Published in final edited form as:

Biochemistry. 2018 June 26; 57(25): 3515–3523. doi:10.1021/acs.biochem.8b00296.

Repurposed HisC Aminotransferases Complete the Biosynthesis of Some Methanobactins

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Abstract

Methanobactins (Mbns) are ribosomally produced, post-translationally modified bacterial natural products with a high affinity for copper. MbnN, a pyridoxal 5'-phosphate (PLP) dependent aminotransferase, performs a transamination reaction that is the last step in the biosynthesis of Mbns produced by several *Methylosinus* (*Ms.*) species. Our bioinformatic analyses indicate that MbnNs likely derive from histidinol-phosphate aminotransferases (HisCs), which play a key role in histidine biosynthesis. A comparison of the HisC active site with the predicted MbnN structure suggests that MbnN's active site is altered to accommodate the larger and more hydrophobic substrates necessary for Mbn biosynthesis. Moreover, we have confirmed that MbnN is capable of catalyzing the final transamination step in Mbn biosynthesis in vitro and in vivo. We also demonstrate that without this final modification, Mbn would exhibit significantly decreased stability under physiological conditions. An examination of other Mbns and Mbn operons suggests that N-terminal protection of this family of natural products is of critical importance, and that several different means of N-terminal stabilization have evolved independently in Mbn subfamilies.

Graphical Abstract

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. Supporting Information. Extended materials and methods, supplemental figures S1–S13, and supplemental tables S1–S4 (PDF).

INTRODUCTION

Methanobactins (Mbns) are copper-chelating peptidic natural products first identified in methanotrophs, bacteria that utilize methane as their sole carbon source.^{1,2} The primary methane-oxidizing enzyme in methanotrophs, particulate methane monooxygenase (pMMO), requires copper,³ necessitating the presence of specialized uptake systems. When bioavailable copper is limited, some methanotrophs produce and secrete Mbns, which acquire copper from the environment,¹ and are reinternalized as the copper-bound form (CuMbn).⁴ Mbns that have been isolated and characterized to date consist of a peptidic backbone modified with two nitrogen-containing heterocycles, either oxazolones or pyrazinediones, and two neighboring thioamide groups that form a high affinity copper binding site (Figure S1).^{1,5–8} Mbns bind both Cu(I) and Cu(II), but Cu(II) is rapidly reduced to Cu(I) by an unknown process.⁹

Mbns are ribosomally produced, post-translationally modified peptide natural products (RiPPs).¹⁰ Similar to the biosynthesis of other RiPPs, Mbn is formed from a precursor peptide, MbnA, comprising a leader peptide that is cleaved during biosynthesis and a core peptide that is post-translationally modified to form mature Mbn.¹¹ The copper ligands derive from two cysteine residues in the MbnA core peptide.⁶ Genes encoding MbnA are found in operons adjacent to genes encoding a range of biosynthetic, regulatory, and transport proteins.¹⁰ Two core proteins, MbnB and MbnC, form the heterodimeric iron-containing enzyme complex MbnBC, which catalyzes the formation of neighboring oxazolone/thioamide groups from specific cysteine residues.¹²

For the Mbns produced by *Methylosinus* (*Ms.*) *trichosporium* OB3b and *Ms.* sp. LW4, another operon-encoded biosynthetic enzyme has been proposed to perform a transamination reaction that is the last step in Mbn biosynthesis,¹⁰ generating a carbonyl group from the N-terminal amino group (Figure 1). This aminotransferase has been termed MbnN.¹⁰ We recently demonstrated that disruption of *mbnN* in *Ms. trichosporium* OB3b (*mbnN*) produces a copper-chelating Mbn biosynthetic intermediate with an absorbance feature at 332 nm and a mass 1 Da higher than that of mature CuMbn.¹² This mass shift is consistent with a biosynthetic intermediate (Mbn-NH₂) that includes the two oxazolone/thioamide moieties observed in the mature compound, but has not undergone the N-terminal transamination reaction that replaces the primary amine with a carbonyl group. The formation of Mbn-NH₂ is consistent with the proposed function of MbnN and contradicts a previously suggested role in formation of the N-terminal oxazolone (Oxa_A).¹³ Mature copper-free (apo) *Ms. trichosporium* OB3b Mbn exhibits an absorbance feature at 392 nm assigned to Oxa_A, which is next to the “N-terminal” carbonyl group. The red shift of this feature upon conversion to mature Mbn is due to the extended conjugation system between the N-terminal carbonyl group and Oxa_A (Figure 1).¹⁴ Biochemical evidence for the proposed role of MbnN has been elusive due to the previous unavailability of the partially mature Mbn substrate, Mbn-NH₂. Here we present the first in vitro characterization of MbnN function. Moreover, bioinformatics analysis provides insights into the origin of MbnN and its interactions with Mbn biosynthetic intermediates.

MATERIALS AND METHODS

Construction of an MbnN model and comparison to HisC

The MbnN sequence from *Ms. trichosporium* OB3b was submitted to the I-Tasser server.¹⁵ The top model was obtained using a histidinol-phosphate aminotransferase HisC enzyme identified in *Mycobacterium (My.) tuberculosis* (PDB: 4R8D). Models were aligned to HisC structures from *My. tuberculosis* (PDB: 4R8D) and *Escherichia (E.) coli* (PDB: 1FG3) in PyMOL using the super command. The charged surfaces were created using the generate/vacuum electrostatics/protein contact potential command.

