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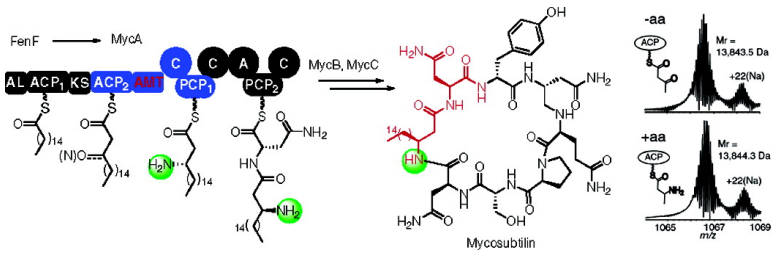

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Characterization of a New Tailoring Domain in Polyketide Biogenesis: The Amine Transferase Domain of MycA in the Mycosubtilin Gene Cluster

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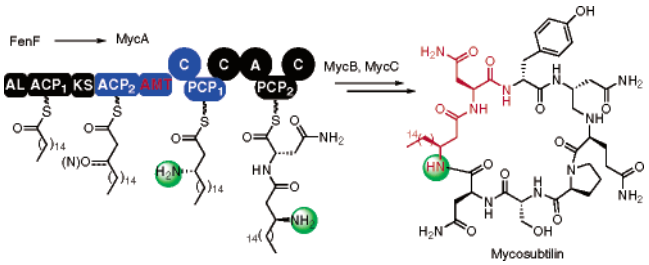
The Iturin family of natural products, isolated from various strains of *Bacillus subtilis*, are characterized by a cyclic lipopeptide structure and possess potent anti-fungal activity.[1] Sequencing of the biosynthetic gene clusters for several members of this family identified both polyketide synthase (PKS) and non-ribosomal peptide synthetase (NRPS) biosynthetic motifs.[2] The gene cluster of mycosubtilin, the first iturin to have its gene cluster identified, encodes four enzymes, one of which (MycA) is particularly complex (Scheme 1).[2a] MycA, a 459 kDa hybrid PKS/NRPS which has extra thiolation (PCP_1) and condensation (C) domains, utilizes an *in trans* acyl transferase domain (fenF) and contains a novel amino transferase (AMT) domain located at the hybrid interface of the PKS and NRPS modules (Scheme 1). The AMT domain of *myc*A shows significant (54%) similarity to glutamate semialdehyde amino transferase (a pyridoxal 5′-phosphate (PLP)-dependent enzyme).[2a]

On the basis of its location, the AMT domain of MycA is proposed to be responsible for the transfer of an amine to the β-position of the growing acyl chain. Using diverse assays, we confirm the function of the AMT domain by identifying the preferred amine donor substrate, dissecting the mechanistic steps of amine transfer, and deciphering the location of amine transfer. The expression and precise characterization of an AMT domain in this paper represents the first detailed study of a PLP-dependent enzyme operating *in cis* within the PKS and NRPS biosynthetic paradigm.

A PLP-dependent AMT domain operating *in cis* in a PKS is striking for two main reasons. First, PLP-dependent domains are rare in thiotemplate assembly lines, having been identified in only three systems: the iturins,[2] the mycrocystins,[3,4] and the prodigiosins.[5] Second, the ability to incorporate amine functionality directly into polyketides would create both a functional handle for later derivitization (e.g., macrocyclization) and a potent hydrogen bond donor to significantly alter the biology of a given compound.

This report focuses on the four-domain fragment of MycA (MycA4N7) shown in blue in Scheme 1. MycA4N7 was cloned and expressed as an N-terminally His_6-tagged 137 kDa protein fragment. This species was shown to incorporate up to two equivalents of radiolabel upon exposure to [^{14}C]-acetyl coenzyme A and Sfp, confirming the presence and activity of the thiolation domains ACP_2 and PCP_1.[6] Examination of the UV absorption spectra of MycA4N7 identified a characteristic absorbance maximum at 420 nm, indicating the presence of an enzyme-bound PLP Schiff base.[7] Titration with PLP indicated that the enzyme maintained ~95% PLP loading upon isolation. The presence of PLP was further confirmed by incubating the enzyme with 5 mM hydroxylamine, resulting in a shift in the absorbance maximum from 420 to 385 nm, consistent with the formation of a PLP−hydroxylamine oxime.[8,9]

Scheme 1. Mycosubtilin Biosynthesis



The proposed AMT-mediated amine transfer involves two distinct mechanistic steps (half-reactions) that can be tracked by changes in the UV absorption maxima of the cofactor (Scheme s1).[10] The first step involves amine transfer from an amino acid to the protein-bound PLP (UV absorption maximum = 420 nm); this forms the corresponding α-ketoacid and pyridoxamine 5′-phosphate (PMP; UV absorbance maximum = 335 nm). The second half-reaction is the transfer of the amine from PMP to a protein-bound β-ketothioester substrate to generate the corresponding protein-bound β-aminothioester, which also regenerates the protein-bound PLP.

Amine transfer to the PLP cofactor bound to the AMT domain was investigated by incubating MycA4N7 with various amino acids, resulting in a shift in the absorbance maximum from 420 to 335 nm (data not shown). In this screen glutamine (Gln) facilitated the fastest initial conversions. The observation that Gln is the preferred amine source is surprising, considering that it is rarely used in this capacity in non-eukaryotic aminotransferases.[11]

Different amino acids gave different overall conversions, with Gln facilitating the largest overall conversion (1 equiv of Gln resulted in ~50% conversion). To probe the reversibility of amine transfer to the PLP cofactor, the PMP form of MycA4N7 was exposed to radiolabeled pyruvic acid. This resulted in the formation of radiolabeled Ala, indicating that the amine-transfer reaction is reversible.[10] The observation that the AMT domain mediates reversible amine transfer with amino acids clearly demonstrates that MycA4N7 has a functional aminotransferase domain. However, it does not provide an indication of how the AMT domain interacts with its proposed substrate, enzyme-bound β-ketothioesters. To answer this question, the full reaction was investigated using large-molecule mass spectrometry.

