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Characterization of Acyl Carrier Protein-Dependent Glycosyltransferase in the Mitomycin C Biosynthesis

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Abstract

Mitomycins are a group of antitumor natural products that are biosynthesized from aminohydroxybenzoic acid (AHBA) and N-acetylglucosamine (GlcNAc). While the biosynthetic gene cluster was reported two decades ago, the mechanism by which the two building blocks, AHBA and GlcNAc, are coupled during biosynthesis remained uncharacterized. Here we report evidence that AHBA is first loaded onto an MmcB acyl carrier protein (ACP) by a MitE acyl ACP synthetase, followed by a transfer of GlcNAc from UDP-GlcNAc by MitB. The results suggest that the early steps of mitomycin biosynthesis proceed via intermediates linked to MmcB.

SYNOPSIS TOC

Mitomycins are antitumor natural products that are reductively activated in hypoxic cells and crosslink CpG sites in DNA, hence their selective activity in hypoxic tumor cells.¹ As a consequence, mitomycin C (MMC, Scheme 1) has been used clinically to treat various cancers including gastro-intestinal, anal, and breast cancers. Despite the medical significance, the mechanism of mitomycin biosynthesis remains largely uncharacterized. Mitomycins are biosynthesized from AHBA and GlcNAc. A biosynthetic gene cluster (BGC) with 47 genes from *Streptomyces lavandulae* was reported in 1999,² and gene knockout studies were performed to demonstrate their essentiality in mitomycin biosynthesis.² While methyltransferases responsible for the late stage modifications have been characterized through genetic and enzymatic approaches,^{3–5} the mechanism of early steps of the biosynthesis is mostly unexplored. Previous gene knockout studies revealed no biosynthetic intermediates from early steps in mitomycin biosynthesis.² As a result, large

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Author Contributions

K.Y. conceived and supervised research. H.P.N. performed experiments. H.P.N. and K.Y. analyzed data and wrote the paper.

The Supporting Information is available free of charge on the ACS Publications website. Methods and materials, SDS-PAGE of MmcB, MitE, and MitB; UV-vis absorption, mass, and NMR spectra of SNAc-AHBA-GlcNAc; mass spectra of AHBA-cystamine and AHBA-GlcNAc-cystamine.

Accession codes: MitB (UniProt ID Q9WW09), MitE (UniProt ID Q9X5R7), and MmcB (UniProt ID Q9X5S0).

majority of the mechanism of backbone construction, including the formation of the fused pyrrolidine and the aziridine rings, remain unknown.

One of the key steps in the MMC biosynthesis is the coupling of AHBA and GlcNAc. Initial insights into this process were obtained from biosynthetic studies on pactamycin. Pactamycin is biosynthesized from GlcNAc and aminobenzoic acid, and some of its biosynthetic genes are homologous to those of mitomycins.⁶ Thus, the two pathways are thought to share similar mechanisms. In pactamycin biosynthesis, a glycosyltransferase, PctL, was reported to catalyze the transfer of GlcNAc to 3-aminoacetophenone.⁶ Based on this observation, a PctL homolog in the mitomycin pathway, MitB, was proposed to catalyze an analogous reaction; transfer of GlcNAc to AHBA free acid (Scheme 1, mechanism A).⁶ 3-Aminoacetophenone, however, was later shown unlikely to be a biosynthetic intermediate based on the absence of incorporation of 3-aminoacetophenone into pactamycin.⁷

On the other hand, an alternative hypothesis was proposed¹ where MitB catalyzes a glycosylation of AHBA covalently attached to ACP based on the presence of a *mmcB* gene that encodes a putative ACP in the mitomycin BGC (Scheme 1, mechanism B). However, no evidence has been reported so far.⁸ In addition, while modifications of ACP-linked intermediates are known for post-polyketide synthase (PKS) modifications,⁹ to our knowledge, the use of ACP as a carrier of biosynthetic intermediates in a pathway independent of PKS or fatty acid synthase (FAS) is unprecedented. Therefore, a significant ambiguity remains about the early steps in the biosynthesis of mitomycins and the timing of the involvement of ACP. Here, we report *in vitro* characterization of MitB and two additional biosynthetic enzymes, MmcB, and MitE (an acyl ACP synthetase), which together provide the first evidence that the glycosylation and likely the following steps of mitomycin biosynthesis proceeds via ACP-linked intermediates. The results are consistent with the absence of accumulation of early intermediates in mitomycin biosynthesis and set foundation for future characterization of subsequent steps.

