Biochemical Studies of Saccharomyces cerevisiae Myristoyl-coenzyme A:Protein N-Myristoyltransferase Mutants*

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Litao Zhang, Emily Jackson-Machelski, and Jeffrey I. Gordon‡

From the Department of Molecular Biology and Pharmacology, Washington University School of Medicine, St. Louis, Missouri 63110

Saccharomyces cerevisiae myristoyl-CoA protein N-myristoyltransferase (Nmt1p) is an essential 455-residue, monomeric enzyme that catalyzes the transfer of myristate from myristoyl-CoA to the NH2-terminal Gly residue of cellular proteins. Nmt1p has an ordered Bi Bi reaction mechanism with binding of myristoyl-CoA occurring before binding of peptide substrates. To define residues important for function, the polymerase chain reaction was used to generate random mutations in the NMT1 gene. A colony color sectoring assay was used to screen a library of 52,000 transformants for nmt1 alleles encoding enzymes with reduced activity. nmt1 alleles were identified that produced temperature-sensitive (ts) growth arrest due to substitutions affecting eight residues conserved in orthologous Nmts: Asn102, Ala202, Cys217, Ser328, Val395, Asn404, Leu420, and Asn426. Ala202 → Thr, Cys217 → Arg, Ser328 → Pro, Asn404 → Tyr, and Asn426 → Ile produced the most severe ts phenotype. Their effects on the functional properties of the enzyme’s myristoyl-CoA and peptide binding sites were defined by purifying each mutant from Escherichia coli and conducting in vitro kinetic analyses with acyl-CoA and peptide substrates and with two competitive inhibitors: S-(2-oxo)pentadecyl-CoA, a nonhydrolizable myristoyl-CoA analog, and SC-58272, a peptidomimetic derived from the NH2-terminal sequence of an Nmt1p substrate (ADP-ribosylation factor-2, Arf2p). None of the substitutions affect the enzyme’s acyl chain length selectivity. When compared with wild type Nmt1p, Cys217 → Arg produces 3- and 6-fold increases in K_i for SC-58272 at 24 and 37 °C but no change in K_i for S-(2-oxo)pentadecyl-CoA, indicating that the substitution selectively affects Nmt1p’s peptide binding site. Asn426 → Ile selectively perturbs the myristoyl-CoA binding site, resulting in the most pronounced reduction in affinity for S-(2-oxo)pentadecyl-CoA (12- and 20-fold). Ala202 → Thr, which conserves the most severe ts phenotype, provides an example of a substitution that affects both sites, producing 3- and 6-fold increases in the K_i for S-(2-oxo)pentadecyl-CoA and 6- and 9-fold increases in the K_i for SC-58272 at 24 and 37 °C. An N-myristoylation-dependent change in the electrophoretic mobility of Arf1p was used to assay the effects of the mutants on cellular levels of protein N-myristoylation under a variety of growth conditions. The ts growth arrest produced by nmt1 alleles correlates with a reduction in myristoyl-Arf1p to 50% of total cellular Arf1p.

Myristoyl-CoA protein N-myristoyltransferase (Nmt1, EC 2.1.3.97) catalyzes the co-translational, covalent attachment of myristate (C14:0) to the NH2-terminal glycine of eukaryotic cellular and viral proteins (1). Nmt is a potential target for antiviral (2–4), antifungal (5–7), and antineoplastic therapy (8–10).

Saccharomyces cerevisiae Nmt1p has been used as a model for examining the enzyme’s kinetic mechanism, substrate specificities, and biological functions. The NMT1 gene is essential for vegetative growth (11, 12). It encodes a 455-residue cytoplasmic protein (13). Nmt1p has no known co-factor requirements (14). The enzyme’s mechanism is ordered Bi Bi (15). Apoenzyme binds myristoyl-CoA, forming a high affinity myristoyl-CoA:Nmt1p complex (K_d = 15 nM; Ref. 16). There is heterotropic cooperativity between the enzyme’s acyl-CoA and peptide binding sites; formation of the binary complex allows synthesis of a peptide binding site (16). After assembly of a ternary myristoyl-CoA:Nmt1p:peptide complex, C14:0 is transferred from CoA to the peptide substrate, and the products are released (CoA followed by myristoylpeptide). Nmt1p’s acyl-CoA and peptide specificities have been defined in vitro using purified enzyme, >300 fatty acid analogs with systematic alterations in chain length, polarity, conformation, and steric bulk, plus >100 octapeptides representing variations in the NH2-terminal sequences of known N-myristoylproteins (reviewed in Ref. 1).

The primary structures of six orthologous Nmts have been determined (11, 17–19). The 450–529-residue proteins contain 105 absolutely conserved amino acids. Searches of current protein data bases with these primary structures has failed to disclose any discernible homology to other sequence entries. Only a modest amount of information exists about Nmt1p’s structure/activity relationships. Deletion analyses suggest that the minimal domain required for myristoyltransferase activity spans Ile69–Phe369 through Gly451–Leu455 (20). Two distinct genetic selections have identified two residues important for function. An allele with a single amino acid substitution, Gly451 → Asp (nmt1–451D) was recovered during a screen for mutations that cause temperature-sensitive (ts) fatty acid auxotrophy (12, 21). nmt1–451D produces growth arrest at various stages of the cell cycle 1 h after shifting from 24 to 37 °C and

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‡ To whom correspondence should be addressed: Dept. of Molecular Biology and Pharmacology, Box 8103, Washington University School of Medicine, 660 S. Euclid Ave., St. Louis, MO 63110. Tel.: 314-362-7243; Fax: 314-362-7058; E-mail: ggordon@pharmdec.wustl.edu.