To directly interrogate the thiolation domains of MycA4N7, peptides containing the active-site serines of both the ACP_2 and PCP_1 domains were identified by ESI−FTMS from fractionated proteolytic mixtures obtained through trypsin digestion.[12] To generate a carrier-domain-bound amine-acceptor substrate, MycA4N7 was treated with acetoacetyl (AcAc) coenzyme A and Sfp.[6] Because

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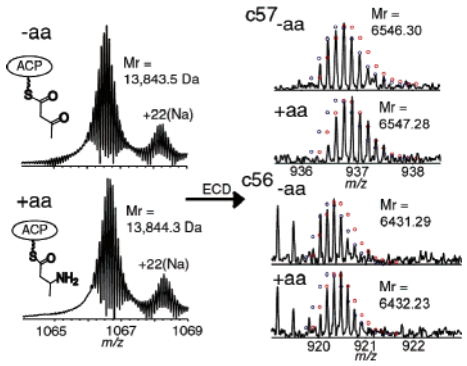

Figure 1. Identification of amine transfer by FTMS. On the left is the intact trypsin fragment. On the right are two fragments obtained by ECD that contain the active site. The blue trace corresponds to a theoretical fit for acetoacetyl-loaded ACP_2, while the red trace is the theoretical distribution of the aminoacetyl-S-ACP_2.

the conversion of a carbonyl to a secondary amine results in a 1 Da increase in mass, ESI−FTMS was used to observe this activity. To observe the initial activity, acetoacetyl-loaded MycA4N7 was incubated with a mixture of proteinogenic amino acids. In this reaction, a newly formed peak eluting at 35.5 min had a mass of 13 844.3 Da, 1 Da larger than the AcAc loaded form of ACP_2 (eluted at 36.5 min, M_r = 13843.5 Da, Figure 1). Using ECD, a tandem mass spectrometric method, 12 c (as example, c57 and c56 are shown in Figure 1) and 3 z ions containing the active site were generated which displayed the 1 Da mass increase. These fragment ions localized the 1 Da increase to the four residues, SIML, containing the active-site serine. In the same reaction, a similar 1 Da mass increase was observed for PCP_1. The increase of the peak corresponding to the aminobutyrate-loaded ACP_2 was used to "screen" for the best amine donor, identifying Gln, the same amino acid identified by the UV−vis assay. Examination of ^{15}N-labeled Gln in the reaction resulted in a mass increase of 2 Da for the ions corresponding to the β-aminobutyryl-S-ACP_2 and β-aminobutyryl-S-PCP_1 domain but only when the ^{15}N label was at the α position. The formation of β-aminobutyrate in a reaction with Gln was further solidified by hydrolysis of β-aminobutyrate from the carrier domain and coelution of the *o*-phthaldehyde derivative with an authentic sample.[13] Monitoring the time course by FTMS of a reaction containing MycA4N7 and α-^{15}N-Gln indicated that amine transfer takes place on ACP_2 (~40% conversion in 4 min) before some +2 Da could be detected for PCP_1 (est. <10% in 32 min). The +2 Da on PCP_1 is likely the result of transfer of the β-aminobutyrate from ACP_2 to the of holo form of PCP_1, which inadvertently resulted from non-enzymatic hydrolysis of the acetoacetyl-S-PCP_1 during the course of the reaction.

The reversibility of the second step of the amine transfer was also examined *in cis* using FTMS. Samples of MycA4N7 were phosphopantethienylated using Nvoc-protected β-aminobutyryl coenzyme A.[6] Removal of the carbamate protecting group resulted in the formation of β-aminobutyryl-S-PCP_1, β-aminobutyryl-S-ACP_2, and acetoacetyl-S-ACP_2. There was no acetoacetyl-S-PCP_1 detected. These results indicate that the second step is reversible and provide additional evidence that the amine-transfer reaction is taking place while the β-keto-thioester substrate is attached to ACP_2.

These results paint a clear picture of the function and mechanism of the AMT domain of MycA: Reversible amine transfer from Gln to enzyme-bound PLP results in the formation of an enzyme−PMP complex. At this point, displacement of the amino-acid-derived α-ketoacid by an ACP-bound β-ketothioester and reversible amine transfer generates the corresponding covalently tethered β-aminothioester. As all of the steps of this reaction are reversible, it is not known what drives the reaction to completion in vivo; it is likely that the reaction is driven by transfer of the β-aminothioester to the downstream Gln loaded on the PCP_2 domain of MycA.

The specific location of the AMT domain at the hybrid interface of PKS and NRPS may be of significance either as a point of insertion of additional domains in the assembly line or for functional communication with the catalytic modules upstream and downstream. This demonstration of a functioning AMT domain operating *in cis* with a PKS ACP module represents a powerful domain for combinatorial biosynthesis, potentially allowing direct access to amine-containing polyketides.

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Supporting Information Available: Experimental details of protein preparation and analysis of amine transfer, including detailed descriptions of FTMS studies. This material is available free of charge via the Internet at http://pubs.acs.org.

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