Alignment of MbnN sequences against related aminotransferases

MbnN and the other sequences listed in Figure S2 were aligned against the TIGR01141 (HisC) hidden Markov model using HMMALIGN.¹⁶ The sequences were chosen to represent a range of structurally and biochemically characterized HisC enzymes along with representatives of related aminotransferase families.

Identification of MbnN relatives via BLAST

Ms. trichosporium OB3b MbnN (MettrDRAFT_3425) and *Komagataibacter oboediens* 174bp2 MbnN2 (Gobo1_010100018566) were used as the seeds for blastp searches against all isolates in the JGI/IMG database using a 1E-5 E-value cutoff. The top 50 results are presented in Tables S1 and S2.

Gene cloning

The *mbnN* genes from *Ms. trichosporium* OB3b were cloned directly from gDNA into either pSGC-His vectors (encoding a TEV-cleavable N-terminal His₆ tag) or pNYCOMPS-His vectors (encoding a C-terminal TEV-cleavable His₆ tag). For both of these species, gDNA was prepared as previously described.¹⁷ The *Ms. trichosporium* OB3b gene was cloned via the forward (TACTTCCAATCCATGACGGCGATCCCATGTGAAACG) and reverse (TATCCACCTTTACTGTTATTCCGACCGAGGCGCGTG) primers for the pSGC-His construct and the forward (TTAAGAAGGAGATATACTATGACGGCGATCCCATGTGAAACG) and reverse (TGAAATAGAGGTTTTTCGGCTTCCGACCGAGGCGCGTG) primers for the pNYCOMPS-His construct.

Protein expression and purification

pSGC-His-OB3b-MbnN and pNYCOMPSC-His-OB3b-MbnN were transformed via heat shock into chemically competent cells from the BL21-derived *E. coli* expression strain NiCo21 (DE3) (New England Biolabs). A colony was transferred to a 5 mL liquid culture of LB media with 30 µg/ml kanamycin (MilliporeSigma) overnight at 37 °C with agitation at 200 rpm. Then, 3 mL of the overnight culture was inoculated into 100 mL of LB media with 30 µg/ml kanamycin (MilliporeSigma) for 4 h at 37 °C with agitation at 200 rpm. For MbnN protein expression, 12 L of auto-induction medium¹⁸ were prepared in 2 L baffled flasks with 30 µg/ml kanamycin. Growth was initiated by the addition of 8 mL volumes of cells in logarithmic growth (from fresh 100 mL cell cultures) at 37 °C and 180 rpm, and when the

OD₆₀₀ reached 0.3–0.5, the temperature was decreased to 18 °C. Cells were harvested after 24 h via centrifugation at 5000 x g and 4 °C for 20 min, flash frozen in liquid nitrogen, and stored at –80 °C. Cells were thawed in Buffer A (25 mM phosphate buffer, pH 8.0, 250 mM NaCl, 10 mM imidazole, 10% glycerol) in the presence of phenylmethylsulfonyl fluoride (PMSF) (250 μM, stock solution (250 mM) was prepared in EtOH) and DNase (MilliporeSigma), and lysed via sonication for 15 min with 1 s pulses and 3 s breaks at 4 °C using a sonic dismembrator (Thermo Fisher Scientific). The lysate was centrifuged at 25,000 x g for 1 h at 4 °C and the resulting supernatant was loaded on to a 5 mL Ni-loaded HiTrap Chelating Column (GE Healthcare Life Sciences). The flow-through was discarded. After washing the column with Buffer A, MbnN with a C-terminal His₆-tag was eluted via a 15-column volume gradient of 0–50% Buffer B (25 mM phosphate, pH 8.0, 250 mM NaCl, 1 M imidazole, 10% glycerol) against Buffer A. Concentration and buffer exchange into Buffer A was then performed using Amicon Ultra-15 concentrators with a 30 kDa molecular weight cutoff (MilliporeSigma).

To remove the C-terminal His₆-tag, MbnN with a C-terminal His₆-tag was added to 5 mL of Buffer A supplemented with 1 mM DTT and 0.5 mM EDTA, followed by the addition of His₆-tagged TEV protease, prepared as previously described. The sample was dialyzed against Buffer A overnight and at 4 °C in a 10 kDa MWCO Slide-A-Lyzer dialysis cassette (Thermo Fisher Scientific). After concentration and buffer exchange into buffer A, the sample was loaded onto a 5 mL Ni-loaded HiTrap Chelating Column (GE Healthcare Life Sciences) to remove the TEV protease and the cleaved His₆ tag. The flow-through, which contained tagless MbnN, was collected and concentrated again in 30 kDa MWCO Amicon spin concentrators (MilliporeSigma). The protein concentration was determined by absorbance at 280 nm using the calculated extinction coefficient (25900 M⁻¹ cm⁻¹), as measured on an Agilent 8453 UV-vis spectrophotometer in a BRAND disposable UV-compatible cuvette (MilliporeSigma).

