Crystal Structures of Saccharomyces cerevisiae N-Myristoyltransferase with Bound Myristoyl-CoA and Inhibitors Reveal the Functional Roles of the N-terminal Region*

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Protein N-myristoylation catalyzed by myristoyl-CoA:protein N-myristoyltransferase (NMT) plays an important role in a variety of critical cellular processes and thus is an attractive target for development of antifungal drugs. We report here three crystal structures of Saccharomyces cerevisiae NMT: in binary complex with myristoyl-CoA (MYA) alone and in two ternary complexes involving MYA and two different non-peptidic inhibitors. In all three structures, the majority of the N-terminal region, absent in all previously reported structures, forms a well defined motif that is located in the vicinity of the peptide substrate-binding site and is involved in the binding of MYA. The Ab loop, which might be involved in substrate recognition, adopts an open conformation, whereas a loop of the N-terminal region (residues 22–24) that covers the top of the substrate-binding site is in the position occupied by the Ab loop when in the closed conformation. Structural comparisons with other NMTs, together with mutagenesis data, suggest that the N-terminal region of NMT plays an important role in the binding of both MYA and peptide substrate, but not in subsequent steps of the catalytic mechanism. The two inhibitors occupy the peptide substrate-binding site and interact with the protein through primarily hydrophobic contacts. Analyses of the inhibitor-enzyme interactions provide valuable information for further improvement of antifungal inhibitors targeting NMT.

Myristoyl-CoA:protein N-myristoyltransferases (NMTs)3; EC 2.3.1.97) are a family of enzymes belonging to the GCN5-related N-acetyltransferase superfamily (for reviews, see Refs. 1 and 2). They catalyze the covalent linkage of myristate, a rare 14-carbon saturated fatty acid (tetradecanoate, C14:0), from myristoyl-CoA (MYA) to the N-terminal glycine of proteins, a process that occurs following the removal of the initiator methionine residue of growing polypeptide chains (3, 4). This co-translational modification is an essential prelude to these altered proteins fully participating in important cellular processes, including signal transduction cascades and vesicular and protein trafficking (4–8). Typically, the consequences of attaching an extended alkyl chain to the protein result in increased lipophilicity, facilitating its association with cellular/subcellular membranes and mediating interactions with other proteinaceous partners (for reviews, see Refs. 3, 9, and 10).

NMTs have been characterized from a broad range of eukaryotic sources, including Saccharomyces cerevisiae (11), Candida albicans (12), Cryptococcus neoformans (13), Plasmodium falciparum (14), and Homo sapiens (15). Kinetic studies with MYA and peptide substrates or their analogs have shown that NMT catalysis conforms to an ordered Bi Bi reaction mechanism (16). Initial binding of MYA to the enzyme induces a conformational change and subsequent formation of a binding site for the protein substrate. Following the reaction, CoA is released prior to dissociation of the myristoylated peptide.

Genetic and biochemical data have established that NMTs are essential for growth and survival of a number of human yeast strains and parasites, such as C. albicans and C. neoformans (17) and the protozoa Leishmania major and Trypanosoma brucei (18). Sequence comparison and biochemical studies have shown that human and fungal NMTs share high sequence conservation at the MYA-binding site, but have divergent peptide substrate specificities (3). These properties make NMT an attractive therapeutic target for antifungal agents (19–21) designed to occupy the peptide substrate-binding site. In addition, because of its essential role in cell viability, NMT is a potential target for antiviral, antiparasitic, and even antineoplastic chemotherapy (18, 22–24). Thus, understanding the structural basis of the recognition and binding of the substrates with the enzyme should be useful for the development of new therapeutic agents.

Crystal structures of NMTs from two species have been determined, including those of C. albicans NMT in apo-form,
in binary complexes with several non-peptidic inhibitors, and in ternary complex with MYA and the peptide inhibitor SC-58272 (25, 26), and structures of S. cerevisiae (Sc) NMT in binary complex with MYA and in ternary complex with a non-hydrolyzable MYA analog (S-(2-oxo)pentadecyl-CoA) and SC-58272 or a peptide substrate (GLYASKLA) (27, 28). Structural and biochemical data have defined how the substrates are bound relative to each other at the active site and have revealed insights into the substrate binding specificity and the catalytic mechanism. However, the N-terminal region of NMTs, which has been implicated to play a pivotal role in the subcellular localization of NMT in mammalian cells (29), is absent in all of those structures because it is either disordered in the structures or removed to facilitate the structural studies.

