.** Nmtlp was dialyzed exhaustively into binding buffer (100 mM HEPES/NaOH, pH 7.4, 3 mM 2-mercaptoethanol, 0.1 mM EDTA) and its concentration determined using an extinction coefficient of 7.44 10^4 M^-1 cm^-1 at 280 nm, determined by quantitative amino acid analysis. Acyl-CoA and peptide substrates were dissolved in the same buffer and the CoA concentration determined with a 260-nm extinction coefficient of 1.54 10^4 M^-1 cm^-1. Due to the inability of the thioester-CoA compounds, the concentrations of acyl-CoA and free CoA were determined at the time of calorimetric titration by analyzing an aliquot of the ligand solution by C4 reverse phase HPLC. The eluted CoA-thiol and acyl-CoA peaks were quantified at 280 nm with an in-line absorbance detector. Solutions typically contained 50–100% acyl-CoA. Peptide solutions were made in binding buffer and their concentrations calculated from the dry mass and peptide content of the solid peptides (estimated by quantitative amino acid analysis). Enzyme and ligand solutions were prepared in identical buffers to avoid background heat of mixing. Prior to each titration, the solutions of Nmtlp and ligand were thoroughly degassed by stirring under vacuum at room temperature to avoid formation of air bubbles in the calorimeter’s reaction cell or in the injection syringe.

**Isothermal Titration Calorimetry.** Isothermal titration calorimetry was carried out using an OMG calorimetric system (MicroCal Inc.) equipped with a refrigerated circulating water bath (MicroCal Inc.) as described in Wiesenmann et al. (24). The calorimeter reaction cell, control module, and preamplifier were placed in a Faraday cage to reduce interference with the nanovolt level pre-amplified signal by local electric fields. The calorimeter reaction cell was filled with a 1.25 mM Nmtlp solution in a cuvette for each experiment. All calorimetric titrations were carried out at 27.0 ± 0.1 °C (300 K). To insulate the calorimeter against variations in room temperature, the outer jacket was maintained at 7.5 °C. The calorim-
Thermodynamic Studies of Myristoyl-CoA:Protein N-Myristoyltransferase

Table I

Thermodynamic parameters for Nmtlp binary and ternary complex formation

All experiments were performed at 300 K as described under "Experimental Procedures." The values shown are the average ± the S.D. of parameters derived from multiple experiments (the number of experiments is shown in parentheses). Typical error estimates for the parameters derived from individual titrations are shown in Table II.

<table>
<thead>
<tr>
<th>Binding reaction</th>
<th>$K_d$</th>
<th>$\Delta G^*$</th>
<th>$\Delta H^*$</th>
<th>$\Delta S^*$</th>
<th>$n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acyl-CoA + Nmtlp binary complex formation</td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myristoyl-CoA</td>
<td>15 ± 1 6 nm</td>
<td>-10.9 ± 0.5</td>
<td>-24.4 ± 1.7</td>
<td>-13.5 ± 2</td>
<td>1.03 ± 0.07</td>
</tr>
<tr>
<td>Palmitoyl-CoA</td>
<td>37 ± 2 5 nm</td>
<td>-10.3 ± 0.4</td>
<td>-15.8 ± 1.4</td>
<td>-5.5 ± 1.6</td>
<td>0.99 ± 0.11</td>
</tr>
<tr>
<td>Lauroyl-CoA</td>
<td>64 ± 8 nm</td>
<td>-9.9 ± 0.8</td>
<td>-14.9 ± 0.5</td>
<td>-5.0 ± 0.5</td>
<td>1.05 ± 0.08</td>
</tr>
<tr>
<td>S-(2-Oxo)pentadecyl-CoA</td>
<td>4.9 ± 2.2 2 9</td>
<td>-11.5 ± 0.3</td>
<td>-23.3 ± 1.0</td>
<td>-13.9 ± 1.1</td>
<td>1.02 ± 0.13</td>
</tr>
<tr>
<td>S-(2-Oxo)heptadecyl-CoA</td>
<td>45 ± 12 nm</td>
<td>-10.1 ± 0.2</td>
<td>-13.2 ± 0.06</td>
<td>-0.1 ± 0.1</td>
<td>0.98 ± 0.11</td>
</tr>
<tr>
<td>Myristoyl-CoA (3'-dephospho)-CoA</td>
<td>108 ± 34 nm</td>
<td>-8.6 ± 0.2</td>
<td>-16.9 ± 0.7</td>
<td>-7.3 ± 0.8</td>
<td>1.02 ± 0.03</td>
</tr>
<tr>
<td>Peptide + Nmtlp-S-(2-Oxo)alkyl-CoA ternary complex formation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAAPSKIV-NH₂ peptide</td>
<td>3.6 ± 0.3 2 5</td>
<td>-7.47 ± 0.05</td>
<td>-6.54 ± 0.4</td>
<td>0.92 ± 0.4</td>
<td>0.92 ± 0.01</td>
</tr>
<tr>
<td>GAAPSKIV-NH₂ peptide</td>
<td>0.67 ± 0.6 2 5</td>
<td>-8.47 ± 0.05</td>
<td>-8.77 ± 0.5</td>
<td>-0.30 ± 0.46</td>
<td>0.94 ± 0.003</td>
</tr>
</tbody>
</table>

