J|A|C|S COMMUNICATIONS

Published on Web 02/12/2009

A PLP-Dependent Polyketide Chain Releasing Mechanism in the Biosynthesis of Mycotoxin Fumonisins in *Fusarium verticillioides*

Ryan Gerber, Lili Lou, and Liangcheng Du*

Department of Chemistry, University of Nebraska-Lincoln, Lincoln, Nebraska 68588

Received November 20, 2008; E-mail: ldu@unlserve.unl.edu

Filamentous fungi produce numerous polyketide metabolites.[1] Many of the metabolites have important biological activities and fascinating molecular architectures. Fungal polyketides are synthesized by iterative polyketide synthases (PKSs), which can be divided into three groups, nonreducing PKS (NR-PKSs), partially reducing PKS (PR-PKSs), and highly reducing PKS (HR-PKSs).[1] Among them, HR-PKSs are the least understood group. HR-PKSs synthesize economically important metabolites such as mycotoxin fumonisins and anticholesterol lovastatin.[2] The domain architecture of fungal HR-PKSs typically consists of KS-AT-DH-MT-ER-KR-ACP, which does not contain a TE/CLC (thioesterase/Claisen-like cyclase), as seen in other types of PKSs.[1,3] It has been unclear how the HR-PKS offloads the linear polyketide chain. Here, we report a pyridoxal 5′-phosphate (PLP)-dependent mechanism for the chain release in the biosynthesis of fumonisins in *F. verticillioides*. The results show that a 2-oxoamine synthase encoded by *FUM8* is responsible for offloading and elongating the carbon chain and introducing an amino group into the fumonisin backbone (Figure 1).

Previous labeling experiments had shown that 18 carbons (C3−20) of the 20-carbon backbone of fumonisins are derived from acetate, and 2 carbons (C1−2), as well as the C-2 amino, are derived from alanine.[4] The two methyl groups at C-12 and C-16 are derived from methionine.[5] The hydroxyls at C-5, C-10, C-14, and C-15 of FB_1 (**1**) are derived from molecular oxygen.[6] Therefore, the initially synthesized carbon chain is a highly reduced polyketide. In the fumonisin biosynthetic gene cluster, *FUM1* was predicted to encode a seven-domain PKS (Fum1p, 2507 residues), whereas *FUM8* was predicted to encode a protein (Fum8p, 839 residues) that is homologous to L-serine palmitoyltransferase (SPT), which is a member of the 2-oxoamine synthase family.[7] 2-Oxoamine synthases are a small family of enzymes involved in several very important primary biosynthetic pathways, including SPT in ceramide-sphingolipid biosynthesis, 5-aminolevulinate synthase (ALAS) in heme biosynthesis, and 8-amino-7-oxononanoate synthase (AONS) in biotin biosynthesis.[8] The enzymes use PLP as a cofactor to catalyze the decarboxylative condensation between an amino acid and an acyl-CoA. To test if fumonisin biosynthesis has adopted a mechanism used by the primary metabolisms, we first expressed the ACP domain of *FUM1* in *E. coli* and obtained the pure protein for in vitro assays (Figure S1). An acyl chain was then loaded to the ACP from various acyl-CoA's using a "promiscuous" 4′-phosphopantetheinyl transferase (PPTase), Svp.[9] Lauroyl-, myristoyl-, palmitoyl-, stearoyl-, and arachidoyl-CoA were used in the studies. The success of the acyl-*S*-phosphopantetheinylation of the ACP was confirmed by measuring the mass change of the protein using MALDI-TOF and/or the detection of the predicted acyl-*S*-pantetheinyl ions from the phosphopantetheine ejection assay using ESI Q-TOF.[10] As shown in Table-S1 and -S2, the ACP could be loaded with an acyl chain with a length of C12 to 20.