1 The abbreviations used are: Nmt, myristoyl-CoA:protein N-myristoyltransferase; NMT1, S. cerevisiae myristoyl-CoA:protein N-myristoyltransferase gene; Nmt1p, the protein product of NMT1; YPD, yeast/peptone/dextrose medium; SC, synthetic complete medium; 5-FOA, 5-fluoro-orotic acid; ORF, open reading frame; ts, temperature-sensitive; ITC, isothermal titration calorimetry; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis.
produces lethality after an 8–12 h incubation at nonpermissive temperatures (12, 22). The growth arrest and lethality can be rescued at 37 °C by adding myristate but not shorter or longer chain saturated fatty acids. Another allele with a single Leu49 → Pro substitution (nmt1–99p) is associated with undermyristoylation of Gap1p, thereby reducing this α subunit’s affinity for the βγ polypeptides of a heterotrimeric G protein involved in the mating response (23, 24). The resulting free βγ subunits produce constitutive activation of the mating pathway and growth arrest. Leu49 → Pro results in less global alterations in protein N-myristoylation than does Gly453 → Asp (24). Gly453 and Leu49 are conserved in all six known NmTs. Introduction of the Gly → Asp substitution in Cryptococcus neoformans and Candida albicans Nmts also produces temperature-sensitive growth arrest and myristic acid auxotrophy in these fungal pathogens (5, 6).

Site-directed mutagenesis has been used to replace each of human Nmt’s four conserved His residues with Asn and each of its two conserved Cys residues with Ser (25). Site-directed mutagenesis of absolutely conserved amino acids can be very useful for identifying residues critical for substrate recognition, binding, and/or catalysis. In the case of Nmt, designing such experiments is hindered by the high percentage of conserved amino acids, by the lack of homology to other proteins, and by the lack of a rapid screening assay for disabling mutations. In this report, we describe how random PCR mutagenesis and an in vivo screen can be used to recognize mutations of conserved residues in Nmt1p that affect the functional properties of its myristoyl-CoA and/or peptide binding sites.

EXPERIMENTAL PROCEDURES

Yeast Strains and Media

pBB110 is a 2 µ YEp plasmid containing NMT1 (11). The isogenic strains YB332 (MATα NMT1 ura3–52 his3Δ200 ade2–101 lys2–801 leu2–3,112) and YB336 (MATα nmt1–181 ura3–52 his3Δ200 ade2–101 lys2–801 leu2–3,112) were described by Johnson et al. (24). YB105 (MATα nmt1–181 his3Δ200 ade2–101 ade3 leu2–801 leu2–3,112 trp1Δ901, pBB110) was obtained from a cross between YB133 (MATα nmt1–181 his3Δ200 ade2–101 lys2–801 trplΔ901 can1 leu2–pRY181, pBB110) and YB428 (MATα nmt1–181 ura3–52 his3Δ200 ade2–101 ade3 leu2 trplΔ901). YB523 was obtained from YB510 by replacing pBB110 with pBB290, a high copy 2 µ NMT1 ADE3 URA3 plasmid derived from pTSV31A (kindly supplied by Alan Bender, Indiana University, Bloomington, IN). Strains were incubated at 24–40 °C onYPD (1% yeast extract, 2% peptone, 2% dextrose/agar plates with or without myristate (500 µM; NuChek-Prep). Brij 58 (1% w/v; Sigma) was included in media containing myristate. Synthetic complete medium (SC) minus leucine (23, 24) was used for selection of plasmids with tagging Wild Type and Mutant Nmts—

Random PCR Mutagenesis and Colony Color Sectoring Assay

The complete open reading frame (ORF) of NMT1 plus three overlapping subdomains of the ORF were amplified by PCR (Fig. 1A). PCR was performed using the conditions described in Fig. 1B plus the following thermocycling protocol: 94 °C for 1 min (denaturation), 55 °C for 2 min (annealing), and 72 °C for 3 min (extension) for a total of 30 cycles. Each PCR fragment was used to replace a segment of the NMT1 ORF by in vivo recombination with a gapped plasmid (26). Replacement involved the following steps: (i) The PCR product was purified by agarose gel electrophoresis and the GeneClean kit (BIO 101; ii) pBB361 (NMT1 LEU2) was digested with either NcoI and BglII, NcoI and AvaII, SphI and MluI, or BsuI alone (Fig. 1A), and the linearized, gapped plasmids were purified as in the first step; (iii) C. albicans strain YB523 (nmt1Δ3, ura3–52, ade2–101 ade3 leu2–3, pBB290) was co-transformed with the PCR fragment and gapped plasmids (27); (iv) colonies were plated on SC minus leucine and incubated for 3 days at 30 °C.

A colony color sectoring assay was performed as outlined in Fig. 2. Leucine prototrophs were replated on YPD and incubated at 30 °C for 5 days. Individual red colonies were restreaked on YPD and incubated for 5 days at 30 °C. Colonies that continued to exhibit a nonsectoring (red) phenotype due to retention of the 2 µ pBB290 expression plasmid (ADE3 URA3) were plated onto SC/5-FOA minus leucine and incubated at 24 °C and 35–40 °C to identify nmt1 mutations that produced a ts growth phenotype.