This effect was endothermic and constant over the concentration range used in the experiment. The average enthalpy resulting from this effect was subtracted from each integrated data point before the isotherm was fit.
Fig. 1A shows the raw data for a typical titration of myristoyl-CoA to Nmtlp. Fig. 1B shows the integrated heat and the best fit isotherm. The acyl-CoA ligand binds to apo-Nmtlp with an affinity of 15 nM, corresponding to a binding free energy of \(-10.9 \text{ kcal/mol}\) (Table I). This free energy is composed of a remarkably large enthalpic term favoring binding by \(-24 \text{ kcal/mol}\) and a large unfavorable entropic term. This large negative \(\Delta H^0\) is consistent with a conformational change in the enzyme upon ligation. The observed \(\Delta H^0\) may represent, in addition to the intrinsic binding enthalpy, a heat of buffer ionization secondary to uptake or release of protons from the enzyme upon binding of ligand. The negative \(\Delta S^0\) may reflect conformational restriction of functional groups in the enzyme as well as the many rotational and vibrational degrees of freedom in the free acyl-CoA molecule.

The affinity of palmitoyl-CoA (hexadecanoyl-CoA) does not differ significantly from that of myristoyl-CoA (Table I), the preferred ligand in vivo. However, while the net Gibbs free energy change is quite similar for the two binding reactions, the two methylene increase in acyl chain length results in a drastic change in the enthalpic and entropic components of \(\Delta G^0\), each component becoming more positive by approximately 9 kcal/mol relative to the parameters for myristoyl-CoA.

Lauroyl-CoA (dodecanoyl-CoA) contains two fewer methyl-enedes in its acyl chain than myristoyl-CoA. It also binds to its acyl chain is mandatory for CoA to bind to Nmtlp with high affinity. Second, we examined the influence of the CoA 3'-phosphate moiety by synthesizing the 3'-dephospho derivative of myristoyl-CoA. The affinity of the enzyme for myristoyl(3'-dephospho)-CoA is 108 nM, a 7-fold decrease relative to myristoyl-CoA (Table I). The decrease in binding free energy due to the removal of the 3'-phosphate is composed of 7.5 kcal/mol more positive \(\Delta H^0\) and 6.2 kcal/mol more positive \(\Delta T S^0\). These changes in the enthalpic and entropic contributions to \(\Delta G^0\) are slightly smaller than the corresponding changes resulting from 2-carbon changes in acyl chain length, although the net \(\Delta G^0\) is greater (Tables I and V). This finding suggests that there is less enthalpy-entropy compensation in the interactions disrupted by removal of the 3'-phosphate group than in the interactions perturbed by altering the length of the acyl chain, and raises the possibility that the reduction in affinity caused by removal of the 3'-phosphate is not due to disruption of the same intermolecular contacts as those which are disrupted by changes in acyl chain length.

The nonhydrolyzable analogs of myristoyl-CoA and palmitoyl-CoA, S-(2-oxo)pentadecyl-CoA (32) and S-(2-oxo)heptadecyl-CoA (Fig. 2), are inhibitors of S. cerevisiae Nmtlp (1, 9). They represent the insertion of a single methylene carbon in the thioester bond of the corresponding acyl-CoA, resulting in

\[
\text{Nmtlp with an affinity similar to myristoyl-CoA and palmitoyl-CoA. Like palmitoyl-CoA, the binding of lauroyl-CoA is driven by a favorable exothermic enthalpy change which is much smaller than the corresponding parameter for myristoyl-CoA binding. The large differences in binding enthalpy and entropy (\(\Delta H^0\) and \(\Delta T S^0 = 8.9 \text{ kcal/mol}\)) demonstrate that the "off"-length acyl-CoAs bind to Nmtlp in a significantly different energetic fashion from myristoyl-CoA, even though the enzyme does not have a great deal of specificity among these ligands in terms of binding free energy. Table V summarizes the differences in the thermodynamic parameters of binary Nmtlp-acyl-CoA formation due to variations in acyl chain length.}