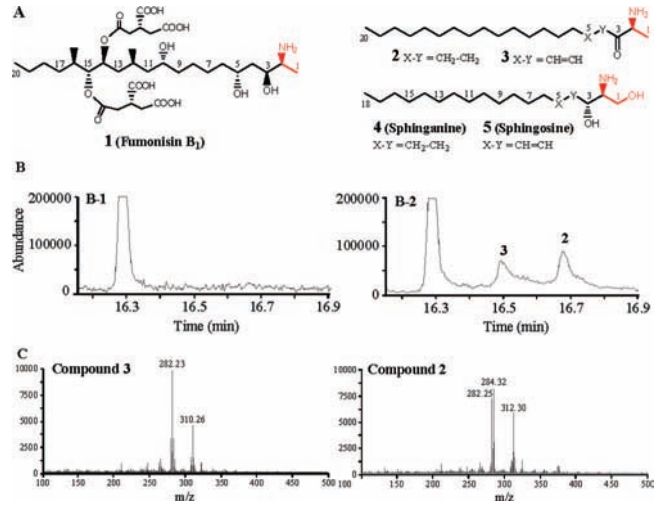

Figure 1. (A) Chemical structure of fumonisin B1, 3-keto products of Fum8p, and related sphingoids. (B) GC analysis of reactions with microsomes from yeast cells with vector alone (B-1) and from yeast cells with pYES2/FUM8 (B-2). (C) MS of the two new peaks appearing in B-2.

To test if *FUM8* is responsible for offloading the acyl chain, we transferred the full length cDNA of *FUM8* into pYES2/NT C to express in yeast INVSc1, after having exhausted various *E. coli* expression systems (see Supporting Information). Microsomes were prepared from yeast cells carrying the vector alone and from cells carrying pYES2/*FUM8*, according to the methods developed for other 2-oxoamine synthases.[11] When C18-*S*-ACP was incubated with the microsomes from pYES2/*FUM8* yeast, two new peaks (retention time 16.5 and 16.7 min) were detected by GC−MS, which were not present in the reaction containing microsomes from pYES2 alone (Figure 1B). Furthermore, these two peaks were not observed if the reactions omitted PLP, C18-CoA, ACP, L-alanine, or the microsomes. The peak at 16.7 min has a $[M+1]^+$ of 312.30 (Figure 1C), which is consistent with the predicted mass (311.32) for the decarboxylative condensation product (**2**) between alanine and C18-CoA. The peak at 16.5 min has a $[M+1]^+$ of 310.26 (Figure 1C), which is coincident with the predicted mass (309.30) for the further dehydrogenated product (**3**) (Figure 1A). Yeast microsomes are known to contain enzymes for sphingolipid biosynthesis, including SPT that synthesizes sphinganine (**4**) from L-serine and C16-CoA and a dehydrogenase that converts sphinganine (**4**) to sphingosine (**5**).[8] It is plausible that the direct product (**2**) of Fum8p was further oxidized to **3** by this dehydrogenase. The results show that Fum8p is able to catalyze the PLP-dependent release of the C18 chain from the C18-*S*-ACP, with a concurrent incorporation of two carbons and one amino group from alanine into the acyl chain. It is noteworthy that the 3-keto group of **2** and **3** was not further reduced by sphinganine 3-ketoreductase in yeast.[12]

10.1021/ja8091054 CCC: $40.75

This is likely due to the difference in stereospecificity of the ketoreductases. Sphingolipids have a 3*R* hydroxyl, whereas fumonisins have a 3*S* hydroxyl (Figure 1A).

To obtain more evidence for the relation of compounds **2** and **3**, we followed a 60-min time course for their production (Figure S2A). Both compounds showed a nearly linear increase in the first 5 min of reaction. Compound **2** gradually decreased after 5 min, while compound **3** increased gradually. Interestingly, the total amounts of the two compounds remained nearly constant after 5 min of reaction. Together with the GC−MS data, the results support that compound **3** is a further oxidized product of compound **2**.