Native top-down mass spectrometry

Samples analyzed by native top-down mass spectrometry (nTDMS) were dialyzed against 100 mM ammonium acetate with a nominal protein concentration of 10–20 μM. A customized Thermo Fisher Q Exactive HF Mass Spectrometer with Extended Mass Range was utilized for nTDMS analyses.¹⁹ The nTDMS platform employs direct infusion of sample into a native electrospray ionization (nESI) source held at +2 kV and coupled to a three-tiered tandem MS process. Intact mass values for MbnN complexes and ejected subunits were determined by charge state deconvolution to convert data from the *m/z* to the mass domain using MagTran.²⁰

Purification of apo Mbn from wild-type *Ms. trichosporium* OB3b and apo Mbn-NH₂ from *Ms. trichosporium* OB3b *mbnN*

Mbn-producing wild-type *Ms. trichosporium* OB3b and *Ms. trichosporium* OB3b *mbnN* were grown as described previously.^{12,21} Wild-type Mbn was also purified as described previously.²¹ However, due to the observed lability of the compound produced from the *mbnN* strain, purification was carried out via an alternate and less extensive method, yielding semi-pure compound. For these samples, after *Ms. trichosporium* OB3b *mbnN*

cells were spun down, 500 mL clarified spent medium were loaded directly onto a methanol-activated and water-equilibrated Sep-Pak tC18 Plus Short Cartridge (Waters), washed with water, and eluted with 60% methanol/40% 10 mM NH₄OAc. The resulting yellow solution was then lyophilized and resuspended in water or buffer as appropriate. This method was also used to quickly clean up some samples after reactions and stabilization with 0.1 mM CuSO₄, and prior to electrospray ionization liquid chromatography–mass spectrometry (ESI-LC-MS) analysis, removing both high-concentration cosubstrates during the wash steps and leaving hydrophobic protein stuck to the column. Very small reactions were cleaned up instead on a C18 Macro Spin Column (The Nest Group) using an analogous protocol. All Mbn masses were confirmed via ESI-LC-MS prior to reactions with MbnN. All solvents used for ESI-LC-MS were Chromasolv HPLC-grade (MilliporeSigma), and aqueous solutions were 0.2 μm filtered prior to use.

Mass spectrometry for Mbn and Mbn-NH₂

ESI-LC-MS analysis was performed using an Agilent 1100 HPLC stack with inline DAD paired with a Bruker AmazonX ion trap mass spectrometer. Initial data analysis was carried out within the Hystar Compass software (Bruker) and figures were prepared in MNovo 9.1.0 (MestrelNova). All solvents used for ESI-LC-MS were Chromasolv HPLC-grade (MilliporeSigma), and aqueous solutions were 0.2 μm filtered prior to use. All samples containing Mbn-NH₂ were stabilized by the addition of 0.1 mM CuSO₄ prior to mass spectrometry.

Reaction mixtures containing protein as well as Mbn were injected onto a Grace Vydac reverse-phase C4 column (214TP5405: 300 Å, 5 μm, 4.6 mm i.d. x 50 mm) with a typical injection volume of 50 μL. After an initial delay of 4.24 min to allow for the elution of salts and other unwanted compounds, samples were analyzed in alternating positive and negative ion modes over a gradient of 0–100% buffer B (75% ACN, 20% *n*-PrOH, 1% formic acid) against buffer A (5% ACN, 1% formic acid) over 13.57 min at a flow rate of 0.5 mL/min.

Aqueous samples of both purified Mbn and post-reaction Mbn in the absence of protein were injected onto a Grace Vydac reverse-phase C18 column (218TP5405: 300 Å, 5 μm, 4.6 mm i.d. x 50 mm) with a typical injection volume of 50 μL. After an initial delay of 4.24 min, samples were analyzed in alternating positive and negative ion modes over a gradient of 5–55% buffer B (80% ACN, 20% 10 mM NH₄OAc) against buffer A (10 mM NH₄OAc) over 13.57 min at a flow rate of 0.5 mL/min.

Aminotransferase activity assay using an aspartate aminotransferase (AST) kit

Purified MbnN (at a final concentration of 25 μM) was assayed for aspartate/α-ketoglutarate transamination activity using an AST Activity Assay kit (MAK055, MilliporeSigma), following the instructions provided by the manufacturer. Reactions were initiated at 37 °C with 20 s of shaking and were monitored for 1 h in a Corning UV-transparent 96-well flat bottom microplate (MilliporeSigma) at 450 nm on a Cytation 5 Cell Imaging Reader (BioTek) and analyzed using Gen5 2.07 (BioTek). Instead of the AST assay buffer, other buffers were utilized to identify reaction conditions with maximal activity, including MOPS buffer (25 mM MOPS, pH 7.2, 250 mM NaCl, 10 mM imidazole, 10% glycerol), Tris buffer

(25 mM Tris, pH 8 or 9, 250 mM NaCl, 10 mM imidazole, 10% glycerol), and phosphate buffer (25 mM phosphate, pH 7 or 8, 250 mM NaCl, 10 mM imidazole, 10% glycerol).

