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Reaction mechanism of 5,8-linoleate diol synthase, 10*R*-dioxygenase, and 8,11-hydroperoxide isomerase of *Aspergillus clavatus*

Fredrik Jernerén [a], Ulrike Garscha [a], Inga Hoffmann [a], Mats Hamberg [b], Ernst H. Oliw [a,*]

[a] *Section of Biochemical Pharmacology, Department of Pharmaceutical Bioscience, Uppsala Biomedical Center, SE-75124, Uppsala, Sweden*
[b] *Department of Medical Biochemistry and Biophysics, Karolinska Institutet, SE-171 77 Stockholm, Sweden*

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ABSTRACT

Aspergilli express fusion proteins of an animal haem peroxidase domain with fatty acid dioxygenase (DOX) activity (~600 amino acids) and a functional or non-functional hydroperoxide isomerase/cytochrome P450 domain (~500 amino acids with EXXR and GPHXCLG motifs). 5,8-Linoleate diol synthases (LDS; ppoA) and 10*R*-DOX (ppoC) of *Aspergillus nidulans* and *A. fumigatus* belong to this group. Our objective was to determine the oxylipins formed from linoleic acid by *A. clavatus* and their mechanism of biosynthesis. *A. clavatus* oxidized linoleic acid to (8*R*)-hydroperoxylinoleic acid (8*R*-HPODE), (10*R*)-hydroperoxy-8(*E*),12(*Z*)-octadecadienoic acid (10*R*-HPODE), and to (5*S*,8*R*)-dihydroxy- and (8*R*,11*S*)-dihydroxylinoleic acids (DiHODE) as major products. This occurred by abstraction of the *pro-S* hydrogen at C-8 and antarafacial dioxygenation at C-8 or at C-10 with double bond migration. 8*R*-HPODE was then isomerized to 5*S*,8*R*-DiHODE and to 8*R*,11*S*-DiHODE by abstraction of the *pro-S* hydrogens at C-5 and C-11 of 8*R*-HPODE, respectively, followed by suprafacial oxygenation. The genome of *A. clavatus* codes for two enzymes, which can be aligned with >65% amino acid identity to 10*R*-DOX and 5,8-LDS, respectively. The 5,8-LDS homologue likely forms and isomerizes 8*R*-HPODE to 5*S*,8*R*-DiHODE. A third gene (ppoB) codes for a protein which carries a serine residue at the cysteine position of the P450 motif. This Cys to Ser replacement is known to abolish P450 2B4 catalysis and the hydroperoxide isomerase activity of 5,8-LDS, suggesting that ppoB of *A. clavatus* may not be involved in the biosynthesis of 8*R*,11*S*-DiHODE.

1. Introduction

Aspergilli are feared pathogens and producers of allergens and toxins, but can also be used as hosts for industrial production of proteins, chemicals and drugs.[1] The genomes of ten aspergilli have been sequenced [1], and nine have been annotated in public databases.[1] Genomic information has allowed comparative analysis of proteins, facilitated gene deletion experiments and production of recombinant enzymes. The present report focuses on fatty acid dioxygenases of aspergilli, important for sexual and asexual reproduction and for biosynthesis of toxins [2,3].

These studies began 30 years ago when Champe et al. discovered oxygenated metabolites of linoleic acid, which induced premature sexual sporulation of *Aspergillus nidulans* [4]. These metabolites (psi factors[2]) are formed by 5,8-linoleate diol synthase (5,8-LDS; also designated ppoA). 5,8-LDS has been identified by gene deletion and by expression of recombinant 5,8-LDS of *A. nidulans* and *A. fumigatus* [3,5].[3] The genomes of *A. fumigatus* and *A. nidulans* contain two additional genes with homology to 5,8-LDS, and one of them was identified by gene targeting and expression to code for linoleate 10*R*-dioxygenase (10*R*-DOX or ppoC) [3,6,7]. The function of the third gene (*ppoB*) of *A. fumigatus* and *A. nidulans* and homologous genes of other aspergilli is unknown. *A. niger* and *A. fumigatus* oxygenate linoleic acid to 8,11-DiHODE, which raised the possibility that the third gene of *A. niger* and *A. fumigatus* may participate in biosynthesis of this metabolite [3,8].

