

## A Role for Old Yellow Enzyme in Ergot Alkaloid Biosynthesis

Johnathan Z. Cheng,<sup>†</sup> Christine M. Coyle,<sup>‡</sup> Daniel G. Panaccione,<sup>\*,‡</sup> and Sarah E. O'Connor<sup>\*,†</sup>

Massachusetts Institute of Technology, Department of Chemistry, 77 Massachusetts Avenue, Cambridge, Massachusetts 02139, and West Virginia University, Division of Plant & Soil Sciences, Morgantown, West Virginia 26506

Received December 2, 2009; E-mail: Dan.Panaccione@mail.wvu.edu; soc@mit.edu

Ergot alkaloids are secondary metabolites produced by filamentous fungi, including certain species of *Aspergillus*, *Claviceps*, and *Neotyphodium*.<sup>1</sup> These compounds elicit a diverse array of pharmacological effects in humans; effects of the semisynthetic derivative ergot alkaloid lysergic acid diethylamide are perhaps the best-known.<sup>1</sup> Naturally occurring ergot alkaloids include the peptide type, which in several *Claviceps* and *Neotyphodium* spp. derive from agroclavine **1**, and clavine type, which in *Aspergillus fumigatus* derives from festuclavine **2**. Extensive isotope feeding studies suggest that chanoclavine-I aldehyde **3** is an intermediate in ergot alkaloid biosynthesis and may be the common precursor to all ergot alkaloids (Scheme 1).<sup>2</sup> Here we demonstrate that a homologue of Old Yellow Enzyme encoded in the *A. fumigatus* ergot gene cluster<sup>3</sup> catalyzes reduction of the  $\alpha,\beta$  unsaturated alkene of **3**. This reduction, which yields dihydrochanoclavine aldehyde **4**, facilitates an intramolecular reaction to generate the immediate precursor to festuclavine **2**. Reduction of the chanoclavine-I aldehyde **3** alkene thus allows formation of the D ring of this natural product class.

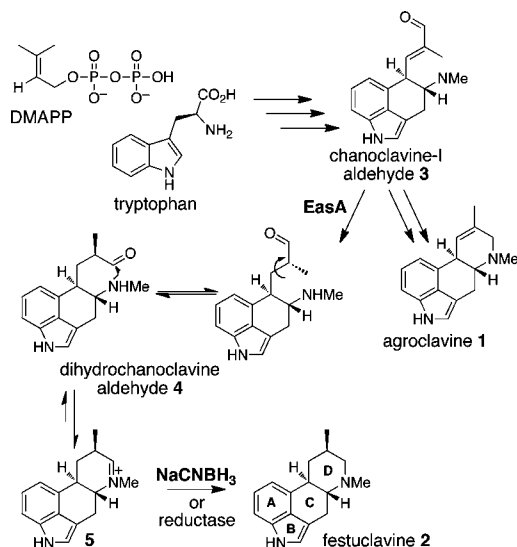
EasA exhibits protein sequence homology to the Old Yellow Enzyme of yeast (Supporting Information, SI), an enzyme that utilizes noncovalently bound FMN and NADPH to perform redox chemistry.<sup>4</sup> Although the physiological substrates of Old Yellow Enzymes remain largely unknown, many studies have demonstrated that these enzymes can reduce the alkene of an  $\alpha,\beta$ -unsaturated carbonyl compound.<sup>4</sup> Therefore, EasA appeared to be a likely candidate enzyme to act on the  $\alpha,\beta$ -unsaturated aldehyde chanoclavine-I aldehyde **3**.

To test this hypothesis, the *easA* gene was amplified from cDNA prepared from the total RNA of *A. fumigatus* tissue. The gene was subcloned for heterologous expression as an N-terminal His<sub>6</sub>-tagged protein in *E. coli* BL21(DE3) cells. Soluble protein was obtained at 2.5 mg/L culture and purified by Ni-NTA affinity chromatography to greater than 90% purity (SI). EasA copurified with a bound flavin cofactor identified to be flavin mononucleotide (FMN) by UV-vis and HPLC analysis (SI). Typically, 20–25% of heterologously expressed EasA protein copurified with the flavin cofactor; efforts to reconstitute apo-EasA with exogenous FMN were not successful.

EasA was assayed in the presence of NADPH with chanoclavine-I aldehyde **3** under aerobic conditions. Starting material and product were monitored by liquid chromatography–mass spectrometry (LC-MS) using selected ion monitoring. This assay demonstrated that, upon the addition of EasA enzyme and NADPH, chanoclavine-I aldehyde **3** ( $[M+H]^+$  255) yielded a product with a mass corresponding to the iminium form of dihydrochanoclavine, **5** ( $[M]^+$  239) (Figure 1a and 1b).