Kinetic studies of synthetic peptides based on the N-terminal sequences of known N-myristoyltransferase indicate that optimum peptide substrates for ScNMT have an absolutely conserved Gly residue at position +1 and a marked preference for Ser and Lys at positions +5 and +6, respectively (9, 30). Based on this preference, a large number of peptide-based and peptidyl-like inhibitors that are effective against C. albicans and C. neoformans (19–21, 31) and that have antiparasitic activity (24) have been developed. Because of the limitations of peptide-based therapeutics, however, alternative small non-peptidic inhibitors have been screened, and some have been identified with very high activity and selectivity against NMTs of pathogenic fungal species (26, 32, 33).

We report here the crystal structures of full-length ScNMT in binary complex with MYA and in ternary complexes with MYA and Compounds I and II, two distinct non-peptidic inhibitors of ScNMT with potent IC_{50} values of 50 and 24 nM, respectively. In all three structures, the majority of the N-terminal region adopts a well defined structure with an α-helix and a loop located near the substrate-binding site and involved in the binding of MYA. The structural and kinetic data indicate that the N-terminal region plays an important role in the binding of both MYA and peptide substrate, but not in the subsequent catalytic reaction. Analyses of the interactions between the protein and the bound inhibitors also provide valuable information for further development of improved therapeutic agents.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification**—The ScNMT gene fragment corresponding to residues 3–455 was cloned into a customized expression plasmid (pBX4) that introduces a His tag (MRGSHHHHHHHSMA) at the N terminus of the expressed protein. The recombinant plasmid was transformed into Escherichia coli strain BL21(DE3) pLysS (Novagen). Bacterial transformants were grown in LB medium supplemented with ampicillin (30 μg/ml) at 37 °C until A_{600} = 0.8, and then protein expression was induced with 0.2 mM isopropyl-β-D-thiogalactopyranoside for 6 h at 23 °C. The cells were harvested by centrifugation at 4500 × g and resuspended in buffer A (20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 2 mM β-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride). The cells were lysed on ice by sonication, and the insoluble cell debris was removed by centrifugation at 15,000 × g at 4 °C.

Protein purification was carried out by a combination of affinity chromatography and ion exchange chromatography at 4 °C. The lysis supernatant was loaded onto a nickel-nitrotriacetic acid-agarose column (Qiagen Inc.) equilibrated with buffer A and then washed with buffer A supplemented with 5 mM imidazole. The target protein was eluted with buffer A supplemented with 100 mM imidazole. The eluted fractions were pooled together and dialyzed against buffer B (20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 2 mM β-mercaptoethanol, and 1 mM EDTA) for 3 h and then loaded onto a DEAE cation exchange column (Amersham Biosciences). The column was washed with buffer B, and the target protein existed in the flow-through fractions. SDS-PAGE and dynamic light scattering analyses indicated that the protein sample had high purity and homogeneity.

Expression plasmids of ScNMTs containing point mutations were generated from the vector containing the wild-type enzyme using the QuikChange site-directed mutagenesis kit (Stratagene). The sequences of these mutant genes were verified by DNA sequencing. Three ScNMT truncates were generated with deletion of residues 1–20 (Δ1–20), 1–30 (Δ1–30), and 1–40 (Δ1–40) of the N-terminal region, respectively. As a negative control, mutant ScNMT encoding the enzyme with deletion of C-terminal residues 452–455 (Δ452–455) was generated by mutating the codon specifying Val^{452} to a stop codon (TAG). Mutant ScNMT proteins were expressed and purified following the procedures used for the wild-type enzyme.

To perform the myristoylation enzymatic activity assay of ScNMT, an octapeptide (P_{ARF1}, GLFASKLF) corresponding to the C-terminal residues of the native substrate ARF1 of ScNMT was synthesized (Shanghai Science Peptide Biological Technology Co., Ltd.). The quality of the peptide was determined by analytical reverse-phase chromatography and mass spectral analysis and shown to have a purity of >95%.