Monitoring the forward reaction of MbnN with semi-purified Mbn-NH₂ via UV-vis spectroscopy

To confirm the production of Mbn-NH₂ and/or Mbn_{Met}-NH₂, the lyophilized sample was dissolved in water, CuSO₄ was added to 0.1 mM to stabilize the compound, and the sample was analyzed by ESI-LC-MS. For activity measurements, the lyophilized sample was redissolved in 90 μ L Tris reaction buffer (25 mM Tris, pH 9, 250 mM NaCl, 10% glycerol, 0.1 mM EDTA) and if necessary was further diluted in reaction buffer until the absorbance at 332 nm was ~0.6 in a quartz cuvette (Hellma Analytics). Prior to the final reaction, 3 mM α -ketoglutaric acid and 0.5 mM DTT were also added. To initiate the reaction, 20 μ M MbnN was added, and the reaction was monitored over 8 h at 30°C using UV-vis absorption spectroscopy on an Agilent 8453 spectrophotometer with an Agilent 89090A Peltier heating/cooling attachment. Spectra were acquired between 200 and 800 nm; the initial spectra were taken 15 s apart, and then the interval between spectra was increased by 1% with every scan. Initial analysis was carried out using ChemStation B.04.02 (Agilent), but extended data analysis was carried out in GraphPad Prism 7 or Igor Pro 6.37 (Wavemetrics). The reaction was quenched by the addition of 0.1 mM CuSO₄, and the final products were analyzed via ESI-LC-MS using a Grace Vydac reverse-phase C4 column as described above.

Identification of L-amino acid cosubstrates for the reverse MbnN reaction

In a Corning UV-transparent 96-well flat bottom microplate (MilliporeSigma), 11 μ M apo Mbn, 10 mM of the target L-amino acid (Table S3), and 300 μ M DTT were added to a final volume of 150 μ L phosphate reaction buffer (25 mM phosphate, pH 8.0, 250 mM NaCl, 10 mM imidazole, 10% glycerol). 25 μ M MbnN was added to initiate the reaction with 5 s of shaking. Afterwards, the absorbance was monitored at 340 nm and 395 nm using a Cytation 5 Cell Imaging Multi-Mode Plate Reader (BioTek), every 5 min for 1.5 h at 37 °C, and analyzed using Gen5 2.07 (BioTek). Since the reverse reaction results in increasing absorbance at 342 nm and decreasing absorbance at 392 nm, an increased ratio of A₃₄₀ to A₃₉₅ (Table S3) was used to identify amino acids that were good cosubstrates for the reverse reaction.

Reverse MbnN reaction with Mbn and L-histidine

Purified and lyophilized apo Mbn was redissolved in phosphate reaction buffer (25 mM phosphate, pH 8.0, 250 mM NaCl, 10% glycerol) to a final concentration of 20 μ M. Other reaction components were added prior to the protein, including 0.5 mM DTT and 10 mM L-histidine. After 25 μ M MbnN was added to initiate transamination, the reaction was monitored by UV-vis spectroscopy on an Agilent 8453 spectrophotometer with an Agilent 89090A Peltier heating/cooling attachment for 8 h at 37 °C. Spectra were acquired between 200 to 800 nm and were taken every 60 s. Initial analysis was carried out using ChemStation B.04.02 (Agilent), but extended data analysis was carried out in GraphPad Prism 7 or Igor Pro 6.37 (Wavemetrics). After 0.1 mM CuSO₄ was added to quench the reaction, the reaction mixture was cleaned via a C18 spin column, lyophilized, re-dissolved in water and further analyzed via ESI-LC-MS as described above.

Acid hydrolysis

After the MbnN reverse reaction was performed, the resulting product was purified using on a C18 Macro Spin Column (The Nest Group, Southborough, MA) and lyophilized. The lyophilized material was resuspended in water for all acid hydrolysis experiments. HCl was added to a final concentration of 100 mM and the reaction was monitored for 24 h at room temperature by UV-vis absorption spectroscopy on an Agilent 8453 spectrophotometer. All spectra were obtained between 200 and 800 nm; the initial spectra were taken 15 s apart, and after this, the interval between spectra was increased by 1% with every scan. Initial analysis was carried out using ChemStation B.04.02 (Agilent), but extended data analysis was carried out in Igor Pro 6.37 (Wavemetrics). At 10 min, 30 min, and 24 h, aliquots were taken and neutralized using NH₄OH; these were analyzed directly via ESI-LC-MS on a C18 column as described above.

RESULTS AND DISCUSSION

Bioinformatics analysis and modeling of MbnN

MbnN belongs to the pyridoxal 5'-phosphate (PLP)-dependent aminotransferase family, members of which catalyze reversible and reciprocal transamination reactions between two cosubstrates (amino group donors and acceptors) with concomitant cycling of the cofactor between PLP and PMP.²² On the basis of sequence similarity, the characterized enzyme that MbnN most closely resembles is the L-histidinol-phosphate aminotransferase HisC, a member of α -aminotransferase Family I β ,²³ which is involved in histidine biosynthesis.²⁴ Despite the large number of microbial genomes sequenced in the five years since the identification of the Mbn operon, almost all genes closely related to *mbnN* can be affirmatively identified as *hisC* on the basis of their genomic neighborhood, while more distant relatives can also be identified as authentic HisC enzymes on the basis of biochemical characterization.^{25–27} This relationship does not result from the duplication of the native *hisC* gene (MettrDRAFT_3084 in *Ms. trichosporium* OB3b): the greatest sequence similarity (as high as 38% identity) is found between MbnN (MettrDRAFT_3425 in *Ms. trichosporium* OB3b) and genes annotated as encoding HisC homologues from a range of unrelated extremophiles, primarily anaerobic, hyperthermophilic, and predominantly chemoautolithotrophic bacteria belonging to the *Aquificae* and *Firmicutes* phyla. Although some of the closest non-MbnN sequences do not come from canonical histidine biosynthesis operons, authentic HisC genes are evident within the top 10 BLAST results, based on the identities of conserved genes in the surrounding operon. Thus, it appears that MbnNs are in fact HisC enzymes that have been repurposed to enact a transamination reaction on a much larger and more structurally complex substrate.