Although acyl chains with C12 to C20 in length could be loaded to the ACP, C18-*S*-ACP is the preferred substrate for Fum8p (Figure S2B). The product that resulted from alanine and C16 condensation showed a similar level (∼41 μM) of production in the first 5 min but quickly dropped to a much lower level (∼10 μM), probably due to the fact the C16 product is closest to the 3-keto intermediate in the sphingolipid pathway and could be quickly metabolized to other sphingoids by the microsomal enzymes.[8] A low level (∼10 μM) of the product expected from alanine and C14 was observed, but no clear product was observed from alanine and C12 or C20. The results are consistent with the proposed biosynthetic mechanism for fumonisins, where a C18 chain is incorporated into the carbon backbone.[2] The biochemical data are also in agreement with our previous studies using genetic approaches.[13] In KS-domain replacement experiments, we switched the fumonisin KS domain with the KS for synthesizing the C41-long chain of T-toxins in *Cochliobolus heterotrophus* and the KS for making the 4-carbon side chain of lovastatin in *Aspergillus terreus*. The results implied that the polyketide chain-releasing enzyme plays an important role in the formation of a distinct product. In PKS functional complementation experiments, we transformed a *FUM1* deletion mutant with the PKS gene (*ALT1*) for AAL-toxin biosynthesis in *Alternaria alternata*. Both fumonisins and AAL-toxins belong to sphinganine-analogue toxins, due to their structural similarity to sphingoids, and they differ mainly in the lengths of their carbon backbones, with fumonisins being from a C18 and alanine while AAL-toxins are from a C16 and glycine.[2] However, the *ALT1*-transformed mutant produced fumonisins, showing that Alt1p is able to synthesize a C18 chain, in spite of the fact that it only synthesizes the C16-derived AAL-toxins in its native host. Since only the products with a specific chain length are produced in these fungi, the results support that the substrate specificity of the chain-releasing enzyme is the key factor for producing a distinct group of fungal polyketides. Now, our in vitro data further confirm that C18 is the preferred substrate among the acyl chains tested, although C18 itself is not the native substrate for fumonisins (12,16-dimethyl C18 is expected to be the native substrate).[2]

Fungal HR-PKSs do not contain a TE domain that is found in bacterial PKSs for offloading reduced polyketides or a CLC domain

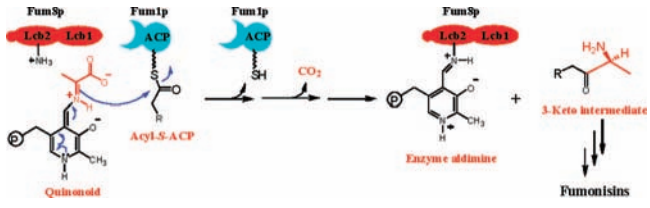

Figure 2. PLP-dependent polyketide chain release in the biosynthesis of fumonisins. Note that Fum8p is predicted to contain a small membrane associated domain, the catalytic domain (Lcb2, long-chain base subunit 2), and the regulatory domain (Lcb1) that are naturally fused in the same protein.[7]

that is found in fungal NR-PKSs for offloading fungal aromatic polyketides.[1,3] TE uses the well-characterized catalytic triad to release a polyketide chain, typically using a nitrogen or an oxygen nucleophile to attack the carbonyl of an acyl-S-ACP. Fungal PR-PKSs that synthesize cyclic compounds like 6-methylsalicylic acid do not contain a TE/CLC, and the products are released via an intramolecular cyclization.[1] However, fumonisins contain a highly reduced, acyclic polyketide chain, and thus the intramolecular cyclization mechanism can not be applied. In this study, our data show that fumonisins use a new mechanism to release the polyketide chain (Figure 2). This mechanism is fundamentally different from the existing mechanisms. First, it uses a PLP cofactor, rather than the common catalytic triad found in thioesterases/cyclases, to facilitate the polyketide chain termination and release. Second, it uses a carbon nucleophile, rather than an oxygen or nitrogen nucleophile found in other polyketide biosyntheses, to attack the terminal carbonyl of acyl-*S*-PKSs to release the polyketide chain. The consequence of this mechanism is not only the release of the chain but also the formation of a new carbon−carbon bond, the elongation of the carbon chain, and the introduction of new functional groups. Finally, the substrate specificity of the PLP-dependent enzyme is important in controlling the polyketide chain length in the final products. These could represent an unprecedented method for generating new structurally diverse polyketides.