Total cellular nucleic acids were extracted (28) from ts isolates and used to transform Escherichia coli strain JS5. Plasmids were purified from bacterial colonies that grew on Luria broth (LB) supplemented with kanamycin (50 µg/ml). The nucleotide sequence of each plasmid’s nmt1 insert was determined using a panel of 14 oligonucleotide primers (11, 12), a DyeDeoxy terminator cycle sequencing kit, and a model 373 Automated DNA Sequencer (Applied Biosystems). The primers cover both strands of NMT1’s ORF at intervals of 200 base pairs. 400–500 base pairs of sequence were obtained per primer, allowing 200–300 base pair overlaps between each primer-driven reaction.

Site-directed Mutagenesis

Twenty-three of the ts nmt1 alleles contained more than one amino acid substitution. These multiply mutated protein sequences were aligned with one another and with the sequences of orthologous Nmts using the algorithm included in GeneWorks (version 2.4). The alignments identified eight residues that were conserved in five or six of the six known Nmts and mutated in the ts nmt1 alleles. Site-directed mutagenesis was used to introduce each of the eight mutations (individually) into NMT1’s ORF: Asp165 → Thr (AAC → ACC); Leu71 → Ser (TTG → TCG); Lys389 → Ile (AAA → ATA); Val395 → Asp (GTT → GAT); Leu453 → Ser (TTG → TCG); Phe413 → Ser (TTC → TCC); Asp477 → Val (GAC → GTC); and Leu695 → Ser (TTG → TCG). The entire ORF of each site-directed mutant nmt1 allele was sequenced to confirm that only the desired nucleotide substitution was present.

Measurement of Steady-state Levels of Wild Type and Mutant Nmts in S. cerevisiae

pBB290 (see above) was removed from strain YB523 (nmt1Δ3) and replaced (29) with low copy centromeric pRS315-derived plasmids (30) that contained a LEU2 selectable marker, kanamycin and ampicillin resistance genes, and a mutant nmt1 ORF under the control of the NMT1 promoter (31). These plasmids include pBB391 (nmt1–1027f; pBB387 (nmt1–2027t); pBB386 (nmt1–217r); pBB380 (nmt1–395p); pBB391 (nmt1–40Y); pBB393 (nmt1–428v); pBB382 (nmt1–451d) and an NMT1 control (pBB361).

One hundred-milliliter cultures of the various transformants were grown at 24 °C in YPD to an A600 = 0.8. Twenty-five-milliliter aliquots were removed and incubated at 24 or 37 °C for 2 h. Cells were subsequently harvested by centrifugation at 1,600 × g for 10 min at 4 °C and washed twice with 15 ml of phosphate-buffered saline. The cell pellet was resuspended in 0.5 ml of lysis buffer (2% SDS, 80 mM Tris pH 6.8, 200 µM β-mercaptoethanol; 2 µM leupeptin; all protease inhibitors from Boehringer Mannheim). Cells were disrupted by vortexing with 0.5 ml of 425–600-µm glass beads (Sigma; vortexing was in four cycles, 1 min/cycle). The mixture was boiled for 10 min. Cellular debris were removed by centrifugation at 10,000 × g for 5 min. The protein concentration in the cleared lysates was determined using the BCA assay kit (Pierce). Equal masses of protein from each sample (100 µg) were resolved, denatured, fractionated by SDS-PAGE (32), and transferred to polyvinylidene difluoride membranes (Amersham Corp.). Western blots were probed with a previously characterized rabbit anti-C. albicans Nmt sera that recognizes the orthologous S. cerevisiae acetyltransferase (Ref. 19; diluted 1:5000 in Blotto). Antibody-antigen complexes were visualized using an enhanced chemiluminescence (ECL) kit and the protocol recommended by its manufacturer (Amersham).

Purification of Nmts from E. coli

Generation of Bacterial Expression Vectors Encoding Polyhistidine-tagged Wild Type and Mutant Nmts—

pBB376 (kindly supplied by Jennifer Lodge, Washington University) was derived from pMON22310 (origin of replication, pBB32; selectable marker, streptomycin resistance gene). pBB376 contains the NMT1 ORF with an NH2-terminal tag of six histidine residues (6XHis). Expression of 6XHis-Nmt1p is controlled by the isopropyl-β-D-thiogalactopyranoside-inducible Ptc promoter. Appropriate restriction enzymes were used to place a fragment of an nmt1 ORF containing an amino acid substitution into similarly digested pBB376, yielding the following plasmids: pBB390 (6XHis-nmt2027p); pBB389 (6XHis-nmt217r); pBB357 (6XHis-nmt328p); pBB359 (6XHis-nmt40Y); pBB388 (6XHis-nmt426l); and pBB377
(6XHis-nmt451Dp). The ORFs contained in each 6XHis-nmt1p expression plasmid were grown in LB/streptomycin (30 µg/ml) and 100 µM isopropyl-β-D-thiogalactopyranoside at 24 °C for 12 h until they reached an A600 = 1. Bacteria were harvested by centrifugation at 5,000 × g for 20 min at 4 °C and resuspended in 50 ml of buffer A (0.3 M NaCl, 5 mM β-mercaptoethanol, 1 mM Pefabloc SC, 2 mM leupeptin, 2 mM pepstatin, 25 mM sodium phosphate buffer, pH 7.0). Cells were lysed with French press at 3,200 p.s.i. The lysates were centrifuged at 20,000 × g for 30 min at 4 °C. Each supernatant was collected and mixed with 5 ml of Ni2+-NTA agarose (Qiagen; the resin was washed three times with buffer A before use). The resulting supernatant was incubated on ice for 2 h with intermittent shaking and then subjected to centrifugation at 3,000 × g for 5 min at 4 °C. The supernatant was removed and discarded. The Ni2+-NTA agarose was washed four times with ice-cold buffer A (30 ml/wash) and loaded into an empty 10-ml Poly-Prep column (Bio-Rad). The column was washed with 10 ml of buffer A containing 40 mM imidazole. Nmt activity was eluted by sequentially washing the column with 3 ml of buffer A containing 60 mM, 80 mM, 100 mM, 120 mM, and finally 150 mM imidazole. Wild type and mutant enzymes eluted with buffer A, 120 mM imidazole and were used on the day of their purification for kinetic studies (see below). Wild type 6XHis-Nmt1p and each mutant 6XHis-nmt1p were purified on at least three separate occasions. The purity of each preparation was verified by SDS-PAGE followed by Coomassie staining.