The closest structurally characterized relative of *Ms. trichosporium* OB3b MbnN is HisC from *My. tuberculosis* (PDB accession code 4R8D).²⁵ Like MbnN, this HisC has a flexible N-terminal region that is slightly longer than that of many other authentic HisC proteins. In HisC structures, which consist of two homodimeric subunits (Figure 2A), this sequence mediates not only interactions between monomers, but also controls access to the active site and engages in interactions with the substrate.²⁷ An I-Tasser model of *Ms. trichosporium* OB3b MbnN against this HisC (with which it has 30% identity) has an estimated RMSD of

5.3 ± 3.7 Å, with much of the variability within the flexible N-terminal region (Figure 2A). A comparison of the extant and predicted surface electrostatic potentials for the two enzymes indicates that both the N-terminal arm and the surface of the protein surrounding the entrance to the active site are notably less charged in MbnN (Figure S3, B and C). This difference is potentially consistent with interactions between MbnN and the uncharged peptidic backbone of Mbn-NH₂. By comparison, interactions between HisC and its substrate occur primarily within the active site cavity, due to the significantly smaller size of histidinol phosphate.

These differences extend to the active site, but only in regions that are involved in histidinol phosphate binding. A wide range of residues are involved either in direct interactions with the PLP/PMP cofactor, or with the second shell hydrogen bonding network that stabilizes that cofactor. Of the twelve most important cofactor- and cosubstrate-supporting residues, all but two are the same in MbnN and the canonical HisC active site (Figure 2, B and C). However, residues that interact with histidinol phosphate in HisC are less well-conserved in MbnN. For example, on the N-terminal loop, an important tyrosine is instead a phenylalanine (Phe29) in *Ms. trichosporium* OB3b (but not *Ms. sp.* LW4) MbnN. In *Corynebacterium* (*C.*) *glutamicum* HisC, replacement of this tyrosine with phenylalanine decreases $k_{\text{cat}}/K_{\text{m}}$ for histidinol phosphate but not alternate substrates, such as L-leucine or L-phenylalanine, which have hydrophobic sidechains that cannot engage in hydrogen bonding.²⁶ Similarly, the N-terminal residue of Mbn-NH₂ has a hydrophobic leucine sidechain. Phe124 in MbnN is also a tyrosine in HisC enzymes, and mutation of that tyrosine to phenylalanine differentially affects activity depending on substrate hydrophobicity.^{25,26}

The adoption of HisC homologues for alternate biochemical pathways is not unprecedented. *Salmonella* (*S.*) *enterica* CobD, a L-threonine-*O*-3-phosphate decarboxylase involved in the cobalamin biosynthetic pathway and a close HisC relative²⁸ lacking aminotransferase activity, exhibits similar sequence alterations to MbnN.²⁹ NikK, an aminotransferase involved in the biosynthesis of the nikkomycin family of natural products, provides an even closer parallel to MbnN.³⁰ This enzyme catalyzes the transfer of an amino group to a ketohexuronic acid intermediate. As with MbnN, close homologues (50% sequence identity) are found only in other nikkomycin operons, while other related sequences (closer to 30% identity) appear to encode authentic HisC enzymes. NikK's N-terminal arm is particularly divergent in sequence, but several key residues in the substrate-binding pocket of the active site, equivalent to Phe29 and Phe124, are also phenylalanine, while the PLP-binding region remains highly conserved. The altered N-terminal domain is predicted to be necessary to accommodate the larger natural product intermediate, which would likely apply to MbnN as well. Taken together, these observations suggest that the sequence differences between MbnNs and HisCs are likely to facilitate binding of the hydrophobic N-terminal leucine side-chain of Mbn-NH₂.

Biochemical characterization of MbnN

For biochemical studies, *Ms. trichosporium* OB3b MbnN with a TEV-cleavable C-terminal His₆-tag was heterologously expressed in the BL21-derived *E. coli* strain NiCo21(DE3)

using auto-induction medium.¹⁸ Expression and purification of an N-terminal His₆-tagged MbnN resulted in the formation of higher order oligomers rather than homodimers, likely due to disruption of the ability of the N-terminal arm to mediate homodimer formation.²⁵ Native PAGE analysis after the C-terminal tag was cleaved confirmed that MbnN is a homodimeric enzyme with a monomeric mass of 42 kDa (Figure S4B). The UV-visible absorption spectrum exhibited a peak at 333 nm (Figure S4C), indicating the presence of a cofactor as a form of pyridoxamine 5'-phosphate (PMP) (PLP λ_{max} , 420 nm; PMP λ_{max} 330 nm)³¹. Further characterization by native Top-Down Mass Spectrometry (nTDMS) shows that the MbnN dimer displays some microheterogeneity with four dominant species observed over an average mass range of 85,000–85,300 Da (Figures S5–S7). To investigate the composition of MbnN subunits, they were ejected from the dimer and their charge states deconvoluted to produce the native mass spectrum² with three major forms of MbnN subunits detected (Figure S6). The first of these MbnN proteoforms is within 1.4 Da error of the theoretical mass for N-terminally acetylated, methionine removed MbnN with a PMP cofactor covalently bound (+248 Da). Additional description of these findings is presented in the Supplemental Information, and the N-terminal processing of MbnN was validated using in-gel digestion and bottom-up proteomics (Figure S8).

