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7,8- And 5,8-linoleate diol synthases support the heterolytic scission of oxygen–oxygen bonds by different amide residues

Inga Hoffmann, Ernst H. Oliw *

Department of Pharmaceutical Biosciences, Division of Biochemical Pharmacology, Uppsala Biomedical Center, Uppsala University, SE-75124 Uppsala, Sweden

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ABSTRACT

Linoleate diol synthases (LDS) are fungal dioxygenase-cytochrome P450 fusion enzymes. They oxidize 18:2*n*-6 sequentially to 8*R*-hydroperoxylinoleic acid (8*R*-HPODE) and 7*S*,8*S*- or 5*S*,8*R*-dihydroxylinoleic acids (DiHODE) by intramolecular oxygen transfer. The P450 domains contain a conserved sequence, Ala-Asn-Gln-Xaa-Gln, presumably located in the I-helices. The Asn938Leu replacement of 7,8-LDS of *Gaeumannomyces graminis* virtually abolished and the Asn938Asp and Asn938Gln replacements reduced the hydroperoxide isomerase activity. Gln941Leu and Gln941Glu substitutions had little effects. Replacements of the homologous Asn^{887} and Gln^{890} residues of 5,8-LDS of *Aspergillus fumigatus* yielded the opposite results. Asn887Leu and Asn887Gln of 5,8-LDS retained 5,8-DiHODE as the main metabolite with an increased formation of 6,8- and 8,11-DiHODE, whereas Gln890Leu almost abolished the 5,8-LDS activity. Replacement of Gln^{890} with Glu also retained 5,8-DiHODE as the main product, but shifted oxygenation from C-5 to C-7 and C-11 and to formation of epoxyalcohols by homolytic scission of 8*R*-HPODE. P450 hydroxylases usually contain an "acid-alcohol" pair in the I-helices for the heterolytic scission of O_2 and formation of compound I ($Por^{+\cdot}$ Fe(IV)=O) and water. The function of the acid-alcohol pair appears to be replaced by two different amide residues, Asn^{938} of 7,8-LDS and Gln^{890} of 5,8-LDS, for heterolysis of 8*R*-HPODE to generate compound I.

Introduction

The cytochrome P450 (P450)[1] superfamily comprises a group of versatile monooxygenases with heme coordinated to a proximal cysteine residue at the active site [1–6]. P450 are present in bacteria, Archae, fungi, plants, insects and vertebrates [2]. Two human P450, prostacyclin synthase (CYP8A1) in the vasculature and thromboxane synthase (CYP5A) in platelets, transform prostaglandin (PG) endoperoxides to biological mediators by intramolecular oxygen transfer mechanisms [7,8]. In fungi, fusion proteins with dioxygenase (DOX) and P450 domains sequentially form hydroperoxides, diols, and allene oxides, which are involved in fungal development and reproduction [9–11].

In the prototype P450 reaction, one oxygen atom from O_2 is incorporated into the substrate and the other one is reduced to water [1,4,6]. In this process, ferryl oxygen ($Por^{+\cdot}$ Fe(IV)=O; P450 compound I) is formed. This powerful oxidant catalyzes hydrogen abstraction and oxygen insertion, designated oxygen rebound, at carbon chains [12]. It was not until recently that P450 compound I could be conclusively identified by oxidation of CYP119 with a peracid [13], which yields compound I in the peroxidase shunt pathway.

Linoleate diol synthases (LDS) are fusion proteins of DOX and P450 domains, and the latter exhibit hydroperoxide isomerase activities [10,14–17]. The DOX domains have catalytic and structural similarities to cyclooxygenases, as both enzymes form a tyrosyl radical for hydrogen abstraction during catalysis [8,18]. The tyrosyl radical of 7,8-LDS abstracts hydrogen at C-8 with formation of 8*R*-hydroperoxylinoleic acid (8*R*-HPODE) [18]. The P450 domain transforms the latter to a diol. One oxygen atom of the hydroperoxide is reduced to an alcohol and the other is inserted at C-5 or C-7, yielding 5,8- or 7,8-DiHODE. The hydroperoxide isomerase activities of LDS do neither require molecular oxygen nor an electron-transfer partner and belong thus to P450 class III, which includes plant AOS, CYP8A1, and CYP5A [1]. The prototype P450 hydroxylases belong to P450 class I and II, and require a redox-partner and molecular oxygen for catalysis [1,4].