Nmt1p without an NH2-terminal 6XHis tag was also expressed in E. coli and purified to apparent homogeneity using a protocol described by Rudnick et al. (33).

Kinetic Studies of Wild Type and Mutant Nmtns

Assessment of Peptide Substrate Specificity—A coupled in vitro Nmt assay system was employed (33, 34). [3H]Myristoyl-CoA was generated using double reciprocal plots. A series of nmt1 alleles encoding mutant enzymes with reduced activity. This assay (Fig. 2) takes advantage of the fact that two mutations within the S. cerevisiae purine nucleotide biosynthetic pathway, when present in different combinations, give rise to colonies with different colors. Mutations in ADE2 cause accumulation of the chromophore phosphoribosylaminomidazole, resulting in red colonies. Strains with ADE3 mutations do not accumulate the chromophore and grow as white colonies. ADE2 mutations also affect an enzymatic step upstream of Ade2p. Therefore, ade2 ade3 strains grow as white colonies. Introduction of a low copy centromeric plasmid containing NMT1 and ADE3 into an ade2 ade3 nmt1Δ strain produces cells that form red colonies (Fig. 2). Since NMT1 is essential, if these cells lose this plasmid they die. Viable colonies containing the epimosaic copy of NMT1 and ADE3 remain red. If a second episome is introduced containing products from the PCR mutagenesis, one of two color phenotypes will be observed. If the mutation does not reduce Nmt1p's activity below the point required to acylate essential cellular N-myristoyltransferases at levels compatible with viability, then there will be no selective pressure to retain the plasmid; i.e. during the course of cell division, 5–20% of newly formed daughter cells will lose the NMT1 ADE3 plasmid, and colonies will appear as red circles containing white sectors (sectoring phenotype). If a mutation inactivates Nmt1p, then the colony will be forced to retain the NMT1 ADE3 plasmid and will

[3] Further details concerning this gel shift assay will be published elsewhere, including its validation using isogenic strains with NMT1 or nmt1–451D plus various combinations of wild type or null alleles of ARF1 and ARF2 (Lodge, J., Jackson-McAuleel, E., Devadas, B., Kishore, N., Freeman, S., McWether, C., Sikorski, J., and Gordon, J., (1997) Microbiology, in press).
remain red (nonsectoring phenotype). Replica plating nonsectoring red colonies onto 5-FOA-containing plates will force the nmt1 \( D \) ade2 ade3 leu2 \( D \) host strain to remove the URA3-containing \( NMT1\text{ADE3} \) episome, thereby revealing the phenotype produced by the remaining \( LEU2 \) episome with its mutant nmt1 allele (Fig. 2).

Three overlapping regions of \( NMT1 \)‘s ORF (Met1\(^{33} \) Leu345, Ala202 \( \rightarrow \) Asn\(^{37} \), and Lys\(^{397} \) \( \rightarrow \) Leu\(^{455} \)) or the entire ORF were replaced by DNA fragments generated using standard conditions for the polymerase chain reaction or conditions designed to increase the error rate of \( Taq \) polymerase. A total of 52,000 transformants were screened; 2122 transformants (4%) had a nonsectoring phenotype, 1093 of the nonsectoring colonies (51%) had a lethal phenotype when incubated at 24 °C on synthetic complete media containing 5-FOA but lacking leucine, and 35 nonsectoring colonies had a ts phenotype based on differences in their growth in this media at 24 and 35 or 40 °C (Fig. 1B).

Polymerase chain reactions 1–3, performed under standard conditions, produced 47 nonsectoring transformants with a lethal phenotype and two with a ts phenotype (Fig. 1A and B). Plasmids were recovered from 38 of the transformants with a lethal phenotype: 25 of the 38 did not contain any \( NMT1 \) sequences and presumably arose from self-ligation of the

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**Fig. 1. Summary of PCR mutagenesis of \( NMT1 \).** A, the locations of unique restriction sites in the \( NMT1 \) ORF are shown at the top. Primers A–E were used for amplifying all or portions of \( NMT1 \) in eight separate polymerase chain reactions. Regions of \( NMT1 \) were replaced by the portions of the PCR products highlighted by shadowed boxes. Replacement was accomplished by co-transformation of \( S.\ cerisevisiae \) with the PCR fragment and a gapped plasmid with homology to both ends of the fragment (homology regions are indicated by lines flanking the shadowed boxes). In vivo recombination repaired the gap with the PCR fragment (see Fig. 2). B, summary of phenotypic analysis of transformants.