Transaminase activity of MbnN

An initial screen for MbnN transamination activity was performed using a commercially available aspartate aminotransferase (AST) kit, which detects the transfer of an amino group from aspartate to α -ketoglutaric acid (the biological amine recipient/carbonyl donor for HisC and many other aminotransferases).³² MbnN transamination activity varied depending on buffer chemistry and pH (Figure S9). Buffers tested included tris(hydroxymethyl)aminomethane (Tris), 3-(*N*-morpholino)propanesulfonic acid (MOPS), and a sodium/potassium phosphate buffer. The highest activity was observed in phosphate buffer and at higher pH, and lower activity was observed using MOPS. Dependence of transaminase activity on pH has been observed previously,³⁰ but inhibition by MOPS has not. However, 1-(*N*-morpholino)ethanesulfonic acid (MES) was recently identified in *My. tuberculosis* as a mild inhibitor of HisC (but possibly not other aminotransferases);²⁵ MES and MOPS are both members of the morpholino subgroup of Good's buffers, and if MbnN is indeed so similar to HisC, it is perhaps unsurprising that these buffers can mildly inhibit it as well.

To generate the native substrate for MbnN, the spent media from the growth of *Ms. trichosporium* OB3b *mbnN* containing the secreted Mbn-NH₂ biosynthetic intermediate was partially purified using a Sep-Pak tC18 column and lyophilized. Further purification was not performed due to the increased acid- and photo-lability of Oxa_A in Mbn-NH₂ when not stabilized by copper.¹² When the spent medium was stabilized by the addition of 0.1 mM CuSO₄ prior to purification, a single major product was detected via electrospray ionization liquid chromatography-mass spectrometry (ESI-LC-MS). This product had an *m/z* of 1085.284 Da, corresponding to the [Mbn_{Met}-NH₂ – 2H + Cu^I][–] species observed previously¹² (Figure S10B); loss of the C-terminal methionine is common for *Ms. trichosporium* OB3b Mbn.^{13,33}

For reactions with MbnN, the lyophilized, partially purified apo Mbn-NH₂ was redissolved in Tris buffer (pH 9) and utilized as a substrate in the presence of excess α -ketoglutaric acid as a cosubstrate. The reaction was monitored by UV-vis over 8 h, revealing an increase at 392 nm accompanied by a decrease at 332 nm (Figure 3A). These spectral changes are consistent with replacement of the N-terminal amino group with a carbonyl group, which results in a red shift of the Oxa_A spectral feature.¹² Copper was then added to stabilize the apo Mbn product prior to analysis via ESI-LC-MS. A new major copper-bound compound was observed at a slightly later retention time than Mbn-NH₂. This species exhibited a -1.039 Da mass shift (predicted: $m = -1.03$ Da) compared to the copper-bound Mbn-NH₂ and an m/z of 1084.245 Da, consistent with the [Mbn_{Met}-2H + Cu]⁻ species of mature Mbn (Figure 3B).

Absent external factors, the reactions catalyzed by PLP-dependent aminotransferases are reversible, and when performed in vitro, the amino and carbonyl forms of both cosubstrates will eventually reach equilibrium. This suggests that it might be possible to catalyze the reverse MbnN reaction, generating Mbn-NH₂ from mature Mbn. For the forward reaction, only a limited number of α -keto amino acid derivatives were readily available, and so α -ketoglutaric acid (the cosubstrate for HisC and a common cosubstrate for many aminotransferases) was used. For the reverse reaction, however, fifteen amino acids were screened as amino group donors (Table S3). In reactions with histidine, an increase was observed in the spectral feature with λ_{max} at 342 nm, accompanied by a shift towards 335 nm (both consistent with an increase in the presence of Oxa_A rings without extended conjugation systems) while the feature with λ_{max} at 392 nm decreased (Figure 3C). These changes are consistent with the loss of the carbonyl group and replacement with an N-terminal amino group. ESI-LC-MS analysis confirmed the formation of a new copper-bound compound with a +1.04 Da mass difference (m/z 1216.293 Da) compared to the mature CuMbn (m/z 1215.250 for [Mbn - 2H + Cu]⁻); this mass is also consistent with the formation of [Mbn-NH₂ - 2H + Cu]⁻ (Figure 3D and Figure S11). Thus, MbnN can react with mature Mbn to produce Mbn-NH₂ in vitro. These results not only confirm the functional role of MbnN, but also provide an alternative pathway to generate the fragile Mbn-NH₂ intermediate as a substrate for future studies of Mbn biosynthesis.

Transamination stabilizes Mbn

A remaining question regarding MbnN is why an N-terminal transamination is required for *Ms. trichosporium* OB3b Mbn, since the key copper-binding ligands are already installed in Mbn-NH₂. The comparative instability of Mbn-NH₂ in the absence of copper suggested that the modification may stabilize the compound. This same instability renders it difficult to isolate sufficient quantities of the full-length apo Mbn-NH₂. To further investigate stability, an acid hydrolysis experiment was performed using Mbn-NH₂ obtained from the reverse reaction of Mbn and MbnN; some mature Mbn remained, due to the constraints of the reverse reaction. For mature *Ms. trichosporium* OB3b Mbn, Oxa_A (392 nm) degrades much more slowly than Oxa_B (342 nm).⁶ Upon treatment of a mixture of Mbn and Mbn-NH₂ with 100 mM HCl, the spectral feature at 332 nm (representing a mix of Oxa_A from Mbn-NH₂ and Oxa_B from both Mbn-NH₂ and mature Mbn) almost disappeared within minutes instead of days, as observed for Oxa_A in mature Mbn (Figure 4). This result also indicates increased