**Crystallization and Diffraction Data Collection**—Crystallization was performed at 4 °C using the hanging-drop vapor diffusion method. MYA was dissolved in deionized water to a concentration of 15 mM, and the non-peptidic inhibitors were dissolved in Me_{2}SO to a concentration of 25 mM. To prepare crystals of the binary complex of ScNMT with MYA, the ScNMT protein solution (~20 mg/ml) was first mixed with the MYA solution at a molar ratio of 1:1.5 and then incubated at 4 °C for 0.5 h before crystallization setup. Sheet-shaped crystals of the binary complex were grown in drops containing equal volumes (2 μl) of the protein mixture solution and the reservoir solution (20 mM HEPES (pH 7.1) and 2.6 mM (NH_{4})_{2}SO_{4}) to a maximum size of 0.2 × 0.2 × 0.3 mm^{3} in 10 days. Diffraction data of the binary complex were collected to 2.9-Å resolution from a flash-cooled crystal at −176 °C at beamline 6A of the Photon Factory (Ibaraki, Japan) and processed with the HKL2000 suite (34). To prepare crystals of the ternary complex of ScNMT with MYA and non-peptidic inhibitors, the ScNMT protein solution was first mixed with the MYA and inhibitor solutions at a molar ratio of 1:1:5:5 and then incubated at 4 °C for 0.5 h before crystallization setup. Single crystals of the ternary complexes were grown under the same conditions as the binary complex to a maximum size of 0.15 × 0.15 × 0.2 mm^{3}. Diffraction data of the ternary complexes were collected to
Structure of N-Myristoyltransferase

RESULTS AND DISCUSSION

Overall Structure of ScNMT—The crystal structures of ScNMT in binary complex with MYA and in ternary complexes with MYA and non-peptidic inhibitors Compounds I and II were solved by molecular replacement and refined to 2.9-, 3.1-, and 3.0-Å resolution, respectively (Fig. 1 and Table 1). The asymmetric unit contains six ScNMT molecules forming two pseudotrimers, which are almost identical with an average root mean square deviation of ~0.45 Å based on superimposition of all C-α atoms. As with other reported NMT structures, the enzyme has a compact saddle-shaped β-sheet that spans the protein core surrounded by several α-helices. The NMT fold has an internal pseudo 2-fold symmetry with each half topologically equivalent to the monomer structure typical of a member of the GCN5-related N-acetyltransferase superfamily. In all three complex structures, there was very good electron density for the bound MYA and the majority of the N-terminal region of the enzyme in all six protomers of the asymmetric unit. In the structure with Compound I bound, there was good electron density for the inhibitor in each ScNMT molecule. However, in the complex with Compound II, there was good electron density for the inhibitor in only two ScNMT molecules, but poor density in the other four molecules. The model of the ScNMT-MYA complex comprises approximately residues 5–30 and 37–455 of each ScNMT molecule. The models of the ScNMT-MYA-Compound I/II complexes contain approximately residues 5–21 and 38–455 of each ScNMT molecule.

The overall structure of ScNMT in all three complexes (Fig. 2) is very similar to that described previously (27, 28). (Hereafter, the nomenclature of the secondary structure of ScNMT is that of Bhatnagar et al. (27)). However, a major difference involves the N-terminal region of the enzyme (residues 4–30). For the first time, it can be seen that this segment adopts a well defined conformation in all three of our complex structures, whereas the equivalent region is either disordered (27) or purposefully removed (28) in previous ScNMT structures. Interestingly, the N-terminal region (residues 1–60) is also invisible in the structures of C. albicans NMT both in the apo-form and in complexes with inhibitors (25, 26). The ordered N-terminal region in the ScNMT structures reported here forms an α-helix (αB) and loop (B’A’) motif (Fig. 3). The αB’ helix packs along a hydrophobic surface patch and appears to stabilize the overall structure. The B’A’ loop is located on the surface of the protein near the MYA- and peptide-binding sites with interactions with the bound MYA and Ab loop. Another marked difference occurs in the Ab loop (residues 103–110), which adopts an open conformation in all three of our complex structures (Fig. 2B and Table 2), but assumes varying conformations in different structures reported previously (27, 28).