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**A** Random PCR mutagenesis

**B** Results

| PCR No. | PCR conditions | No. of colonies obtained from co-transformation | No. of nonsectoring colonies | Phenotype of nonsectoring colonies on SC-leu + 5-FOA |
|---------|----------------|-----------------------------------------------|-----------------------------|--------------------------------------------------|
| 1       | Standard\(^a\) | 10,000                                       | 180                         | 2/20                                             |
| 2       | Standard\(^a\) | 5,000                                        | 108                         | 0/11                                            |
| 3       | Standard\(^a\) | 4,000                                        | 90                          | 0/16                                            |
| 4       | Modified\(^b\) | 10,000                                       | 504                         | 21/245                                          |
| 5       | Modified\(^b\) | 8,000                                        | 400                         | 1/230                                           |
| 6       | Modified\(^b\) | 10,000                                       | 440                         | 0/250                                           |
| 7       | Standard\(^a\) | 2,000                                        | 172                         | 5/151                                           |
| 8       | Modified\(^c\) | 3,000                                        | 228                         | 6/170                                           |
|         |                 | 52,000                                       | 2,122                       | 35/1,093                                        |

\(^a\) [MgCl\(_2\)]=1.5 mM, [Tris, pH=8.3]=10 mM, [dNTP]=0.2 mM, [dBB361(NMT1)]=2 ng/100 \( \mu \)L, [primer]=3 \( \mu \)M, [AmpliTaq polymerase]=2.5 Units/100 \( \mu \)L.

\(^b\) same as \(^a\) except [MgCl\(_2\)]=7 mM, [dCTP]=[dTTP]=1 mM, [MgCl\(_2\)]=0.5 mM, [AmpliTaq polymerase]=5 Units/100 \( \mu \)L.

\(^c\) same as \(^a\) except [MgCl\(_2\)]=7 mM, [AmpliTaq polymerase]=5 Units/100 \( \mu \)L.
Mutagenesis of S. cerevisiae Nmt1p

Alignments of the 24 multiply substituted protein sequences with the six orthologous Nmts revealed a total of 11 substitutions that affected eight conserved residues. Site-directed mutagenesis was used to generate eight nmt1 alleles, each containing a single substitution: Asn102 → Thr, Leu171 → Ser, Lys389 → Ile, Val408 → Asp, Leu408 → Ser, Phe413 → Ser, Asp417 → Val, and Leu420 → Ser (Fig. 3B). (In the three instances where a conserved residue was replaced by more than one amino acid (Lys405, Val405, and Asp417), we selected the replacement that was more frequently encountered in our library of ts nmt1 alleles.)

The eight nmt1 alleles engineered by site-directed mutagenesis were tested to determine whether they conferred a ts phenotype. An nmt1Δ strain, with a low copy centromeric plasmid containing NMT1 (wild type control), nmt1–451D (ts mutant control, see the Introduction), or one of the eight nmt1 alleles, was incubated at 24, 30, 33, 35, 37, and 40 °C on YPDagar. The NMT1 episome supported similar rates of growth over this broad temperature range. An episome with the previously characterized nmt1–451D allele resulted in growth arrest at 40 °C. Asn102 → Thr (nmt1–102T), Val405 → Asp (nmt1–395D), and Leu420 → Ser (nmt1–420S) also produced complete growth arrest at 40 °C (Fig. 4A). In contrast, plasmids containing nmt1 alleles with Leu171 → Ser, Lys405 → Ile, Leu408 → Ser, Phe413 → Ser, or Asp417 → Ser substitutions had no detectable effect on growth, even at 40 °C (data not shown).

Fig. 4A also shows the phenotypes produced by the five ts nmt1 alleles obtained directly from the colony color sectoring screen and found to contain single amino acid substitutions. Growth arrest occurred at 30 °C (Ala202 → Thr, nmt1–202T), 35 °C (Ser238 → Pro, nmt1–328P, and Asn426 → Ile, nmt1–426I), 37 °C (Asn404 → Tyr, nmt1–404Y) or 39 °C (Cys317 → Arg, nmt1–217R).

The remaining 1046 nonsectoring colonies with lethal phenotypes were generated by using modified PCR conditions designed to increase the error rate of Taq polymerase or by targeting the entire NMT1 ORF. There was no obvious difference in the frequency of nonsectoring colonies with lethal phenotypes when the modified PCR conditions were used to target Met3 → Leu4, Ala202 → Asn407, or Lys405 → Leu425 (reactions 4–6 in Fig. 1B). Our analyses of the 35 nonsectoring colonies with a ts phenotype and the 47 nonsectoring colonies with a lethal phenotype suggested that the majority of the nmt1 alleles that would be recovered from these 1046 colonies would contain multiple amino acid substitutions and/or stop codons. Therefore, they were not analyzed further.