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labiality when compared to Oxa_B alone.⁶ Thus, the N-terminal oxazolone is uniquely unstable, and the presence of the carbonyl group does stabilize Mbns from *Ms. trichosporium* OB3b and *Ms. sp. LW4*. Aliquots from the hydrolysis reaction were taken for further analysis with ESI-LC-MS. After 10 min, the major species exhibited a mass shift of -24.78 Da (predicted *m*: -25.98), corresponding to the hydrolysis of a single oxazolone and presumably pH-related protonation of the N-terminal primary amine of the Mbn intermediate (Figures S12 and S13A). When the pH of the sample was increased with ammonium hydroxide, the hydrolyzed Mbn intermediate was also observed without amine protonation (Figure S13B). After 30 min, masses corresponding to the hydrolysis of Oxa_B in mature Mbn as well as fully hydrolyzed Mbn-NH₂ were initially detected (Figure S13C).⁶ Completely hydrolyzed Mbn and Mbn-NH₂ intermediate were observed by ESI-LC-MS after 24 hrs (Figure S13, D and E).

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These results combined with analysis of the compound produced by the *Ms. trichosporium* OB3b *mbnN* strain clearly demonstrate that MbnN catalyzes a transamination reaction that occurs after both oxazolone rings are installed on the precursor peptide MbnA, and after the leader peptide is lost.¹² This conclusion differs from that of a previous study of a *Ms. trichosporium* OB3b *mbnN* strain, in which transamination was proposed to precede formation of Oxa_A.¹³ This proposal was based on the observation of a species in the spent medium that differs in mass from Mbn-NH₂ by -24.98 Da (predicted *m*: -25.98). However, the observed mass corresponds to that of the amine-protonated Mbn intermediate after hydrolysis of Oxa_A. Given the fragility of Mbn-NH₂, hydrolysis rather than incomplete biosynthesis best explains the observed compound.

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MbnN-mediated transamination is the last step of Mbn biosynthesis in *Ms. trichosporium* OB3b and *Ms. sp. LW4*. This modification does not directly affect the copper binding site, but it does significantly stabilize Mbn. Many RiPP natural products have post-translational modifications at or near the N- and/or C-termini that protect the termini from degradation by exopeptidases.³⁴ Protection of the N- and C-termini is a clearly recurring theme for Mbns as well. Perhaps tellingly, Mbns generally lack C-terminal modifications, and C-terminal peptide degradation is correspondingly widespread in structurally characterized Mbns, with the presence of either a disulfide bond or an oxazolone ring seemingly preventing further degradation. By contrast, N-terminal protection is common. In the structurally characterized Group I and II Mbns, N-terminal transamination stabilizes the N-terminal oxazolone in both *Methylosinus* species, while that oxazolone is further modified (possibly by the flavoenzyme MbnF) to form acid-impervious heterocycles in *Methylocystis* species.³⁵ Other stabilizing modifications are predicted to be present in the uncharacterized Mbn families found in non-methanotrophic species. Group III Mbns are predicted to form intramolecular disulfide bonds mediated by an N-terminal cysteine.³⁵ Group V Mbn operons encode an additional uncharacterized biosynthetic protein, MbnX,³⁵ which may play a role in performing additional stabilizing modifications.

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Finally, Group IV Mbn operons appear to have independently recapitulated the strategy described here. These operons also encode PLP-dependent aminotransferases, originally also annotated as MbnN. However, as more sequences have emerged, it is clear that these aminotransferases are not related to the Group I MbnN. Instead, like the HisC-derived

MbnNs, these enzymes are repurposed Class III ω -aminotransferases,³⁶ which carry out transamination reactions on a wide range of substrates including ornithine, lysine, 4-aminobutyrate, adenosylmethionine-8-amino-7-oxanonanoate, alanine, and aspartate. The top non-Mbn-related BLAST results for this Group IV aminotransferase are encoded within proteobacterial operons related to amino acid catabolism, including genes for ABC amine and peptide importers and additional enzymes supporting a role in the metabolism or catabolism of amine-containing amino acids (lysine,³⁷ arginine) or related compounds (GABA,³⁸ ornithine,³⁹ biotin⁴⁰). The reactions catalyzed by the Group IV aminotransferases are likely to be quite distinct from the reactions carried out by their close homologues, just as observed with the Group I MbnN and the HisC-like aminotransferases. A single repurposed aminotransferase gene might have been a happy mistake, but the independent incorporation of two distinct aminotransferase families into two distinct subgroups of Mbn operons underscores how important it is to protect the N-termini and stabilize the N-terminal oxazolones of this unusual family of natural products.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding Sources

No competing financial interests have been declared. Funding for this work was provided by NIH grants GM118035 (A.C.R.) and T32GM105538 (L.F.S.), and AHA grant 14PRE20460104 (G.E.K.). L.F.S. is a Gilliam Fellow of the Howard Hughes Medical Institute.