The I-helices of P450 are crucial for enzyme activity. They form the distal substrate pocket over the heme iron and participate in

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

[1] *Abbreviations used:* AOS, allene oxide synthase(s); CYP, cytochrome(s) P450; DiHODE, dihydroxy-9(*Z*),12(*Z*)-octadecadienoic acid(s); DOX, dioxygenase(s); ESI, electrospray ionization; 8*R*-HODE, 8(*R*)-hydroxy-9(*Z*),12(*Z*)-octadecadienoic acid; 8*R*-HPODE, 8(*R*)-hydroperoxy-9(*Z*),12(*Z*)-octadecadienoic acid; 10*R*-HPODE, 10(*R*)-hydroperoxy-8(*E*),12(*Z*)-octadecadienoic acid; HPODE, hydroperoxyoctadecadienoic acid(s); LDS, linoleate diol synthase(s); MS, mass spectrometry; NP, normal phase; P450, cytochrome(s) P450; PG, prostaglandin; Por, porphyrin; RP, reversed phase; TIC, total ion current.

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hydrogen transfer during the hydroxylation reaction with aid of an acid-alcohol residue pair, which is present in over 80% of all hydroxylases in the consensus sequence (Ala/Gly)-Gly-Xaa-(Glu/Asp)-Thr-(Ser/Thr) [1,5,6]. Partial sequences of the I-helices of P450 hydroxylases and sequences of peroxide isomerases (P450 class III), which catalyze heterolytic and homolytic scission of O–O bonds, are shown in Fig. 1. The acid-alcohol pair of P450cam (CYP101), P450BM3 (CYP102), and fatty acid ω-hydroxylase (CYP4A11) (Fig. 1) is absent in peroxide isomerases of class III, but the Asn residues of CYP8A1 and AOS may facilitate the scission of the O–O bond of the substrates close to the heme iron [15,19,20].

The crystal structure of CYP8A1 demonstrates that Asn^{287} could be important for the homolytic scission of PGH_2. The carbonyl oxygen atoms of Ala^{283} and Gly^{286} may form hydrogen bonding to water molecules, which are necessary for protonation of the ferric peroxo intermediate [19]. A 3D homology model of 7,8-LDS of *Gaeumannomyces graminis* aligned Asn^{938} and Ala^{934} of 7,8-LDS with Asn^{287} and Ala^{283} of CYP8A1 (*vide infra*). The amide residue which is present in the conserved Ala-Asn-Gln-Xaa-Gln sequence and Ala^{934} may therefore merit further investigation.

The first goal of the present study was to investigate whether amide residues in the conserved LDS sequence (Ala-Asn-Gln-Xaa-Gln) or Ala^{934} could alter the hydroperoxide isomerase activity of 7,8-LDS. The second goal was to identify important residues in the sequence of 5,8-LDS of *Aspergillus fumigatus* for comparison with 7,8-LDS of *G. graminis* and with 5,8-LDS of *Aspergillus terreus* [11].

Materials and methods

Materials

Fatty acids (99%) were from Merck, Sigma, and Larodan, and were stored at 50–100 mM in ethanol (−20 °C). Champion pET Directional TOPO Kit was from Invitrogen. Restriction enzymes and chemically competent *Escherichia coli* (NEB5α) were from New England BioLabs, Invitrogen, and Fermentas. Gel extraction kit was from Qiagen and Fermentas. *Pfu* DNA polymerase was from Fermentas and Phusion DNA Polymerase was from Finnzymes. RNaseA and ampicillin were from Sigma. Sequencing was performed at Uppsala Genome Center (Rudbeck Laboratories, Uppsala University).