Structure of the MYA-binding Site—Previous analyses of the crystal structures of ScNMT in binary complex with MYA and in ternary complexes with an MYA analog and a peptide substrate or peptidomimetic have identified structural elements involved in the binding of MYA, including helices αA’ and αC; strands βe, βf, and βn; and loops fg, eC, and Ab (27, 28). However, because of the absence of the N-terminal region of the enzyme in those complexes, full definition of the MYA-binding
site appears to be incomplete. As described above, the majority of the N-terminal region is well resolved in our structures of ScNMT both in binary complex with MYA and in ternary complexes with MYA and Compounds I and II, which allows us to define the MYA-binding site more completely.

The MYA in our complexes assumes a "question mark" conformation similar to that observed in the ScNMT complexes reported previously. There are four bends at the pyrophosphate group, the C-6–C-7 positions of pantetheine, the C-1 position of myristate, and the C-5–C-6 positions of myristate, respectively. In our ScNMT-MYA complex, MYA maintains the interactions with residues of the structural elements described previously (27, 28). In addition, the N-terminal region also forms part of the MYA-binding site, and several residues of the B’A’ loop are directly or indirectly involved in the binding of MYA (Fig. 3). Specifically, the side chain N-atom of Gln29 forms a hydrogen bond with the 2-hydroxyl group of the ADP-ribose moiety (2.8 Å); the side chain N-atom of His38 forms a hydrogen bond with one 3-phosphate oxygen of ADP (2.6 Å); and the side chain N-atom of His38 forms a hydrogen bond with the side chain O-atom of Glu11 (2.6 Å). The aromatic side chain of Phe27 makes -stacking interaction with the adenine moiety of ADP. These interactions further stabilize the binding of MYA. Moreover, the B’A’ loop has several hydrophilic interactions with the Ab loop, which was also suggested to play a critical role in the binding of MYA (27, 28). The main chain carbonyl of Lys26 forms a hydrogen bond with the side chain O-atom of Glu105, and the side chain O-atom of Ser25 forms hydrogen bonds with the main chain amide of Glu105 and main chain carbonyl of Tyr103. These interactions appear to stabilize the conformation of the Ab loop and its interactions with MYA. It is noteworthy that omission of MYA in the
crystallization solution still resulted in the structure of ScNMT with bound MYA and a well resolved N-terminal region, indicating that MYA has a very tight binding affinity with the enzyme and can be co-purified with the enzyme from the expression system and that the presence of the N-terminal region might stabilize the binding of MYA.

Structure of the Non-peptidic Inhibitor-Binding Site—The non-peptidic inhibitors Compounds I and II are bound at the peptide-binding site and have primarily hydrophobic interactions with the surrounding residues (Fig. 4). Structural comparisons indicate that the overall structures of the inhibitor-bound complexes are similar to that of the ScNMT-MYA complex and that binding of the inhibitors does not induce substantial conformational change in the peptide-binding site, except minor adjustment of the side chains of a few residues. These results suggest that this site has a rigid structure and that the inhibitor has to have proper structural and chemical properties to bind most effectively. Analysis of the chemical properties of the inhibitors indicated that Compound II is more hydrophobic than Compound I, consistent with the former having a relatively higher ClopP value (5.42 for Compound II versus 3.62 for Compound I). Structure comparison showed that Compound II binds slightly deeper toward the catalytic active site and has more hydrophobic contact with surrounding residues than Compound I (Fig. 4). Binding of Compound II with the protein buries 70.0% (393.8 Å²) of the surface area of the inhibitor, whereas binding of Compound I with the protein buries 53.8% (295.5 Å²) of the surface area of the inhibitor.