Two additional experiments were performed to assess the relative contributions of the acyl chain and CoA moieties to the interactions between Nmtlp and its acyl-CoA ligand. First, we attempted to measure thermodynamic parameters for the interaction of free CoA with Nmtlp. No interaction was detected between CoA and Nmtlp at enzyme concentrations of 30 and 70 nM, and appropriate ligand concentrations. The absence of a detectable heat of binding implies either that the affinity of the interaction is so weak (\(K_w > 70 \mu M\)) that the amount of binding which occurs under these conditions is insignificant, or that the entropy of binding is very small, in which case binding cannot be detected calorimetrically. It is unlikely that the acyl chain alone is responsible for the large \(\Delta H^0\) of acyl-CoA binding to Nmtlp. Therefore, this result suggests that the presence of an acyl chain is mandatory for CoA to bind to Nmtlp with high affinity. Second, we examined the influence of the CoA 3'-phosphate moiety by synthesizing the 3'-dephospho derivative of myristoyl-CoA. The affinity of the enzyme for myristoyl(3'-dephospho)-CoA is 108 nM, a 7-fold decrease relative to myristoyl-CoA (Table I). The decrease in binding free energy due to the removal of the 3'-phosphate is composed of 7.5 kcal/mol more positive \(\Delta H^0\) and 6.2 kcal/mol more positive \(\Delta T S^0\). These changes in the enthalpic and entropic contributions to \(\Delta G^0\) are slightly smaller than the corresponding changes resulting from 2-carbon changes in acyl chain length, although the net \(\Delta G^0\) is greater (Tables I and V). This finding suggests that there is less enthalpy-entropy compensation in the interactions disrupted by removal of the 3'-phosphate group than in the interactions perturbed by altering the length of the acyl chain, and raises the possibility that the reduction in affinity caused by removal of the 3'-phosphate is not due to disruption of the same intermolecular contacts as those which are disrupted by changes in acyl chain length.

The nonhydrolyzable analogs of myristoyl-CoA and palmitoyl-CoA, S-(2-oxo)pentadecyl-CoA (32) and S-(2-oxo)heptadecyl-CoA (Fig. 2), are inhibitors of S. cerevisiae Nmtlp (1, 9). They represent the insertion of a single methylene carbon in the thioester bond of the corresponding acyl-CoA, resulting in

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Acyl-CoA</th>
<th>(K_{\text{app}})</th>
<th>(V_{\text{max}})</th>
<th>(V_{\text{app}}/K_{\text{m}})</th>
</tr>
</thead>
<tbody>
<tr>
<td>GNAARRNH₂</td>
<td>Myr-CoA</td>
<td>0.37 ± 0.04</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Palm-CoA</td>
<td>0.70 ± 0.13</td>
<td>15 ± 2</td>
<td>7.9</td>
</tr>
<tr>
<td>GGAAPSKIV-NH₂</td>
<td>Myr-CoA</td>
<td>0.13 ± 0.08</td>
<td>13 ± 0.02</td>
<td>0.37</td>
</tr>
<tr>
<td></td>
<td>Palm-CoA</td>
<td>2.0 ± 0.9</td>
<td>0.8 ± 0.7</td>
<td>0.15</td>
</tr>
</tbody>
</table>

\(V_{\text{app}}\) is expressed as a percentage of the value obtained with myristoyl-CoA and GNAARR-NH₂ (100% \(V_{\text{app}} = 2.43 \pm 0.48 \times 10^5\) pmol min\(^{-1}\) mg\(^{-1}\)).

\(V_{\text{app}}/K_{\text{m}}\) is scaled relative to catalytic efficiency of myristoyl-CoA and GNAARR-NH₂ (100% \(V_{\text{app}}/K_{\text{m}} = 6.57 \times 10^5\) pmol min\(^{-1}\) mg\(^{-1}\)).

Table V

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Acyl-CoA</th>
<th>(K_{\text{app}})</th>
<th>(V_{\text{max}})</th>
<th>(V_{\text{app}}/K_{\text{m}})</th>
<th>Peptide specific</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>(\mu M)</td>
<td>%</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>GNAARRNH₂</td>
<td>Myr-CoA</td>
<td>13.6 ± 3.9</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Palm-CoA</td>
<td>109 ± 32</td>
<td>22 ± 9</td>
<td>2.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Myr-(3'-dephospho)-CoA</td>
<td>159 ± 45</td>
<td>732 ± 378</td>
<td>65</td>
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</tr>
<tr>
<td></td>
<td>Palm-CoA</td>
<td>0.72 ± 0.93</td>
<td>7.5 ± 5.3</td>
<td>4250</td>
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</tr>
<tr>
<td></td>
<td>Myr-(3'-dephospho)-CoA</td>
<td>0.10 ± 0.025</td>
<td>22 ± 4.9</td>
<td>287</td>
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<tr>
<td>GGAAPKIV-NH₂</td>
<td>Myr-CoA</td>
<td>1.5 ± 0.4</td>
<td>4.1 ± 0.4</td>
<td>37</td>
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</table>

\(V_{\text{app}}\) is expressed as a percentage of the value obtained with myristoyl-CoA and GNAARR-NH₂ (100% \(V_{\text{app}} = 3.27 \pm 0.95 \times 10^5\) pmol min\(^{-1}\) mg\(^{-1}\)).