Steady-state Levels of Mutant Nmts at Permissive and Nonpermissive Temperatures

nmt1Δ cells, containing episomal copies of one of the eight ts nmt1 alleles with single amino acid substitutions, were incubated in YPD broth at the permissive temperature (24 °C) until they reached mid-log phase. The temperature was then raised to 37–40 °C for 2 h. Western blots of total cellular proteins were prepared and probed with a previously characterized rabbit anti-Nmt sera (19). The steady-state levels of six of the mutant acyltransferases were equivalent to each other and to Nmt1p (Fig. 1B). The concentration of nmt1–420Sp at 24 °C was equal to Nmt1p but was 50% that of the wild type enzyme at 37–40 °C. nmt28Sp was ~3-fold less abundant than Nmt1p at permissive and nonpermissive temperatures (Fig. 4B). Thus, with the exception of nmt1–328P and nmt1–420S, differences in temperature sensitivity produced by the mutant nmt1 alleles could not be simply ascribed to differences in the steady-state levels of their protein products.
In Vitro Kinetic Studies of Purified Wild Type and Mutant Nmts

As noted above, five of the eight ts nmt1 alleles produced growth arrest on YPD at \(39^\circ\)C. To define the effects of their Ala\(^{202}\) \(\rightarrow\) Thr, Cys\(^{217}\) \(\rightarrow\) Arg, Ser\(^{328}\) \(\rightarrow\) Pro, Asn\(^{426}\) \(\rightarrow\) Ile substitutions on substrate recognition and/or catalysis, each mutant was expressed in \(E. coli\), a bacterium with no endogenous Nmt activity (37). Nmt1p and nmt451p were used as reference controls. A genetically engineered NH\(_2\)-terminal tag of six histidine residues (6XHis) allowed rapid purification of wild type and mutant enzymes by Ni\(_2\)-NTA-agarose affinity chromatography. The purification protocol produced a 100-1000-fold increase in specific activity over what was observed in unfractionated bacterial lysates and yielded apparently homogeneous preparations of each enzyme based on Coomassie staining of SDS-polyacrylamide gels (data not shown).

Effects of the Amino Acid Substitutions on the Functional Properties of Nmt’s Myristoyl-CoA Binding Site—
The acyl chain length selectivity of each mutant was examined in an in vitro assay system that used purified enzyme, unlabeled C\(_{12}:0\)-CoA, C\(_{14}:0\)-CoA, or C\(_{16}:0\)-CoA, and a fixed concentration of an extensively characterized radiolabeled octapeptide substrate of Nmt1p (GAR\(-[3H]ASVLS-NH\(_2\); Refs. 34, 38, and 39).

Mutagenesis of \(S. cerevisiae\) Nmt1p

Fig. 3. Mutations that produce ts nmt1 alleles. A, summary of nucleotide and amino acid substitutions. B, alignment of six orthologous Nmts. Absolutely conserved residues are boxed. Amino acid substitutions that produce ts growth arrest in \(S. cerevisiae\) are indicated by residue number and the substituted amino acid (e.g., 102T \(\rightarrow\) Asn). The \(C. elegans\) Nmt sequence was defined from a cDNA recovered from a Bristol N2 strain library. The protein product was expressed in, and purified from, \(E. coli\) and found to have Nmt activity (E. Anderson, J. Lodge, E. Jackson-Machelski, and J. Gordon, unpublished observations).

The acyl chain length selectivity of each mutant was examined in an in vitro assay system that used purified enzyme, unlabeled C\(_{12}:0\)-CoA, C\(_{14}:0\)-CoA, or C\(_{16}:0\)-CoA, and a fixed concentration of an extensively characterized radiolabeled octapeptide substrate of Nmt1p (GAR\(-[3H]ASVLS-NH\(_2\); Refs. 34, 38, and 39).

Neither the addition of the 6XHis tag to Nmt1p nor any of the amino acid substitutions had statistically significant effects on

\[\text{FIG. 3. Mutations that produce ts nmt1 alleles.} \]
the specificity of the acyltransferase reaction at 24 or 37 °C (range of lauroyltransferase activity = 2–15% of myristoyltransferase activity; palmitoyltransferase activity = 0–8%; n = 2 independent preparations of each mutant, each assayed on two separate occasions in triplicate).

Having established that all of the mutants “retained” their character as myristoyltransferases, we examined the effects of the amino acid substitutions on the enzyme’s affinity for S-(2-oxo)pentadecyl-CoA (40). The rationale was as follows. None of the mutants could be purified in sufficient quantity or were sufficiently stable at 24 or 37 °C to permit thermodynamic analysis of myristoyl-CoA binding by isothermal titration calorimetry (ITC), as has been done with wild type apo-Nmt1p (16). For an ordered reaction such as the one catalyzed by Nmt1p, a competitive inhibitor for the first of the two substrates, obtained at any concentration of the second substrate, is a dissociation constant for E + I. S-(2-Oxo)pentadecyl-CoA is a competitive Nmt inhibitor containing a single methylene insertion between the CoA sulfur and the fatty acyl carbonyl carbon of myristoyl-CoA. Determination of its Ki required less enzyme and could be performed more rapidly than ITC analysis of myristoyl-CoA binding. Moreover, ITC has established that purified apo-Nmt1p perceives S-(2-oxo)pentadecyl-CoA as a close approximation of myristoyl-CoA: the enthalpy of binding of the nonhydrolyzable inhibitor (~25.8 kcal/mol) is equivalent to that of myristoyl-CoA (~24.8 kcal/mol) (Ref. 16).3

The Ki for S-(2-oxo)pentadecyl-CoA was defined at 24 and 37 °C using the purified mutant Nmts, varying amounts of [3H]myristoyl-CoA, and an octapeptide substrate derived from the NH2-terminal sequence of Pp2p, a protein phosphatase that suppresses the cell lysis defect produced by a protein kinase C null allele (41, 42). The extent of the Ki change at 24 °C (Ki 5 4–7 nM) and from 15- to 300-fold at 37 °C (Ki 5 5 25 30 nM) was determined using purified enzyme, S-(2-oxo)pentadecyl-CoA, varying amounts of [3H]myristoyl-CoA, and an octapeptide substrate derived from the NH2-terminal sequence of Pp2p. The averages of duplicate determinations are shown. Duplicate values reported in Tables I–III varied by <30% (see “Experimental Procedures”).