We started our investigation by characterizing recombinant MitB using AHBA and an SNAc analog of MmcB-linked AHBA (SNAc-AHBA, Fig. 1a). Initially, we were unsuccessful in purifying soluble MitB with the deposited amino acid sequence.² Thus, we revisited the sequence of MitB and found that the deposited sequence was missing the N-terminal 43 amino acid residues that are highly conserved among closely related homologs. When we investigated the upstream region of the *mitB* gene, we identified an alternative start codon. When MitB was re-cloned from the upstream start codon, the resulting protein was highly soluble and purified to homogeneity with the yield of ~12 mg per g cell paste (Fig. S1).

SNAc-AHBA was chemically synthesized and used as a substrate of MitB assay in the presence of UDP-GlcNAc as a glycosyl donor substrate. When the MitB assay solutions were analyzed by anion exchange HPLC (Fig. 1b, trace 1), formation of UDP (retention time 4.9 min) and another peak was observed at a retention time 1.7 min with a concomitant consumption of UDP-GlcNAc. Neither of these two product peaks was observed when MitB or SNAc-AHBA was omitted from the reaction (traces 2 and 4). When the assay was performed using AHBA as the sugar acceptor substrate, neither UDP nor any modified AHBA was formed. These observations suggested a glycosyltransferase activity of MitB

with SNAc-AHBA and not with free AHBA. The 1.7 min peak in the MitB assay with SNAc-AHBA was isolated and structurally characterized by UV-vis absorption, NMR and mass spectrometry (Table S1, Fig. S3–S8). The isolated compound exhibited an UV absorption band centered at 336 nm, characteristic for AHBA thioesters. The high-resolution MS showed m/z of 458.161, which is consistent with SNAc-AHBA with GlcNAc (calculated $m/z = 458.160$). The HMBC correlations observed between C3-H1' and C3-NH1' were consistent with a transfer of GlcNAc to the N-3 of AHBA. The $^3J_{H1'-H2'}$ coupling constant of 8.0 Hz was consistent with a β -glycosidic linkage. Based on these observations, we assigned the structure of the MitB product as SNAc-AHBA-GlcNAc (Fig. 1a), which suggests that MitB catalyzes a transfer of GlcNAc to N-3 of SNAc-AHBA with an inversion of configuration to form the N - β -glycoside. The absence of activity with free AHBA suggests that the free acid is unlikely the physiological substrate, and MitB acts on AHBA covalently linked to MmcB (MmcB-AHBA).

The steady-state kinetics of MitB catalyzed glycosylation of SNAc-AHBA was characterized under pseudo-first order conditions. SNAc-AHBA exhibited a very high K_m value (10 ± 3.3 mM, Fig. 1c), suggesting that SNAc is only a partial mimic of the phosphopantetheine arm of *holo*-MmcB, and the native substrate is likely AHBA linked to MmcB. The K_m and apparent k_{cat} values for UDP-GlcNAc were determined at an unsaturated concentration of SNAc-AHBA (2 mM) due to the high K_m value of SNAc-AHBA. The observed K_m value (0.94 ± 0.15 mM, Fig. 1d) was consistent with the reported physiological concentration of UDP-GlcNAc in bacteria (0.1 – 9.2 mM).^{10, 11} Overall, these kinetic characterizations were consistent with the model that UDP-GlcNAc and MmcB-AHBA are likely the physiological sugar donor and acceptor substrates for MitB.

To obtain further evidence that MmcB-AHBA is the substrate of MitB, reactions with MmcB-loaded substrates were investigated. In the MMC biosynthetic gene cluster, MitE was the only enzyme that has a significant sequence homology to CoA ligases or any other enzymes that could potentially activate and load AHBA onto MmcB. Therefore, we first characterized the functions of recombinant MmcB and MitE. The thiol quantitation of MmcB using Ellman's reagent suggested that only 29% of as-isolated MmcB was phosphopantetheinylated. The partial phosphopantetheinylation of MmcB was also confirmed by MS (Fig. S9). Therefore, as-isolated MmcB was incubated with CoA and phosphopantetheinyltransferase, *sfp*, after which 100% of MmcB was phosphopantetheinylated.