\(V_{\text{app}}/K_{\text{m}}\) is scaled relative to catalytic efficiency of myristoyl-CoA and GNAARR-NH₂ (100% \(V_{\text{app}}/K_{\text{m}} = 2.4 \times 10^5\) pmol min\(^{-1}\) mg\(^{-1}\)).

Peptide specific \(V_{\text{app}}/K_{\text{m}}\) is scaled relative to the catalytic efficiency obtained with myristoyl-CoA and each peptide to highlight the similarity of the acyl-CoA effect on both peptides.

**Summary of peptide kinetic parameters for Nmtlp**
compounds that are, overall, one methylene unit longer than the corresponding hydrolyzable acyl-CoA. Nevertheless, the length from the carbonyl carbon to the ω-terminal methyl of their acyl chains is the same as for myristoyl-CoA and palmitoyl-CoA. We used these compounds to form stable Nmtlp.alkyl(thioether)CoA-peptide ternary complexes that do not turn over. This allows us to study the thermodynamics of substrate peptide binding to Nmtlp in the context of a preformed binary complex. However, before initiating these studies we characterized the thermodynamics of formation of the Nmtlp.alkyl(thioether)-CoA binary complex so that we could assess whether binding of the nonhydrolyzable CoA ligands was similar to binding of myristoyl-CoA and palmitoyl-CoA.

S-(2-Oxo)pentadecyl-CoA binds to Nmtlp with an affinity of 5 nM (Table I). This extremely high affinity is at the limit of the reported sensitivity range of the OMEGA titration calorimeter (24). However, the large enthalpy of the ligation permits the use of very low concentrations of enzyme and analog, resulting in a significant population of free ligand in the cell, and hence a binding isotherm which is sufficiently shallow to allow accurate fitting (Fig. 1C). Like myristoyl-CoA, S-(2-oxo)pentadecyl-CoA binds to Nmtlp with a remarkably large enthalpy of −25 kcal/mol and a large unfavorable entropic component (Table I). S-(2-Oxo)heptadecyl-CoA binds to Nmtlp with affinity that is 10-fold lower than S-(2-oxo)pentadecyl-CoA and nearly identical to the affinity of palmitoyl-CoA. The binding of the nonhydrolyzable palmitoyl-CoA analog is enthalpy driven, with a ΔHo of −13 kcal/mol, similar to the −16 kcal/mol ΔF of palmitoyl-CoA binding. Thus, the nonhydrolyzable analogs of myristoyl-CoA and palmitoyl-CoA bind to Nmtlp with similar energetics to the corresponding hydrozable acyl-CoAs (Table V). (The similar thermodynamics of Nmtlp binding to the natural acyl-CoA ligands and the nonhydrolyzable thioether analogs argues against the formation of a stable covalent acyl-enzyme complex, but does not rule out the transient formation of such a complex during catalysis.)

**Binding of Peptide Substrates to Nmtlp**—To test the order of ligand binding predicted from earlier kinetic studies, we examined the interactions of a high affinity peptide substrate with apo-Nmtlp and Nmtlp complexed with the nonhydrolyzable acyl-CoA analogs. GAAPSKIV-NH2 represents the amino terminus of S. cerevisiae Cnblp, a protein which is highly homologous to the mammalian regulatory component (β-subunit) of the Ca2+/calmodulin-dependent phosphoprotein phosphatase calcineurin B (29). This octapeptide has the lowest Kao of any known Nmtlp substrate (24 nM; cf. Table IV). Titration calorimetric analysis revealed no detectable heat of interaction between Nmtlp and GAAPSKIV-NH2 even at enzyme concentrations as high as 115 μM (data not shown). This indicates that the dissociation constant of Nmtlp-peptide is significantly lower than those for the acyl-CoA binding sites.