Acknowledgment. This work was supported in part by the NSF (MCB-0614916) and NSF China (No. 30428023). We thank Sara Basiaga, Ashraf Raza, Ron Cerny, and Kurt Wulser for technical supports. The cDNA of *FUM8* was a gift from Robert Proctor, and Yousong Ding and Kathia Zaleta-Rivera were involved with the early *FUM8* expression work.

Supporting Information Available: Details of experimental procedures, protein expression, MS data, and time courses. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Cox, R. J. *Org. Biomol. Chem.* **2007**, *5*, 2010–2026.
(2) (a) Du, L.; Zhu, X.; Gerber, R.; Huffman, J.; Lou, L.; Jorgenson, J.; Yu, F.; Zaleta-Rivera, K.; Wang, Q. *J. Ind. Microbiol. Biotechnol.* **2008**, *35*, 455–464. (b) Kennedy, J.; Auclair, K.; Kendrew, S. G.; Park, C.; Vederas, J. C.; Hutchinson, C. R. *Science* **1999**, *284*, 1368–1372.
(3) Fujii, I.; Watanabe, A.; Sankawa, U.; Ebizuka, Y. *Chem. Biol.* **2001**, *8*, 189–197.
(4) (a) Blackwell, B. A.; Edwards, O. E.; Fruchier, A.; ApSimon, J. W.; Miller, J. D. *Adv. Exp. Med. Biol.* **1996**, *392*, 75–91. (b) Branham, B. E.; Plattner, R. D. *Mycopathol.* **1993**, *124*, 99–104.
(5) Plattner, R. D.; Shackelford, D. D. *Mycopathol.* **1992**, *117*, 17–22.
(6) Caldas, E. D.; Sadilkova, K.; Ward, B. L.; Jones, A. D.; Winter, C. K.; Gilchrist, D. G. *J. Agric. Food Chem.* **1998**, *46*, 4734–4743.
(7) Proctor, R. H.; Brown, D. W.; Plattner, R. D.; Desjardins, A. E. *Fungal Genet. Biol.* **2003**, *38*, 237–249.
(8) (a) Hanada, K. *Biochim. Biophys. Acta* **2003**, *1632*, 16–30. (b) Ferreira, G. C.; Vajapey, U.; Hafez, O.; Hunter, G. A.; Barber, M. J. *Protein Sci.* **1995**, *4*, 1001–1006. (c) Webster, S. P.; Alexeev, D.; Campopiano, D. J.; Watt, R. M.; Alexeeva, M.; Sawyer, L.; Baxter, R. L. *Biochemistry* **2000**, *39*, 516–528.
(9) Sanchez, C.; Du, L.; Edwards, D. J.; Toney, M. D.; Shen, B. *Chem. Biol.* **2001**, *8*, 725–738.
(10) Dorrestein, P. C.; Bumpus, S. B.; Calderone, C. T.; Garneau-Tsodikova, S.; Aron, Z. D.; Straight, P. D.; Kolter, R.; Walsh, C. T.; Kelleher, N. L. *Biochemistry* **2006**, *45*, 12756–12766.
(11) Han, G.; Gable, K.; Yan, L.; Allen, M. J.; Wilson, W. H.; Moitra, P.; Harmon, J. M.; Dunn, T. M. *J. Biol. Chem.* **2006**, *281*, 39935–39942.
(12) Beeler, T.; Bacikova, D.; Gable, K.; Hopkins, L.; Johnson, C.; Slife, H.; Dunn, T. *J. Biol. Chem.* **1998**, *273*, 30688–30694.
(13) (a) Zhu, X.; Yu, F.; Bojja, R. S.; Zaleta-Rivera, K.; Du, L. *J. Ind. Microbiol. Biotechnol.* **2006**, *33*, 859–868. (b) Zhu, X.; Yu, F.; Li, X. C.; Du, L. *J. Am. Chem. Soc.* **2007**, *129*, 36–37. (c) Zhu, X.; Vogeler, C.; Du, L. *J. Nat. Prod.* **2008**, *71*, 957–960.

JA8091054