In the ScNMT structure with bound Compound I, the inhibitor has mainly hydrophobic interactions with a number of aromatic residues, including Tyr^{103}, Phe^{111}, Phe^{113}, Tyr^{219}, Phe^{234}, Phe^{334}, and Tyr^{349} (Fig. A4). The benzene ring of the inhibitor stacks perpendicularly with the aromatic side chain of Phe^{234}, whereas the thiazolidine moiety interacts with Phe^{111}, Phe^{113}, Phe^{334}, and Tyr^{349}. The nitrophenol moiety is located at the center of the catalytic active site and points toward the carboxylate group of the C-terminal Leu^{455} residue (~7.5 Å) and the thioester carbonyl group of MYA (~8.0 Å). It is also well positioned to make both hydrophobic and hydrophilic interactions (possibly via water molecules) with several residues nearby, including Tyr^{103}, Phe^{113}, Gly^{207}, Tyr^{219}, Leu^{455}, and the pantothenic group of CoA.

His^{221} and Asp^{417} have been implicated to be crucial in binding of the peptide substrate. Steady-state kinetic studies have shown that His^{221} plays an important role in enzymatic activity and that mutation H221A incurs a marked increase in $K_m$ values and the 2-thionyl group of the thiazolidine moiety of the inhibitor. These peaks could be water molecules, which

### Table 1

Summary of diffraction data and structure refinement statistics

|                | MYA          | MYA-Compound I | MYA-Compound II |
|----------------|--------------|----------------|-----------------|
| **Statistics of diffraction data** |              |                |                 |
| Wavelength (Å) | 1.0000       | 1.5418         | 1.5418          |
| Resolution range (Å) | 50.0–2.90 (3.00–2.90) | 15.0–3.10 (3.21–3.10) | 50.0–3.00 (3.10–3.00) |
| Space group    | C2           | C2             | C2              |
| Cell parameters |              |                |                 |
| a (Å)          | 188.2        | 188.8          | 188.8           |
| b (Å)          | 150.9        | 151.5          | 151.3           |
| c (Å)          | 134.7        | 133.9          | 134.2           |
| β              | 107.7°       | 107.5°         | 107.6°          |
| Unique reflections | 273,862     | 180,222        | 259,132         |
| Mosaicity     | 1.12         | 0.35           | 0.33            |
| Average redundancy | 3.5 (3.4)  | 3.0 (2.7)      | 4.0 (3.8)       |
| Average I/σ(I) | 11.0 (3.8)   | 4.5 (2.2)      | 4.1 (2.1)       |
| Completeness (%) | 98.6 (97.2)  | 97.5 (97.7)    | 97.7 (94.2)     |
| $R_{merge}$ (%) | 10.5 (36.8)  | 15.4 (32.9)    | 18.1 (36.4)     |

### Statistics of refinement and model

|                | MYA          | MYA-Compound I | MYA-Compound II |
|----------------|--------------|----------------|-----------------|
| Resolution (Å) | 20.0–2.9     | 15.0–3.1       | 15.0–3.0        |
| No. of reflections ($F_o > 0τ(F_o)$) | 73,997       | 59,849         | 66,404          |
| Free R set     | 3883         | 3222           | 3550            |
| R factor (%)   | 24.8         | 27.0           | 26.0            |
| No. of atoms   | 22,163       | 21,988         | 21,848          |
| Average B factor of all atoms (Å²) | 51.2          | 37.2           | 33.4            |
| r.m.s.$^a$ bond lengths (Å) | 0.007         | 0.007          | 0.006           |
| Ramachandran plot (%) | 1.125°       | 1.158°        | 1.078°          |
| Most favored regions | 86.2          | 82.5           | 85.4            |
| Allowed regions | 12.6         | 15.9           | 13.5            |
| Generously allowed regions | 1.2          | 1.6            | 1.1             |

$^a$ Numbers in parentheses refer to the highest resolution shell.

$^b$ $R_{merge} = |F_o| - |F_c|/|F_o|$

$^c$ $R$ factor $= |F_o| - |F_c|/|F_o|$

$^d$ r.m.s., root mean square.
might mediate the interaction between these two residues and the inhibitor, stabilizing inhibitor binding. Clearly, this is a feature of the inhibitor that might be altered to further enhance the hydrophilic interaction with the enzyme and to improve binding affinity. In addition, the 4’-carbonyl group of the thiazolidine moiety has no contact with the surrounding residues in the hydrophobic environment. Thus, substitution of this polar group with a hydrophobic group might improve its interactions and thus binding. Moreover, additional groups on the nitrophenol moiety might also prove beneficial.