|          | 24 °C | 37 °C |
|----------|-------|-------|
| Nmtlp    | 5     | 5     |
| 6XHis-Nmtlp | 7   | 4     |
| 6XHis-nmt202Tp | 23 | 24    |
| 6XHis-nmt217Rp | 5   | 5     |
| 6XHis-nmt328Pp | 17  | 32    |
| 6XHis-nmt404Yp | 4   | 21    |
| 6XHis-nmt426lp | 85  | 79    |
| 6XHis-nmt451Dp | 25  | 30    |

For Nmtlp, the Ki is 5 nM (Table I), a value identical to the Ki obtained from ITC (16). The addition of an NH2-terminal 6XHis tag to Nmtlp has no appreciable effect on its affinity for S-(2-oxo)pentadecyl-CoA (Ki 5 4–7 nM). The Cys217 → Arg substitution in nmt217Rp does not produce any detectable alteration in affinity for the inhibitor (5 nM at 24 and 37 °C). In contrast, Asn404 → Tyr produces no change in affinity at 24 °C but a 5-fold reduction at 37 °C (Ki 5 21 nM). The other substitutions result in 3–5-fold increases in Ki at 24 °C and 6–8-fold increases at 37 °C (Table I).

Effects of the Amino Acid Substitutions on the Functional Properties of Nmtlp’s Peptide Binding Site—As noted in the Introduction, once a binary myristoyl-CoA/Nmtlp complex forms, an allosteric transition occurs that allows formation of a functional peptide binding site. Previous in vitro and in vivo studies using myristic acid analogs have emphasized that changes in interactions between apo-Nmtlp and its acyl-CoA substrates can produce changes in the functional characteristics of the enzyme’s peptide binding site (16, 43, 44). Therefore, we surveyed myristoylpeptide production at 24 and 37 °C using Nmtlp, 6XHis-Nmtlp, each of the five mutants, saturating concentrations of [3H]myristoyl-CoA, and a panel of five octapeptide substrates. Two of the peptides had been used for defining each enzyme’s acyl chain length selectivity and its affinity for S-(2-oxo)pentadecyl-CoA (GARASVLS-NH2 from human immunodeficiency virus type I Pr55cag and GNSGSKQH-NH2 from Pp2p, respectively).

The addition of a 6XHis tag to Nmtlp has no demonstrable effect on peptide specificity as judged by myristoylpeptide production at 24 and 37 °C. When compared with 6XHis-Nmtlp, the specific activity of each 6XHis-nmtlp mutant is reduced at 24 and 37 °C for all peptides surveyed. For example, in the case of the Pp2p peptide, the decrease ranges from 10– to 50-fold at 24 °C and from 15- to 300-fold at 37 °C. The extent of the

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3 R. Bhatnagar, O. Schall, E. Jackson-Machelski, J. Sikorski, G. Gokel, and J. Gordon, submitted for publication.
Two peptides were selected for more detailed kinetic analysis: GNSGSQKHP-NH$_2$ (Ppz2p) and GAAPSKIV-NH$_2$ (Cnb1p) (representing the NH$_2$-terminal sequence of Cnb1p, the N-myristoylated regulatory subunit of yeast Ca$^{2+}$/calmodulin-dependent phosphoprotein phosphatase (45, 46)). They were chosen because all of the mutants were able to generate readily detectable amounts of these myristoylpeptides at 24 and 37 °C and because Ppz2p had been used when computing the NH$_2$-terminal eight residues of a yeast N-myristoylprotein, ADP-ribosylation factor-2 (Arf2p; Ref 7). Amino acids 1–4 were replaced with a para-(2-methylimidazole-N-buty1) phenylacetic acid derivative, amino acids 7 and 8 (Leu-Ser-NH$_2$) were deleted, and the 2-cyclohexylethyl amide derivative of lysine was used as the C-terminal residue (see legend to Table I). SC-58272 is competitive for binding of peptide substrates but not for myristoyl-CoA (7). ITC has been used to determine that the $K_c$ of SC-58272 for myristoyl-CoA-Nmt1p binary complexes is 29 nM.

Table III presents $K_c$ values for SC-58272 defined in the presence of saturating amounts of [3H]myristoyl-CoA and varying amounts of the Ppz2p peptide. With Nmt1p, the $K_c$ at 24 °C was very similar to the $K_c$ defined calorimetrically (45 versus 29 nM). The addition of a 6XHis tag to Nmt1p produces <2-fold changes in the $K_c$ at 24 and 37 °C. Ala$^{202}$ → Thr results in $K_c$ increases of 6- and 9-fold at 24 and 37 °C compared with 6XHIS-Nmt1p. Cys$^{17}$ → Arg produces 3- and 6-fold increases at these two temperatures.