**Table V**

<table>
<thead>
<tr>
<th>Ligand</th>
<th>ΔAG°</th>
<th>ΔMH°</th>
<th>TΔAS°</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myristoyl-CoA</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Palmitoyl-CoA</td>
<td>0.6</td>
<td>8.6</td>
<td>8.0</td>
</tr>
<tr>
<td>Lauril-CoA</td>
<td>1.0</td>
<td>9.5</td>
<td>8.5</td>
</tr>
<tr>
<td>S-(2-Oxo)pentadecyl-CoA</td>
<td>−0.6</td>
<td>−0.9</td>
<td>−0.4</td>
</tr>
<tr>
<td>S-(2-Oxo)heptadecyl-CoA</td>
<td>0.8</td>
<td>12.1</td>
<td>10.4</td>
</tr>
</tbody>
</table>

**Fig. 2. Structure of S-(2-oxo)pentadecyl-CoA.** The methylene group (arrow) inserted between the acyl carbonyl and the CoA sulfur replaces the labile thioester linkage of acyl-CoAs with a stable thioether bond, making this myristoyl-CoA analog "nonhydrolyzable" and preventing catalytic transfer of the acyl chain to a peptide substrate by Nmtlp. The palmitoyl-CoA analog, S-(2-oxo)heptadecyl-CoA, has two more methylenes in its acyl chain.

**Fig. 1. Calorimetric titration of Nmtlp with myristoyl-CoA and S-(2-oxo)pentadecyl-CoA.** Panel A, raw heat signal (with base line subtracted) from 25 injections of 2.5-μl aliquots of a 94 μM solution of myristoyl-CoA into a cell containing 1.35 ml of 3.5 μM Nmtlp at 300 K. Panel B, the integrated total heat of each injection normalized to the amount of ligand injected (filled circles) and the fit (line) to the model described under "Experimental Procedures." The parameters derived from the fit line are given in the first row of Table II. Panel C, integrated heats and fit line for 25 injections of 6 μl of a 69 μM solution of S-(2-oxo)pentadecyl-CoA into a cell containing 1.35 ml of 3.5 μM Nmtlp. The parameters defining the fit line are K‡ = 2.3 × 10⁶ M⁻¹, ΔF = −24.2 kcal/mol, n = 1.14.

**Thermodynamic Studies of Myristoyl-CoA:Protein N-Myristoyltransferase**
may be due to the release of ordered water molecules from the peptide’s backbone and side chains which must be at least residual motion in the ternary complex.

The positive entropy change that is greater than 1.0, probably reflecting errors in our calculations of the entropy (0.9 kcal/mol; Table I). The best fit binding stoichiometry for the formation of the ternary complex is slightly less than 1.0, probably reflecting errors in our calculations of the concentration of peptide. The positive entropy change that occurs upon binding of this octapeptide substrate is surprising, considering the number of rotational degrees of freedom in the peptide’s backbone and side chains which must be at least partially restricted upon binding. This positive entropy change may be due to the release of ordered water molecules from the peptide binding site, to release of conformational restrictions elsewhere in the complex, to removal of hydrophobic peptide surface area from contact with solvent, and/or to significant residual motion in the ternary complex.

The binary complex of Nmt1p-S(2-oxo)pentadecyl-CoA can serve as a model for the behavior of the binary complex formed by Nmt1p and palmitoyl-CoA. Surprisingly, GAAPSKIV-NH2 binds to this binary complex with 5-fold higher affinity than to the Nmt1p-S(2-oxo)pentadecyl-CoA binary complex. The Kd of 0.67 μM observed with GAAPSKIV-NH2 and Nmt1p-S(2-oxo)heptadecyl-CoA is enthalpy driven, with an exothermic heat of binding of -8.8 kcal/mol. This is 2.3 kcal/mol more favorable than the enthalpy of binding of the peptide to the nonhydrolyzable myristoyl-CoA analog Nmt1p binary complex. The positive entropy of binding observed with the nonhydrolyzable myristoyl-CoA analog is lost with the nonhydrolyzable palmitoyl-CoA analog, but ΔH° is still remarkably favorable. Thus, the energetic differences observed in the formation of Nmt1p-acyl-CoA binary complexes are reflected in the subsequent interaction of the complex with this peptide substrate.

Changing residues in a substrate peptide that are known to be important for recognition results in large decreases in affinity of that peptide for the binary complex. For example, the α-methylene Gly is known to be essential for peptide N-myristoylation (20). A Ser is present at position 5 in all known yeast N-myristoylproteins. Kinetic studies have shown that substitution of this Ser with an Ala (i.e. replacement of hydroxyl with a proton) decreases affinity (Km) for Nmt1p by 2-3 orders of magnitude in several sequence contexts (see Refs. 20

**DISCUSSION**

Thermodynamic measurements provide information about the energetic basis of macromolecular interactions and complement studies of enzyme activity and structure: defining binding affinities allows systematic identification of determinants of binding energy; partitioning the binding free energy into enthalpic and entropic components facilitates understanding the forces underlying molecular recognition. We have used titration calorimetry to quantify the energetic consequences of varying acyl chain length and removing the 3'-phosphate group of CoA on the interactions of Nmt1p with acyl-CoA ligands. We have also explored the effects of varying acyl chain length on peptide binding to Nmt1p-acyl-CoA complexes. These studies provide insights about how this acyltransferase selects its substrates and about its catalytic mechanism.