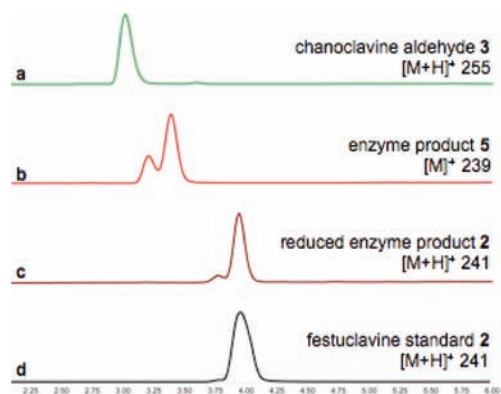
High resolution mass spectrometry of the enzymatic product also corresponded with the expected theoretical formula of compound

**Scheme 1.** Proposed Role of EasA in the Biosynthesis of the Ergot Alkaloids



**5** (SI). Control experiments, using either heat-inactivated enzyme or assay conditions lacking NADPH, resulted in no formation of any product (SI).

The enzymatic activity of EasA was further substantiated by trapping the enzyme product with sodium cyanoborohydride (NaCNBH<sub>3</sub>). Addition of NaCNBH<sub>3</sub> to the assay mixture after turnover of chanoclavine-I aldehyde **3** resulted in the conversion of iminium ion **5** to the expected reduction product festuclavine **2** ( $[M+H]^+$  241) (Figure 1c). The reduced enzymatic product comigrated with an authentic standard of festuclavine **2** (Figure 1d). The identity of the reduced enzymatic product was further



**Figure 1.** LC-MS chromatograms. (a) Starting substrate chanoclavine-I aldehyde **3**. (b) Product resulting from incubation of EasA with NADPH and **3**. (c) Product in chromatogram b reduced with NaCNBH<sub>3</sub>. (d) Festuclavine **2** standard.

<sup>†</sup> Massachusetts Institute of Technology.

<sup>‡</sup> West Virginia University.

confirmed by high resolution mass spectrometry and  $^1\text{H}$  NMR characterization (SI).

Recent genetic studies indicate that the  $\Delta$ *easA* deletion mutant of *A. fumigatus* fails to accumulate downstream ergot alkaloids, while upstream precursor intermediates chanoclavine-I and chanoclavine-I aldehyde **3** increased in abundance.<sup>5</sup> Therefore, the function of EasA *in vivo* is consistent with the biochemical properties observed *in vitro*.

The immediate product of the chanoclavine-I aldehyde reduction is expected to be dihydrochanoclavine aldehyde **4** ( $[\text{M}+\text{H}]^+$  257) (Scheme 1). However, analysis of the enzyme reaction by LC-MS indicated that **4** accumulates only in trace amounts. The reaction of the aldehyde and amine moieties of **4** to form cyclized iminium ion **5** is therefore rapid, and the iminium ion form of dihydrochanoclavine aldehyde, **5**, appears to be favored.

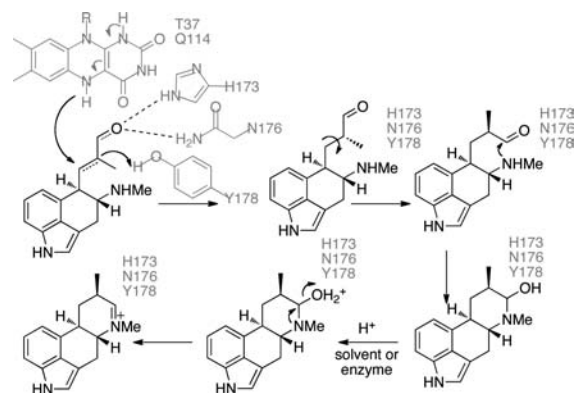
A minor compound with  $[\text{M}]^+$  239 is also formed over the time course of the assay (Figure 1b). Although this compound could not be isolated in quantities sufficient for structural characterization, we speculate that it is a diastereoisomer of **5** resulting from imine–enamine tautomerization that occurs after the cyclized iminium ion is formed (SI).

Steady state kinetic constants for EasA were measured by monitoring the disappearance of starting material by LC-MS. Data were fit to a ping-pong bi-bi kinetic model, which is typical for Old Yellow Enzymes.<sup>6</sup> Enzyme concentration was calculated using flavin absorption, ensuring that only holo-enzyme was considered in the protein concentration measurement (SI). The  $K_m$  value of chanoclavine-I aldehyde **3** and NADPH was  $4.8 \pm 1.1$  and  $192 \pm 40.1 \mu\text{M}$ , respectively, with a  $k_{\text{cat}}$  value of  $2310 \pm 282 \text{ min}^{-1}$ .