**Conclusions from the Kinetic Studies**—The finding that Cys$^{17}$ → Arg produces an increase in the $K_c$ for SC-58272 but no change in the $K_c$ for S-(2-oxo)pentadecyl-CoA indicates that this substitution of a conserved Cys has selective effects on the functional properties of the enzyme’s peptide binding site. In contrast, substitution of the conserved Asn$^{126}$ with Ile appears to selectively affect the functional properties of the enzyme’s myristoyl-CoA binding site. Ala$^{202}$ → Thr, which produces the most severe ts phenotype, provides an example of a substitution that affects the functional properties of both sites, resulting in increases in the $K_c$ for S-(2-oxo)pentadecyl-CoA and SC-58272. Such a substitution may perturb specific contacts between the enzyme and the myristoyl or CoA moieties, which, in turn, could disturb the subsequent allostERIC transition required for creation of a fully functional peptide binding site. Alternatively, Ala$^{202}$ → Thr may produce more global changes in the conformation of Nmt1p.
growth of all but one of the ts mutants (Fig. 4). Arf1p was determined. Fig. 6 shows representative results (Table II), and a 6-fold decrease in affinity for the myristoyl-CoA analog.)

TABLE III

Inhibition of wild type and mutant Nmts by the peptidomimetic SC-58272.

| Nmtlp  | 24 °C | 37 °C |
|--------|-------|-------|
| 43     | 14    |
| 6XHis-Nmtlp | 24   | 9     |
| 6XHis-nmt202Tp | 138  | 82    |
| 6XHis-nmt217Rp | 74   | 55    |

**Defining Levels of Acylation of an N-Myristoylprotein in NMT1 and nmt1 Cells**

At 37 °C, adding 500 μM myristate to YPD media rescues growth of all but one of the ts nmt1 mutants (data not shown). (nmt1–328P is the exception. Recall that at 37 °C nmt328Pp has the lowest steady-state cellular concentration among the mutants (Fig. 4B), the greatest reduction in peptide V max in vitro (Table II), and a 6-fold decrease in affinity for the myristoyl-CoA analog.)

At 39–40 °C, adding 500 μM myristate to YPD only partially rescues nmt1Δ cells with nmt1–426I (lowest affinity for S-(2-oxo)pentadecyl-CoA among the mutants), nmt1–202T (lowest affinity for SC-58272 plus moderate reductions in affinity for S-(2-oxo)pentadecyl-CoA, or nmt1–404Y (most marked change in S-(2-oxo)pentadecyl-CoA affinity between 24 and 37 °C).

Levels of cellular protein N-myristoylation produced by the wild type and mutant nmt1 alleles were defined under these various growth conditions using an Arf gel mobility shift assay. Arf1p and Arf2p are two functionally interchangeable but essential N-myristoylproteins involved in vesicular trafficking (47, 48). They depend upon N-myristoylation for expression of their biological functions (49). Arf1p represents ~90% of cellular Arfs (47). N-Myristoylation produces a change in the electrophoretic mobility of Arf1p during SDS-PAGE; the acylated species migrates more rapidly than the nonmyristoylated species (19). We therefore reasoned that Arf1p could be used to report the extent of cellular protein N-myristoylation by noting the ratio of its distinctively migrating N-myristoylated and nonmyristoylated forms.

nmt1Δ cells containing plasmids with NMT1 or ts nmt1 inserts were grown in YPD at 24 °C to mid-log phase. Ailiquots of the cultures were then incubated for an additional 2 h at 24 °C or 33–40 °C in YPD alone or in YPD plus 500 μM myristate. Western blots of total cellular proteins were prepared and probed with rabbit antibodies that react with Arf1p but not Arf2p, and the ratio of N-myristoylated to nonmyristoylated Arf1p was determined. Fig. 6 shows representative results obtained with NMT1, nmt1–404Y, and nmt328P. Only myristoyl-Arf1p is detectable in NMT1 cells, whether they are cultured in YPD at 24, 37, or 40 °C. Defects in protein N-myristoylation are evident in nmt1–404Y cells when cultured in YPD alone. Even at the permissive temperature of 24 °C, only ~60% of Arf1p is acylated; at 37 °C, the value falls below 50%. In nmt1–328P cells, >95% of Arf1p is N-myristoylated when grown at 24 °C on YPD; however, between 33 and 37 °C, myristoyl-Arf1p decreases to 40% of total Arf1p. The ability of myristate to increase levels of myristoyl-Arf1p to >50% of total cellular Arf1p can be directly correlated with its ability to rescue the growth of a given nmt1 strain. At 37 °C, myristoyl-Arf1p levels increase in nmt1–404Y cells from <50% to >95% 2 h after exposure to myristate (Fig. 6). These cells are able to sustain growth in this media at this temperature. In contrast, myristate has no detectable beneficial effects on the efficiency of Arf1p acylation in nmt1–328P cells at 37 °C (Fig. 6), a temperature where they are unable to survive on YPD/myristate. The gel shift assay was used to establish that a reduction in levels of myristoyl-Arf1p to <50% is also associated with a failure of cells containing the other ts nmt1 alleles to survive (data not shown).

**Prospectus**

A panel of mutant Nmts, with defined defects in the functioning of their myristoyl-CoA and/or peptide binding sites has been generated using an error-prone PCR strategy. A full understanding of the structural significance of their amino acid substitutions must await determination of the atomic structure of Nmt1p with and without bound substrates or substrate analogs. Nonetheless, the temperature-sensitive growth arrest produced by various members of this panel offers an opportunity to conduct genetic screens for factors that affect Nmt activity, to examine the adaptive responses of cells to under-myristoylation of cellular proteins, and to identify the functions of cellular N-myristoylproteins in dividing and nondividing yeast cells.

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