We reported previously that two 32P-labeled photoactivatable octapeptide substrates are able to specifically bind to a Nmt1p-S(2-oxo)pentadecyl-CoA complex but not to the apoenzyme (10). This finding provided physical evidence supporting the ordered Bi Bi reaction mechanism inferred from enzyme kinetic and fluorescence binding studies (9, 33). Our energetic characterization of the formation of a ternary complex of Nmt1p with a peptide substrate and this alkyl(thioether)-CoA provides definitive evidence that the acyltransferase is capable of binding peptide, acyl chain, and CoA simultaneously. Our inability to detect formation of a Nmt1p-peptide binary complex adds to a body of evidence supporting ordered ligation (see Refs. 5, 9, 10, and 33), and clearly indicates strong heterotropic cooperativity between the enzyme’s acyl-CoA and peptide binding sites. This cooperativity may reflect a conformational change by the enzyme upon acyl-CoA ligation, generating a
high affinity binding site for peptide substrates. This is consistent with the large negative $\Delta \Delta G$ of acyl-CoA binding (Table I). Although the kinetic mechanism has a preferred order, it is likely that at least some peptide substrates are able to bind to apo-Nmtlp, albeit with much lower affinity than to the binary complex. Future thermodynamic studies may define this affinity and thus the extent of ordering, or the energetic value of the cooperative interaction between the sites.

A long standing question concerning the regulation of protein $N$-myristoylation is how Nmtlp is able to catalyze the transfer of myristate with high selectivity in vivo, given the relative rarity of this acyl-CoA compared to palmitoyl-CoA. The affinities of Nmtlp for myristoyl-CoA, palmitoyl-CoA, and lauroyl-CoA (Table I) provide little specificity for binding one acyl-CoA rather than another. This suggests that for the enzyme to avoid catalyzing the transfer of other acyl chains or being competitively inhibited by them, there must be some form of functional segregation of the acyl-CoA pools so that Nmtlp is not exposed to the bulk cellular concentrations of other acyl-CoAs. One possible mechanism of segregation is suggested by the results of recent measurements by Pietsch and McLaughlin (34) of the free energy of association of acylated peptides and myristoyl-CoA with model membrane systems. They demonstrated that the free energy of association of acyleptides with uncharged membranes depends only on the length of the acyl chain, with a contribution of 0.8 kcal/mol/methylene, and that the affinity is independent of the chemical nature of the polar head of the amphipathic molecule for several myristoylpeptides and myristoyl-CoA. They concluded that myristate is used with high selectivity against longer acyl chains for protein $N$-myristoylation because the hydrophobicity of this chain length may promote reversible membrane association, while longer chains likely serve as permanent membrane anchors. Likewise, myristoyl-CoA is predicted to associate with membranes with a 1.6 kcal/mol less favorable free energy than palmitoyl-CoA, equivalent to approximately 15-fold greater partitioning of palmitoyl-CoA into membranes at 300 K. This could provide an additional level of selectivity for the enzyme so that it could avoid binding to the more prevalent palmitoyl-CoA. It is also possible that cellular acyl-CoA pools are bound by other proteins, and that delivery of the proper length acyl-CoA to Nmtlp is mediated by protein-protein interactions and the relative affinity of carrier proteins and Nmtlp for each acyl-CoA.