In the Compound II-bound ternary complex, the inhibitor makes mainly hydrophobic contacts with aliphatic residues Leu332, Ile347, and Val395 and aromatic residues Tyr103, Phe111, Phe113, Tyr115, Phe119, Tyr219, Phe234, Tyr330, Phe334, and Tyr349 (Fig. 4B). The methyltetrahydrocarbazole moiety of the inhibitor has hydrophobic interactions with Tyr219, Phe234, and Val395. In addition, both His221 and Asp417, which are involved in the recognition and binding of the peptide substrate, make hydrophobic rather than hydrophilic interactions with the cyclohexane ring. The amine group of the inhibitor is in a position to form a hydrogen bond with the hydroxyl group of Tyr349. In addition, the adjacent hydroxyl group also makes a hydrophilic interaction with the hydroxyl group of Tyr349.

The terminal cyclohexane ring of Compound II is surrounded by a number of aromatic residues, including Tyr103, Phe113, Tyr115, Phe119, Tyr330, and Tyr349. It is located in proximity to the carboxylate group of Leu455 (~3.5 Å). Because there is some room between the cyclohexane ring of the inhibitor and Leu455, modification of the ring to augment its interaction with the C-terminal Leu455 residue might further stabilize inhibitor binding.

**Functional Roles of the N-terminal Region of ScNMT**—In all previously reported structures of NMTs
from S. cerevisiae and C. albicans, the N-terminal region of the enzyme is either disordered or removed during structural studies (25–28). In the ScNMT structures reported here, the majority of the N-terminal region is defined and folds as an α-helix (αB’) and a loop (B’A’). Analysis of our ScNMT complexes and comparison with other S. cerevisiae and C. albicans NMT structures allow us to discuss, for the first time, the potential functional roles of the N-terminal region of NMT.

Structural comparison showed that the Ab loop (residues 103–110) adopts different conformations (open, semi-open, and closed) in the previously reported NMT structures (Fig. 2B and Table 2). In the structure of ScNMT in complex with an MYA analog and the peptidic inhibitor SC-58272, the Ab loop assumes a closed conformation, and residues of the Ab loop (Val\textsuperscript{104}–Gly\textsuperscript{110}) cover the peptide-binding site and interact with the peptide substrate (27). In particular, the charged e-amino group of the crucial Lys residue at position +6 is tightly surrounded by the side chains of acidic residues Asp\textsuperscript{106}, Asp\textsuperscript{108}, and Asp\textsuperscript{117} (Fig. 2C). These results led to the suggestion that the Ab loop plays an important role in the recognition and binding of the peptide substrate (27). However, in the structure of ScNMT complexed with only MYA, the Ab loop assumes an open conformation in one monomer and a semi-open conformation in the other (28). In the open conformation, the Ab loop is pointed toward the solvent, and the tip of the loop is ~5 Å away from the peptide-binding site. The semi-open conformation is between the closed and open conformations, with the tip of the Ab loop being ~5 Å away from the peptide-binding site. Also, in the structure of ScNMT in complex with an MYA analog and a peptide substrate, the Ab loop adopts a semi-open conformation rather than the closed conformation, and residues of the Ab loop have no interaction with the side chain of the crucial Lys residue of the peptide (28). In the structures of C. albicans NMT, the Ab loop assumes a closed conformation in its complex with MYA and SC-58272, but is disordered in the apo-form and in its complex with a non-peptidic inhibitor (25, 26). Interestingly, in all three of our ScNMT complex structures, the Ab loop assumes the open conformation with well defined electron density and has
no interaction with the bound non-peptidic inhibitors. These results indicate that the Ab loop has a high flexibility and is unlikely to be directly involved in the recognition and binding of the peptide substrate. Moreover, sequence comparison also indicated that most of the residues forming the Ab loop are strictly conserved in all known NMTs from different species and that these conserved residues, such as Asp106 and Asp108, are less likely to participate in the recognition of the variable sequences of species-specific peptide substrates (Fig. 2A).