LDS and 10*R*-DOX appear to be fusion proteins with an N-terminal dioxygenase domain and a C-terminal hydroperoxide isomerase/P-450 domain[1] [5,9], which is catalytically active in LDS. The first characterized oxygenase in this group was 7,8-LDS of *Gaeumannomyces graminis*, the take-all fungus of wheat [10,11]. The oxygenation mechanism of 7,8-LDS with formation of a tyrosyl radical [11] suggested catalytic similarities with cyclooxygenases, which was extended by sequencing, expression and site-directed mutagenesis to

* Corresponding author. Tel.: +46 18 4714455; fax: +46 18 4714847.
E-mail address: Ernst.Oliw@farmbio.uu.se (E.H. Oliw).

[1] The aspergilli web page: www.aspergillus.org.uk/.

[2] *Abbreviations used*: CP, chiral phase; CYP, cytochrome P450; DiHODE, dihydroxyoctadecadienoic acid; DOX, dioxygenase; HPODE, hydroperoxyoctadecadienoic acid; LDS, linoleate diol synthase; NP, normal phase; psi, premature sexual sporulation inducer, ppo, psi producing oxygenase; RP, reversed phase

[3] Hoffmann I., F. Jernerén, and E.H. Oliw (2009), Expression and site-directed mutagenesis of 5,8-LDS of *Aspergillus fumigatus*, manuscript (presented at Regulatory Oxylipins: An International Symposium, Lausanne, Switzerland, 4–6 June 2009).

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include structural similarities [10,12–14]. The tyrosyl radical likely abstracts the *pro-S* hydrogen at C-8 and forms a carbon centered radical. The latter reacts with molecular oxygen and forms 8*R*-HPODE, which can be isomerized to 7*S*,8*S*-DiHODE by suprafacial intramolecular oxygenation at C-7 [15]. The N-terminal hydroperoxide isomerase domain of 7,8-LDS and 5,8-LDS shows homology to P450 with conserved ExxR and FxxGPHxCLG motifs[3], as first described by Lee et al. [9] and recently experimentally confirmed with 5,8-LDS of *A. nidulans* [5].

A. clavatus is a potent lung allergen, but it rarely causes invasive disease [16]. In analogy with *A. fumigatus* and *A. nidulans*, the genome of *A. clavatus* codes for three putative oxygenases, which appear to be homologues of 5,8-LDS, 10*R*-DOX, and ppoB. The deduced protein sequence of ppoB of *A. clavatus* differs in the hydroperoxide isomerase/P450 domain from all known 5,8-LDS and related ppoB sequences with a serine residue at the expected position of cysteine. Previous work has established that replacement of Cys with Ser in this domain of CYP2B4 abolished or strongly (>95%) reduced its hydroxylation capacity [17]. The 8,11-hydroperoxide isomerase activities of *A. clavatus*, if catalyzed by ppoB, could therefore be expected to be nil or at least markedly diminished in comparison with *A. fumigatus* and *A. niger*. We therefore decided to study *A. clavatus*.

The present manuscript focuses on the mechanisms of oxygenation by *A. clavatus*. Our first objective was to determine whether *A. clavatus* oxidized linoleic acid and to determine the major products and their oxygenation mechanism. Our second objective was to confirm by nucleotide sequencing the serine residue in the hydroperoxide isomerase/P450 domain of ppoB. Finally, we aligned the amino acid sequences of the three putative oxygenases of *A. clavatus* with known 10*R*-DOX and 5,8-LDS enzymes, and report that the latter two constitute two groups with high percentage amino acid identities (>65%), whereas alignment of the remaining putative enzymes suggested a heterogenous group.

2. Materials and methods

2.1. Materials

18:2n-6 (99%) and HPLC solvents were from VWR. [5*S*-^{2}H]18:2n-6, [8*R*-^{2}H]18:2n-6, [11*S*-^{2}H]18:2n-6, and [11*R*-^{2}H]18:2n-6 were prepared as described [3,15]. Fatty acids were dissolved in ethanol and stored in stock solutions (50–100 mM) at −20 °C. Chemically competent *Escherichia coli* (Top10) and DNA were from Invitrogen. Phusion DNA polymerase was from Finnzymes. GeneJET cloning kit was from Fermentas. Restriction enzymes were from New England BioLabs and Fermentas. Qiagen quick gel extraction kits were from Qiagen. *A. clavatus* (CBS 514.65) was from Centraalbureau voor Schimmelcultures (Baarn, Holland).