The resulting *holo*-MmcB was first used to investigate a loading of AHBA by MitE. MitE was originally annotated as Co-A ligase,² but our bioinformatic analysis suggested that it more closely aligns with acyl-ACP synthetases, which catalyze adenylation of an acyl group followed by the transfer of the acyl group to the phosphopantetheine arm of ACP. Thus, we investigated the ability of MitE to load AHBA to *holo*-MmcB in the presence of ATP. The loading of AHBA to MmcB was assessed by two means. In the first approach, the reactions were quenched by precipitating the proteins with ethanol, followed by an incubation with potassium hydroxide (KOH) to hydrolyze the thioester bond. The resulting solution was analyzed by HPLC. In the complete reaction, AHBA was observed to an amount 21% of MmcB (Fig. 2a). In the negative controls without ATP or MitE, the amounts of AHBA

observed were less than 1.5% of *holo*-MmcB, which is likely a carryover via adventitious binding of AHBA. In the negative control without *holo*-MmcB, we observed slightly higher level of AHBA (4% of *holo*-MmcB), which we attributed to the presence of adenylated AHBA in the MitE active site.

To obtain further evidence of the loading of AHBA to MmcB, we performed another assay based on a cysteamine thiol exchange originally developed for characterization of the product of CalE8, an iterative enediyne PKS.¹² In this approach, an ACP-loaded biosynthetic intermediate is first released from ACP by a thioester exchange with cysteamine followed by an intramolecular S,N-acyl shift and oxidation to form a cystamine amide conjugate¹² (Fig. 2b). Indeed, when the MitE assay solution was incubated with cysteamine, we observed a compound with an absorption band at 310 nm characteristic for AHBA-related compounds, which co-migrated with a synthetic standard of AHBA-cystamine amide conjugate (AHBA-cystamine, Fig. 2c). The HR-MS characterization of this compound was also consistent with AHBA-cystamine (Fig. S10). Quantitation of AHBA-cystamine formed in the MitE assay accounted for 45% of *holo*-MmcB. We also investigated the ability of MitE to catalyze the ATP-dependent ligation of CoA and AHBA, but observed no evidence of such activity (Fig. S11). These evidences in combination suggest that MitE catalyzes the loading of AHBA onto *holo*-MmcB.

Based on these observations, we performed MitEMitB coupled assay. When the KOH release method was used, the complete reaction condition yielded AHBA-GlcNAc with the amount 30% of *holo*-MmcB (Fig. 2d and S12). The omission of UDP-GlcNAc resulted in a complete abolishment of AHBA-GlcNAc formation, and instead resulted in AHBA formation, which suggested the accumulation of MmcB-AHBA. When the cysteamine release method was used, the complete reaction condition yielded AHBA-GlcNAccystamine based on HPLC and HR-MS analyses (Fig. 2e and Fig. S13). The amount of AHBA-GlcNAccystamine was 67% of *holo*-MmcB. The difference in the amount of product release between the KOH release and cysteamine release may be due to the difference in the efficiency of thioester cleavage between the two methods.

The intact MmcB with and without its incubation with MitE and MitB was also characterized by MS. The *holo*-MmcB was observed as an N-terminal methionine cleaved peptide as well as its gluconoylated derivative, a known posttranslational modification at the N-terminus of His-tagged proteins¹³ (Fig. 3a). When this *holo*-MmcB was incubated with MitE and MitB at 30 °C for four hours in the presence of ATP, AHBA, and UDP-GlcNAc, a mass shift of 338.3 – 338.6 Da from *holo*-MmcB was observed (Fig. 3b). These mass shifts are within the error of measurements to the predicted mass shift upon modification of *holo*-MmcB with AHBA-GlcNAc (addition of 338.1 Da). These comprehensive characterization of MitB and MitE together suggests that MitE catalyzes the loading of AHBA onto MmcB, which is subsequently *N*-glycosylated by MitB.