EasA was compared to the Old Yellow Enzyme homologue from *Saccharomyces carlsbergensis*, OYE1, which has been well studied and structurally characterized.<sup>7</sup> Mechanistic studies of OYE1 suggest that T37<sup>8a</sup> and Q114<sup>8b</sup> are critical for flavin binding; H191<sup>6a</sup> and N194<sup>6a</sup> interact with NADPH; and H191,<sup>6a</sup> N194,<sup>6a</sup> and Y196<sup>8c</sup> are involved in reduction of the substrate alkene. These residues align with T31, Q106, H173, N176, and Y178 in the EasA protein sequence. Based on the high sequence identity of EasA with OYE1 (40%) (see SI for alignment with multiple OYE family members), a mechanism for chanoclavine-I aldehyde **3** reduction that is analogous to the OYE1 mechanism can be proposed (Scheme 2). A hydride is transferred from the reduced FMN to the  $\beta$  carbon of chanoclavine-I aldehyde **3**, where H173 and N176 could act as hydrogen bond donors to the carbonyl oxygen of chanoclavine-I aldehyde **3**.<sup>6a</sup> The Y178 residue likely serves as a proton donor to the  $\alpha$  carbon concurrently with or after transfer of the hydride, as in OYE1.<sup>8c</sup> Notably, in the EasA catalyzed reaction, the resulting reduced product, dihydrochanoclavine aldehyde **4**, also cyclizes to form the iminium species **5**. It remains to be determined whether this cyclization is spontaneous or catalyzed enzymatically. The extensive mechanistic work on Old Yellow Enzymes<sup>4,6–8</sup> will provide an excellent foundation for further study of EasA.

Several genes involved in ergot alkaloid biosynthesis have been recently cloned,<sup>9</sup> but the mechanism of D ring formation in ergot biosynthesis has not been previously reported. This biochemical

**Scheme 2.** Proposed Mechanism of EasA Based on the Known Mechanism of OYE1



study of EasA demonstrates that reduction of the alkene in **3** facilitates an intramolecular reaction that leads to the D ring, thereby completing the common structural framework found in all ergot alkaloids. This work highlights a new role for an Old Yellow Enzyme in natural product biosynthesis.

**Acknowledgment.** We gratefully acknowledge support from MIT and from NRI Grant 2008-35318-04549 from USDA NIFA. We thank Hyang-Yeol Lee for assistance with NMR spectroscopy and Xudong Qu for assistance in cloning *easA* cDNA. Published as Scientific Article 3062 from the WV Agricultural and Forestry Experiment Station.

**Supporting Information Available:** Experimental details for enzyme expression and assay, isolation of the substrate, characterization of products, and protein sequence alignments. This material is available free of charge via the Internet at <http://pubs.acs.org>.

## References

- (a) Schardl, C.; Panaccione, D.; Tudzynski, P. In *The Alkaloids*; Cordell, G., Ed.; Elsevier: 2006; Vol. 63, pp 45–86. (b) Floss, H. G. *J. Nat. Prod.* **2006**, *69*, 158–169. (c) Haarmann, T.; Rolke, Y.; Giesbert, S.; Tudzynski, P. *Mol. Plant Path.* **2009**, *10*, 563–577.
- (a) Floss, H.; Tcheng-Lin, M.; Chang, C.; Naidoo, B.; Blair, G.; Abou-Chaar, C.; Cassidy, J. *J. Am. Chem. Soc.* **1974**, *96*, 1898–1909. (b) Naidoo, B.; Cassidy, J. M.; Blair, G. E.; Floss, H. G. *J. Chem. Soc., Chem. Commun.* **1970**, 471–472.
- Coyle, C. M.; Panaccione, D. G. *Appl. Environ. Microbiol.* **2005**, *71*, 3112–3118.
- (a) Karplus, P. A.; Fox, K.; Massey, V. *FASEB J.* **1995**, *9*, 1518–1526. (b) Williams, R. E.; Bruce, N. C. *Microbiology* **2002**, *148*, 1607–1614.
- Coyle, C. M.; Cheng, J. Z.; O'Connor, S. E.; Panaccione, D. G. Submitted.
- (a) Brown, B. J.; Deng, Z.; Karplus, P. A.; Massey, V. *J. Biol. Chem.* **1998**, *273*, 32753–32762. (b) Meah, Y.; Brown, B. J.; Chakraborty, S.; Massey, V. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 8560–8565.
- Fox, K.; Karplus, P. A. *Structure* **1994**, *15*, 1089–1105.
- (a) Xu, D.; Kohli, R.; Massey, V. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 3556–3561. (b) Brown, B. J.; Hyun, J.; Duwuri, S.; Karplus, P. A.; Massey, V. *J. Biol. Chem.* **2002**, *277*, 2138–2145. (c) Kohli, R.; Massey, V. *J. Biol. Chem.* **1998**, *273*, 32763–32770.
- (a) Tsai, H.-F.; Wang, H.; Gebler, J. C.; Poulter, C. D.; Schardl, C. L. *Biochem. Biophys. Res. Commun.* **1995**, *216*, 119–125. (b) Panaccione, D. G.; Johnson, R. D.; Wang, J.; Young, C. A.; Damrongkool, P.; Scott, B.; Schardl, C. L. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 12820–12825. (c) Correia, T.; Grammel, N.; Ortel, I.; Keller, U.; Tudzynski, P. *Chem. Biol.* **2003**, *10*, 1281–1292. (d) Wallwey, C.; Matuschek, M.; Li, S.-M. *Arch. Microbiol.* (online DOI 10.1007/s00203-009-0536-1).

JA910193P