Although there is little overall variation in binding affinity among lauroyl-CoA, myristoyl-CoA, and palmitoyl-CoA, there is clearly a large difference in the enthalpic and entropic components of binding acyl-CoA of different chain lengths, which is also reflected in the reduced catalytic activity when palmitoyl-CoA is the first ligand bound. These findings indicate that Nmtlp is able to perceive a 2-carbon deviation in chain length. Given the large number of functional groups and amount of hydrophobic surface area in CoA that is potentially available for interaction with Nmtlp, it is remarkable that such seemingly modest changes in the acyl chain would have such a large impact on the interaction of acyl-CoA with the aminoglycine and subsequently the binary complex or peptide substrates. Table V shows that a 2-carbon deviation in either direction from the optimal C14:0 reduces the favorable enthalpy of binding by approximately 9 kcal/mol, with a nearly equal compensating change in the entropic contribution to binding free energy, meaning that the Nmtlp-lauroyl-CoA and Nmtlp-palmitoyl-CoA binary complexes exist in a much broader and shallower potential energy well than myristoyl-CoA. Such a large difference in the energetics of ligation is unlikely to be the result of simply adding or subtracting two methylene units without otherwise changing the conformation of bound myristoyl-CoA, but rather a significant difference in the conformation of a binary complex containing myristoyl-CoA and an off-length acyl-CoA. This result initially raised the possibility that when Nmtlp binds lauroyl-CoA or palmitoyl-CoA, it cannot undergo the putative conformational change necessary for the generation of a functional peptide binding site. However, the observed high affinity binding of a peptide substrate to the Nmtlp-S-(2-oxo)heptadecyl-CoA binary complex refuted this notion. Instead, the altered energetics of binding the off-length acyl-CoAs seems to allow generation of a high affinity peptide binding site, but a less catalytically productive active site, as indicated by the reduced kinetic parameters obtained with palmitoyl-CoA compared to myristoyl-CoA. This may result from relatively small differences in the backbone conformation of the protein and acyl-CoA, but significant alterations in the specific contacts between macromolecule and ligand, resulting in failure to properly position catalytically important functional groups. Thus, a model for Nmtlp's chain length specificity is suggested in which C14:0, but not C12:0 or C16:0, allows formation of a precise set of "tight" electrostatic contacts involving the CoA moiety, resulting in much greater immobilization of this ligand and the interacting protein residues, and giving rise to the observed favorable enthalpy and unfavorable entropy, while making the key intermolecular contacts that provide catalytic destabilization. The inability of CoA to form the requisite contacts with Nmtlp when it is linked to shorter or longer acyl chains may result in either unpaired polar functional groups or formation of intramolecular contacts in the CoA.

Five protein structures have been reported to date with bound CoA (35-40). Each structure includes a large number of specific individual contacts between CoA and the protein's functional groups, suggesting that a great deal of binding enthalpy is available for forming these complexes. Molecular recognition of CoA shares several common features among these five proteins, including a similar bent conformation, certain hydrogen bonds between the adenine group and protein atoms, and hydrophobic and pi-pi interactions involving the adenine and the pantetheine and cysteamine peptide bonds. However, there are significant differences, including the relative importance of electrostatic interactions with the three phosphate groups of CoA, internal hydrogen bonds, and the pattern of hydrogen bonding to polar groups outside the purine ring. Thus, these proteins have evolved overlapping yet distinct ways of making use of the great potential binding energy of CoA. This is particularly noticeable in comparing chloramphenicol acetyltransferase type III (36) and the catalytic subunit of dihydrolipoyl transacetylase (E2p) (39), two proteins which share significant primary sequence homology and identical secondary structure topology but recognize CoA by different sets of interactions (40-42). In addition, E2p has been crystallized in a second, apparently nonproductive complex with CoA in which the adenine head group binds in a fashion identical to that found within the productive complex, but the pantetheine arm is in an entirely different conformation, away from the active site with several internal hydrogen bonds and fewer contacts with the enzyme (40).

The structural basis of chain length specificity has been addressed in two protein structures containing bound acyl-CoA (37, 38, 43). One of these proteins is a carrier having no catalytic functions (acyl-CoA binding protein, ACBP) while the other must not only bind acyl-CoA but also catalyze the dehydrogenation of the C2-C3 bond in C6-C12 acyl-CoAs (medium chain acyl-CoA dehydrogenase) (44). Palmitoyl-CoA binds to ACBP with a 180° turn in the pantetheine arm of its CoA moiety so that the cysteamine and proximal part of the acyl chain make hydrophobic contacts with the remainder of the CoA molecule (36). Kragelund et al. (38) suggest that the acyl
chain length specificity of this 89-residue protein is based on the hydrocarbon chain being long enough to fold into such a conformation but short enough so that its ω terminus does not protrude beyond ACBP's hydrophobic core. As noted in the Introduction, studies of the activities of myristoyl-CoA analogs with single cis or trans double bonds or single triple bonds placed at various positions along methylene chain indicate that the C14-CoA binds to Nmtlp with a bend located in the vicinity of C5-C6 (carboxyl carbon = C1; 12, 18). This is similar to the conformation of palmitoyl-CoA bound to ACBP. Moreover, introducing a bend in the vicinity of C5-C6 of palmitoyl-CoA (e.g. as with 2,5-hexadecanoyl-CoA) yields a C16-CoA which is almost as active a substrate for Nmtlp as myristoyl-CoA (18), raising the possibility that Nmtlp and ACBP may both measure the length between carboxyl carbon and a principal bend at C5-C6 (although ACBP is more tolerant of variations in the overall number of methylenes, binding C14-C22-CoAs with high affinity, cf. Ref. 45).