The studies described here provide comprehensive characterization of MitB activity, which together demonstrates that MitB acts on AHBA covalently linked to MmcB, suggesting that the early steps of MMC biosynthesis proceed with the intermediates linked to MmcB. MitB did not catalyze the glycosylation of free AHBA, suggesting that the presence of a negative

charge prevents AHBA from being accepted as a substrate of MitB. Similar ACP-dependent biosynthesis mechanism is also conceivable for pactamycin. The reported activity of PctL was assayed with 3-aminobenzophenone that does not have carboxylate, which might have allowed PctL to accept free 3-aminobenzophenone and catalyze the transglycosylation. In fact, while this manuscript was in revision, a paper by Kudo et al.¹⁴ was published online, in which PctL was shown to catalyze a transfer of GlcNAc to ABA loaded to the cognate ACP (PctK). Thus, the two pathways are likely unique examples of natural product biosynthesis that proceed with ACP-linked biosynthetic intermediates. Especially, MMC pathway is unique in that ACP is used independent of PKS or FAS. Intriguingly, our preliminary phylogenetic analysis suggests that MmcB and PctK are phylogenetically distinct from ACP for PKS, or peptidyl carrier proteins for nonribosomal peptide synthetases, and closer to ACPs for bacterial fatty acid synthases (Fig. S14). Further understanding in mitomycin and pactamycin pathways will extend our understanding in the role of ACP in non-PKS/FAS/NRPS pathways.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

MMC	mitomycin C
AHBA	3-amino-5-hydroxybenzoic acid
ACP	acylcarrier protein
PKS	polyketide synthase
FAS	fatty acid synthase
NMR	nuclear magnetic resonance
MS	mass spectroscopy
HMBC	heteronuclear multiple bond correlation
CoA	coenzyme A
UDP	uridine 5'-diphosphate
GlcNAc	N-acetylglucosamine

UDP-GlcNAc	uridine 5'-diphospho- <i>N</i> -acetylglucosamine
ATP	adenosine 5'-triphosphate

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Figure 1. Characterization of MitB activity using SNAc-AHBA. (a) MitB assay using an SNAc analog of AHBA. (b) Anion exchange HPLC analysis of MitB assays. Traces shown are the assay with SNAc-AHBA as substrate (trace 1), without MitB (trace 2), an assay with AHBA as substrate (trace 3), and an assay without AHBA or SNAc-AHBA (trace 4). (c and d) Pseudo-first order steady-state kinetic analysis with varying concentration of UDP-GlcNAc (0.10, 0.20, 0.40, 0.80, 1.6, 3.2, 6.4, and 10.0 mM) in the presence of 2 mM SNAc-AHBA (c), or with varying concentration of AHBA-SNAc (0.10, 0.20, 0.40, 0.80, 1.6, 3.2, 6.4, and 10.0 mM) in the presence of 2 mM UDP-GlcNAc (d). The solid lines represent non-linear fit to the Michaelis-Menten equation. Each point represents an average of two or three repeats, and error bars are standard deviation.

Figure 2.

MitE and MitB activity assays. HPLC analyses of MitE (a and c) and MitE-MitB coupled assay (d and e) using the KOH release method (a and d) or cystamine release method (c and e). AHBA and AHBA-GlcNAc were detected by anion exchange HPLC (a and d), and AHBA-cystamine and AHBA-GlcNAc-cystamine were analyzed by reverse phase HPLC (c and e). The identity of AHBA-cystamine and AHBA-GlcNAc-cystamine was confirmed by co-injection with the synthetic standards as well as HR-MS. (b) Release of AHBA and AHBA-GlcNAc from MmcB as cystamine adduct in the cystamine release method.

Figure 3. Deconvoluted mass spectra of *holo*-MmcB (a) and *holo*-MmcB incubated with MitE and MitB in the presence of AHBA, ATP, and UDP-GlcNAc (b). Shown are the molecular weights determined after deconvolution of multiply charged ions and stable isotopes. The mass accuracy is within 1.0 Da. Signals are labeled with assigned species with observed and theoretical molecular weights. Only the most abundant mass was assigned.

Scheme 1.
Possible mechanisms of AHBA glycosylation.