On the other hand, detailed analysis of our ScNMT structures showed that the B’A’ loop of the N-terminal region takes the position of the Ab loop in the closed conformation and has interactions with the bound inhibitors (Fig. 2). In particular, Asp22, Asp23, and Thr24 of the B’A’ loop occupy the corresponding positions of Asp106 and Asp108 of the closed Ab loop and are well positioned to interact with the side chain of Lys at position +6 of the peptide substrate, suggesting that these residues might be involved in the recognition and binding of the peptide substrate. Sequence comparison also showed that the residues forming the aB’ helix are moderately conserved in most NMTs from different species, but that the residues forming the B’A’ loop are not conserved (Fig. 2A), suggesting that the B’A’ loop might be the determinant of species-specific function(s).

To investigate further the functional roles of these various residues and the N-terminal region in the

![Figure 4](image-url)
myristoylation reaction, we carried out mutagenesis studies and truncation analysis and determined the kinetic parameters of the altered ScNMTs (Table 3). Both single and double mutations of Asp\textsuperscript{106} and Asp\textsuperscript{108} to Ala or Lys in the Ab loop had no significant effect on the $K_m$ value. On the other hand, whereas single mutations of Asp\textsuperscript{22}, Asp\textsuperscript{23}, and Thr\textsuperscript{24} to Ala or Lys had minor effects on the $K_m$ value, double mutations D22A/D23A and D22K/D23K caused a substantial increase in $K_m$ (7- and 11-fold, respectively). Consistently, truncation of the first 30 or 40 residues of the N-terminal region, which removed Asp\textsuperscript{22} and Asp\textsuperscript{23}, caused an ~12-fold increase in $K_m$, whereas truncation of the first 20 residues had a much smaller effect (2-fold). However, none of these mutations had any significant effect on the $K_{cat}$ value. These data further substantiate our structural results that Asp\textsuperscript{106} and Asp\textsuperscript{108} of the AB loop are not involved in the binding of the peptide substrate, whereas Asp\textsuperscript{22} and Asp\textsuperscript{23} of the N-terminal B’A’ loop are involved in the binding of the peptide substrate, but do not participate in catalysis, consistent with previous biochemical data showing that removal of the N-terminal 34 residues of ScNMT has no significant effect on enzymatic activity (43).

Together, our structural and kinetics data indicate that Asp\textsuperscript{106} and Asp\textsuperscript{108} of the AB loop are not directly involved in the recognition and binding of the peptide substrate. Instead, Asp\textsuperscript{22} and Asp\textsuperscript{23} of the N-terminal B’A’ loop assume this role. These two residues appear to provide the negatively charged electrostatic environment to recognize and bind the positively charged side chain of the lysine residue at position +6 of the peptide substrate. Thus, the closed conformation of the Ab loop seen in the ScNMT-MYA analogoinhibitor complex (27) could be an artifact resulting from the absence of the N-terminal region.

In addition to the involvement in the recognition and binding of the peptide substrate, the N-terminal region of ScNMT appears to have other functional role(s). As discussed above, the N-terminal region forms part of the MYA-binding site. In particular, several residues of the B’A’ loop have both hydrophilic and hydrophobic interactions with MYA and help to stabilize the Ab loop of the enzyme, which is also involved in MYA binding. Thus, it is evident that the N-terminal region plays an important role in the recognition and binding of MYA. Structural comparison also showed that, in the absence of the N-terminal region, Phe\textsuperscript{49}, Ile\textsuperscript{208}, Val\textsuperscript{209}, Pro\textsuperscript{213}, and Phe\textsuperscript{419} form a hydrophobic patch near the MYA-binding site that is directly exposed to the solvent, conceivably making the enzyme less stable. With the N terminus in place, the oB’ helix covers this hydrophobic patch and forms both hydrophobic and hydrophilic contacts with the rest of the protein. Therefore, it is very likely that the N-terminal region helps to stabilize the overall structure of the enzyme.