Medium chain acyl-CoA dehydrogenase binds acyl-CoA’s in a drastically different conformation than ACBP; in medium chain acyl-CoA dehydrogenase the pantetheine arm and acyl chain extend away from the adenosine head (37, 43). Acyl chains having more than 12 carbons atoms are poor substrates for medium chain acyl-CoA dehydrogenase (44). This specificity is represented in the crystal structure by a tightly defined binding pocket which cannot accommodate the longer acyl chains without significant distortion of the surrounding protein backbone or movement of the CoA (43). However, medium chain acyl-CoA dehydrogenase binds alkyl(thioether)-CoA analogs containing chain lengths of C2-C16 with linearly increasing favorable binding free energy (46), indicating a segregation of binding specificity and catalytic activity similar to that observed with Nmtlp. The sensitivity of Nmtlp to sterically bulk at the ω terminus of the acyl chain (12, 19) suggests that like medium chain acyl-CoA dehydrogenase, myristoyl-CoA:protein N-myristoyltransferase may sense chain length by binding the acyl chain in an apolar pocket of limited depth.

Fierke and Jencks (47) have suggested that CoA consists of two functional domains, the pantetheine-cysteamine arm, which makes contacts responsible for transition state stabilization, and the adenosine-3’-monophosphate-5’-diphosphate moiety, which provides binding energy necessary to position the catalytically important portion of the molecule. Their theory is supported by structural studies of E2p where a side chain essential for stabilizing a model transition state is ideally positioned by an H-bond involving the pantetheine amide and by structural studies of a nonproductive E2p-CoA complex where the adenine head group does not change its position while the pantetheine arm does (39, 40). The formulation of Fierke and Jencks (47) is consistent with a model for the chain length specificity of Nmtlp (and medium chain acyl-CoA dehydrogenase) in which acyl-CoA’s outside the catalytic specificity of the enzyme are able to bind with high affinity owing to the very favorable binding of the adenosine head group, but the pantetheine arm is displaced from its catalytically relevant position due to the improper length of the acyl chain. In the case of Nmtlp, the observed formation of a high affinity peptide binding site with palmitoyl-CoA indicates that the energy of binding the off-length acyl-CoA is sufficient to induce the cooperative transition which allows peptide binding but not generation of a highly efficient active site. The subsequent binding of GAAPSKIV-NH₂ with higher affinity to the Nmtlp-S(2-oxo) heptadecyl-CoA complex than to the myristoyl-CoA analog-Nmtlp complex may reflect this poorly formed active site: electrostatic strain, facilitating catalysis in the productive ternary Nmtlp-myristoyl-CoA-peptide complex, may not be present with the longer acyl chain.

Our thermodynamic studies of Nmtlp reveal that the effect of removing the CoA 3’-phosphate group from myristoyl-CoA is similar to the effect of a 2-carbon change in acyl chain length: i.e. an enthalpy dominated reduction in binding affinity. This suggests that the 3’-phosphate moiety of CoA participates in electrostatic interactions which are important for binding to Nmtlp as is the case with citrate synthase (35), chloramphenicol acetyltransferase (42), acetyl-CoA binding protein (38, 45), and dihydrolipoyl transacetylase (40). However, removing the 3’-phosphate from myristoyl-CoA has little effect on Nmtlp’s catalytic efficiency just as with: (i) medium chain acyl-CoA dehydrogenase, in which the 3’-phosphate group is solvent exposed and does not appear to contribute to either binding (37) or to catalysis (48); and (ii) chloramphenicol acetyltransferase, where the binding defect of the 3’-dephospho derivative of acetyl-CoA is not accompanied by a large diminution in Vₚmax (42). In addition, the reduction in catalytic efficiency of Nmtlp utilizing myristoyl(3’-dephospho)-CoA relative to myristoyl-AC is much smaller than the defect resulting from a 2-carbon increase in chain length. The greater ΔΔG°', with smaller ΔΔH° and ΔΔS° components, observed by removing the 3’-phosphate compared to increasing chain length suggests that eliminating the 3’-phosphate removes a source of free energy that is different from that disrupted by altering the chain length. Taken together, these findings indicate that the 3’-phosphate group of myristoyl-CoA may contribute binding free energy but little catalytic destabilization and that it is not the interactions of the 3’-phosphate which are primarily disrupted when palmitoyl-CoA is substituted for myristoyl-CoA. This latter possibility can be tested directly by examining the thermodynamic and catalytic effects of removing the 3’-phosphate moiety from acyl-CoAs of varying chain lengths.

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REFERENCES