2.2. Assay of enzyme activity

A. clavatus was grown in liquid culture with 1.5% malt extract medium for 2–3 days at 37 °C, and then at room temperature. Mycelia were harvested by filtration and washed with saline. Mycelia were either incubated with linoleic acid (0.5–1 mg/ml) in 5 vol of 0.1 M $NaBO_3$ (pH 8.0) for 4–5 h at room temperature as described [3] or grinded with liquid nitrogen to a fine powder, which was stored at −80 °C. The nitrogen powder was homogenized (glass–teflon, 10 passes; +4 °C) in 10 vol (w/v) of 0.1 mM $KHPO_4$ buffer (pH 7.3)/2 mM EDTA/0.04% Tween-20, and centrifuged at 13,000×*g* (10 min, +4 °C). The supernatant was used immediately for studies of enzyme activities. An aliquot (0.5 ml) was incubated with 100 μM linoleic acids for 30–40 min on ice. The incubation was terminated by ethanol and metabolites were extracted using SepPak/C_{18} (Waters), as described [3,18]. Triphenylphosphine (10 μg) or $NaBH_4$ was used to reduce hydroperoxides to alcohols.

2.3. LC–MS analysis

Reversed phase-HPLC (RP-HPLC) with MS/MS analysis was performed with Surveyor MS pump (ThermoFisher) and with an octadecyl silica column (5 μm; 2.1×150 mm; Phenomenex), which was eluted at 0.3 ml/min with methanol/water/acetic acid (Suprapur, Merck), usually 750/250/0.05. The effluent was subject to electrospray ionization in a linear ion trap mass spectrometer (LTQ, ThermoFisher). The heated transfer capillary was set at 315 °C, the ion isolation width at 1.5, and the collision energy at 25–35 (arbitrary scale). For analysis of products formed from [$^2H_1$]18:2n-6 we used the zoom scan function and an isolation width of 6. $PGF_{1\alpha}$ (100 ng per min) was infused for tuning.

Normal phase-HPLC (NP-HPLC) with MS/MS analysis was performed with a silicic acid column (5 μm; Kromasil 100 Å, 250×2 mm, ChromTech) using 3–5% isopropanol in hexane at 0.3–0.6 ml/min, and the effluent was mixed with isopropanol/water from a second pump (Surveyor MS pump) as described [18]. The combined effluents were introduced by electrospray into the ion trap mass spectrometer (LTQ). Chiral phase-HPLC (CP-HPLC) of 10-HODE was performed with Reprosil Chiral-NR and 8-HODE with Chiralcel OB-H as described [18]. The effluent was mixed with isopropanol/water (3/2; 0.3 ml/min) and subject to MS/MS analysis of carboxylate anions.

2.4. Partial sequencing of the putative 8,11-LDS of *A. clavatus* (XP_001270527)

Genomic DNA was prepared from *A. clavatus* by isopropanol precipitation and used as template for amplification by PCR with forward primer 5′-cagctgctaagaggcgggaag and reverse primer 5′-gcgaacctatcaaaaagaatc. This amplicon (784-bp) contained the C-terminal sequence of XP_001270527. The fragment was cloned into pJET1.2/blunt and sequenced (Uppsala Genome Center, Uppsala, Sweden).

2.5. Analysis of sequence homology

Sequence alignment and phylogenetic relationships were analyzed using *MEGA* version 4 [19]. Protein sequence data of aspergilli dioxygenases were obtained from GenBank (www.ncbi.nlm.nih.gov) and aligned by the ClustalW algorithm. A bootstrap consensus tree was created using the neighbor-joining method and bootstrap test of phylogeny with 500 replications.

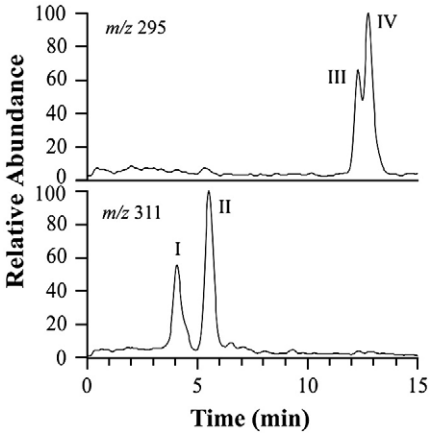

Fig. 1. RP-HPLC–MS/MS analysis of hydroxy and dihydroxylinoleic acids formed by *A. clavatus*. Peaks I and II contained 8,11- and 5,8-DiHODE, and peaks III and IV 8-HODE and 10-HODE (m/z 295→full scan), respectively. The MS/MS spectra were as previously reported [3].