Moreover, protein N-myristoylation appears to be a tightly regulated reaction involving coordinated participation of several different enzymes/proteins (e.g. N-methionyl aminopeptidase, fatty-acid synthetase, long chain acyl-CoA synthetase, acyl-CoA-binding proteins, etc.), access of NMT to pools of MYA, and timely N-myristoylation of nascent polypeptide substrates to avoid potential interfering reactions (e.g. N-acetylation and polypeptide folding) (for reviews, see Refs. 44–47). The ability of NMT to function within such a complicated process implies the existence of mechanisms designed to ensure targeting of the enzyme to the appropriate protein synthesis machinery, possibly involving interactions with other cooperating components that facilitate the recognition and efficient N-myristoylation of the rapidly growing polypeptide substrates. However, so far, no information is available regarding the mechanism(s) regulating either the specific association of NMT with the cytoplasmic protein synthesis machinery or its direct participation during protein synthesis. Both are presumably required for ScNMT to accomplish the co-translational N-myristoylation of proteins in yeast cells. Some experiments have shown that the N-terminal region of human NMT plays an important role in targeting the enzyme to the site of protein synthesis on ribosomes, thereby facilitating the participation of the enzyme in the co-translational N-myristoylation of proteins in mammalian cells (29). Because the N-terminal region of ScNMT is located on the molecular surface near both the MYA- and peptide-binding sites, it is possible that the N-terminal region and other structural elements might provide a potential binding site for other associated proteins in the subcellular localization of the enzyme and thus coordinated control of its catalytic activity.

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| Table 3 | Kinetic data for myristoylation of P\textsubscript{ARP1} by wild-type and mutant ScNMTs |
|---|---|---|---|
| ScNMT | $K_m$ | $k_{cat}$ | $k_{cat}/K_m$ |
| Wild-type | 13.68 ± 1.88$^A$ | 3.06 ± 0.28 | 2.34 × 10\textsuperscript{2} |
| D22A | 19.04 ± 2.54 | 1.72 ± 0.15 | 9.03 × 10\textsuperscript{4} |
| D22K | 23.79 ± 3.89 | 2.50 ± 0.38 | 1.05 × 10\textsuperscript{4} |
| D23A | 21.37 ± 2.71 | 2.71 ± 0.35 | 1.27 × 10\textsuperscript{4} |
| D23K | 50.16 ± 5.92 | 3.64 ± 0.40 | 7.26 × 10\textsuperscript{4} |
| T24A | ND$^B$ | ND | ND |
| T24D | 41.56 ± 3.87 | 2.77 ± 0.51 | 6.67 × 10\textsuperscript{4} |
| D106A | ND | ND | ND |
| D106K | 30.63 ± 3.24 | 2.65 ± 0.25 | 8.68 × 10\textsuperscript{4} |
| D108A | 7.89 ± 1.12 | 1.94 ± 0.15 | 2.45 × 10\textsuperscript{4} |
| D108K | 13.88 ± 1.15 | 1.94 ± 0.34 | 1.40 × 10\textsuperscript{4} |
| D22A/D23A | 92.38 ± 12.23 | 3.83 ± 0.28 | 4.15 × 10\textsuperscript{4} |
| D22K/D23K | 149.80 ± 7.62 | 4.73 ± 0.81 | 3.16 × 10\textsuperscript{4} |
| D106A/D108A | 38.50 ± 4.49 | 2.55 ± 0.12 | 6.62 × 10\textsuperscript{4} |
| D106K/D108K | ND | ND | ND |
| Δ1–20 | 27.73 ± 2.54 | 3.58 ± 0.43 | 1.29 × 10\textsuperscript{4} |
| Δ1–30 | 158.7 ± 8.59 | 3.85 ± 0.48 | 2.43 × 10\textsuperscript{4} |
| Δ1–40 | 162.5 ± 7.63 | 4.03 ± 0.46 | 2.48 × 10\textsuperscript{4} |
| Δ452–455 | ND | ND | ND |

$^A$ All kinetic parameters are the average of duplicate determinations.
$^B$ ND, not determined. Data for these mutants could not be fitted to the Michaelis-Menten equation by nonlinear regression to determine the $K_m$ and $k_{cat}$ values.
$^-^-$, the enzymatic activity was too low to be detected.