The Northwestern Keck Biophysics Facility is supported in part by NCI CCSG P30 CA060553. Proteomics services were performed by the Northwestern Proteomics Core Facility, supported by NCI CCSG P30 CA060553 and the National Resource for Translational and Developmental Proteomics supported by P41 GM108569. The Integrated Molecular Structure Education and Research Center (IMSERC) at Northwestern University is supported in part by the Soft and Hybrid Nanotechnology Experimental (SHyNE) Resource (NSF ECCS-1542205), the State of Illinois, and the International Institute for Nanotechnology (IIN). Constructs for MbnN expression were provided by Anthony S. Gizzi and Steven C. Almo at the Albert Einstein Anaerobic Structural and Functional Genomics Resource (Albert Einstein College of Medicine), supported by the Price Family Foundation and NIH grants U54-GM094662, U54 GM093342, P01 GM118303. We thank Dr. Steven Patrie for helpful discussions.

ABBREVIATIONS

Mbn	methanobactin
CuMbn	copper-bound methanobactin
pMMO	particulate methane monooxygenase
RiPPs	ribosomally produced, post-translationally modified peptides
Ms	<i>Methylosinus</i>
HPLC	high-performance liquid chromatography
PLP	pyridoxal 5'-phosphate
PMP	pyridoxamine 5'-phosphate

PAGE	polyacrylamide gel electrophoresis
TEV	tobacco etch virus
AST	aspartate aminotransferase
MOPS	3-(N-morpholino)propanesulfonic acid
MES	1-(N-morpholino)ethanesulfonic acid
ESI-LC-MS	electrospray ionization liquid chromatography-mass spectrometry

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Figure 1.

The transamination reaction catalyzed by MbnN and its role in the biosynthesis of Mbn from *Ms. trichosporium* OB3b. The oxazolone ring at the N-terminus (Oxa_A) has an absorption feature with λ_{max} at 332 nm before transamination and at 392 nm after transamination. The oxazolone ring near the C-terminus (Oxa_B) has an absorption feature with λ_{max} at 342 nm. The C-terminal methionine (gray) is sometimes absent in Mbn after recovery from spent medium.

Figure 2. The active site and N-terminal arm of MbnN are modified to accommodate a substrate with a different shape and charge from histidinol phosphate

(A) A model of MbnN from *Ms. trichosporium* OB3b (red) generated by I-Tasser and aligned against HisC from *My. tuberculosis* (blue, PDB: 4R8D). (B) Stereoview of the aligned active sites of MbnN (white) against PLP-loaded HisC (grey (chain B), dark grey (chain A), yellow (PLP), PDB: 4R8D) from *My. tuberculosis*. Residue labels followed by a single ' correspond to residues that are present on chain A (and are not modeled for MbnN). Underlined residues are altered in MbnN: F29 and F124 are tyrosines in *My. tuberculosis* HisC, while T100 is an aspartate residue. These residues form a more hydrophobic pocket but do not affect interactions with the PLP/PMP cofactor or an α -ketoglutarate-like cosubstrate. (C) Stereoview of the aligned active sites of the MbnN model (white) against PLP- and histidinol-phosphate loaded HisC from *E. coli* (gray (chain B), dark gray (chain A), yellow (PLP), teal (Hsp), PDB: 1FG3). Residue labels that are followed by a single ' are present on chain A (and are not modeled in for MbnN). Underlined residues are altered in MbnN: F29 and F124 are tyrosines in 1FG3, while S99 is an alanine, T100 is an aspartate

residue, and T222 is a leucine while S224 is a threonine. None of the H-bonding interactions with the histidinol group present in the *E. coli* enzyme are possible in the hydrophobic MbnN active site.

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Figure 3. Observing the forward and reverse reactions of MbnN via UV-vis spectroscopy and mass spectrometry

(A) Monitoring the forward MbnN reaction via UV-vis spectroscopy. Absorption spectra were obtained over 8 h during the reaction between semi-purified apo Mbn-NH₂, 20 μ M MbnN and 3 mM α -ketoglutaric acid. 0.5 mM DTT was also added to reduce the disulfide bond in Mbn-NH₂. Inset: difference spectra against the initial timepoint. (B) Negative ion mode ESI-LC-MS spectrum after the reaction described in (A), after the addition of 0.1 mM CuSO₄ to stabilize the resulting compound. The characteristic copper isotopic distribution is visible. (C) Monitoring the reverse reaction between MbnN and Mbn via UV-vis spectroscopy. Absorption spectra were collected over an 8 h reaction between 20 μ M apo Mbn with 25 μ M MbnN and 10 mM L-histidine, including 0.5 mM DTT to reduce the disulfide bond in Mbn. Inset: difference spectra against the initial timepoint. (D) Negative ion mode ESI-LC-MS spectrum of the reaction described in (C) after stabilization with 0.1 mM CuSO₄, revealing the presence of copper-bound Mbn-NH₂ (CuMbn-NH₂) along with unreacted and copper-bound mature Mbn (CuMbn).

Figure 4.

Acid hydrolysis of a mixture of Mbn and the Mbn-NH₂ intermediate. Mbn-NH₂ was generated by the reverse reaction of MbnN with L-histidine; this reaction is incomplete, but the instability of the intermediate precludes an extended cleanup step. Acid hydrolysis of the compounds was carried out in 100 mM HCl solution and monitored for 24 hrs. Inset: decrease in absorbance at 342 nm (red, representing both oxazolones in the intermediate and Oxa_B in the mature Mbn) and at 392 nm (blue, Oxa_A in the mature Mbn) over time.