			I-Helix
Class I and II			
Heterolytic scission	CYP4A11	321	EGHDTT
	P450BM3	264	AGHETT
	P450cam	248	GGLDTV
Class III			
Homolytic scission	CYP8A1	283	ATQGNMGPA
	CYP74A	317	ATCFNTWGG
	CYP6003B	960	GVVANVLQY
Heterolytic scission	7,8-LDSgg	934	AGTANQTQL
	7,8-LDSmo	942	AGTANQTQI
	5,8-LDSaf	883	GMVANQAQL
	5,8-LDSan	883	GMVANQAQL
	5,8-LDSat	874	GMVANQGQL

Fig. 1. Partial alignment of the I-helices of P450 hydroxylases and peroxide isomerases. The three hydroxylases of P450 class I and II contain the acid-alcohol pairs marked in red, which facilitate the heterolytic cleavage of O_2 [1]. The peroxide isomerases of P450 class III support homolytic scissions of PGH_2 (CYP8A1), hydroperoxides (AOS), and heterolytic scission of hydroperoxides (LDS). The Asn residues (marked red) of CYP8A1 and AOS (CYP74A; CYP6003B) facilitate the homolytic cleavage of O–O bonds. Conserved residues of LDS from *G. graminis* (gg), *M. oryzae* (mo), *A. fumigatus* (af), *A. nidulans* (an) and *A. terreus* (at) are marked in blue and red, and the red residues may facilitate heterolytic cleavage of 8*R*-HPODE. The activities of 7,8-LDSmo have been confirmed by recombinant expression (Hoffmann, Jernerén, and Oliw, unpublished observation) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

Expression of LDS in E. coli and site-directed mutagenesis

Full-length constructs of the open reading frames of 5,8-LDS of *A. fumigatus* and 7,8-LDS of *G. graminis* in pIZ/V5-His and pET101D-TOPO were obtained as previously described [17]. Site-directed mutagenesis was performed by PCR technology (16 cycles) with *Pfu* polymerase according to the QuickChange Protocol (Stratagene). 10 ng of pIZ/V5-His constructs served as templates and the oligonucleotide primers, which are listed in Table S1, introduced the desired substitutions. PCR products were analyzed by agarose gel electrophoresis to confirm amplification of one distinct product before digestion of maternal DNA with DpnI (37 °C, 2 h) and transformation. All mutations were confirmed by sequencing before expression and subcloning into pET101D-TOPO [17]. Two substitutions (N938Q·7,8-LDS and N938D·7,8-LDS) were obtained in a different way. Two 611-bp long DNA sequences were purchased from GeneScript (Hong-Kong, China) coding for the N938Q and N938D replacements, respectively (Table S1). The sequences covered Bsu36I and AscI restriction sites which were used for cloning into equally digested pIZ/V5-His_7,8-LDS, prior to subcloning into pET101D-TOPO.

For expression, transformed BL21 cells were grown to A_{600} 0.6–0.8 in 2xYT medium and protein expression was induced by 0.1 mM isopropyl-β-D1-galactopyranoside for 5 h at room temperature with moderate shaking (~100 rpm). The cells were harvested by centrifugation and stored at −80 °C or processed immediately as described [17].

Enzyme assays

Recombinant proteins, expressed in *E. coli,* were incubated with 100 μM 18:2*n*-6 for 30 min on ice. At least three independent experiments were performed for each mutant. The reaction (0.3–0.5 ml) was terminated with methanol (2–4 vols.), and proteins were removed by centrifugation. The metabolites were extracted on octadecyl silica (SepPak/C_{18}), evaporated to dryness, diluted in ethanol (40 μl), and 10 μl were subject to LC–MS/MS analysis.

LC–MS/MS analysis of oxylipins

Reversed phase-HPLC (RP-HPLC) with MS/MS analysis was performed with a Surveyor MS pump (ThermoFisher) and an octadecyl silica column (5 μm; 2.0 × 150 mm; Phenomenex), which was usually eluted at 0.3 ml/min with methanol/water/acetic acid, 800/200/0.05, or 750/250/0.05. The effluent was subject to electrospray ionization (ESI) 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 amu, the collision energy at 35 (arbitrary scale), and the tube lens varied between 90 and 120 V. $PGF_{1\alpha}$ was infused for tuning. Samples were injected manually (Rheodyne 7510) or by an autosampler (Surveyor Autosampler Plus, ThermoFisher).

Normal phase-HPLC (NP-HPLC) with MS/MS analysis was performed with pump P2000 (Thermo Separation Products) and a silicic acid column (5 μm; Kromasil 100SI, 2 × 250 mm, ChromTech), which was eluted with hexane/isopropyl alcohol/acetic acid, 95/5/0.01, at 0.5 ml/min. The column effluent was mixed in-line at 0.25 ml/min with isopropyl alcohol/water, 60/40, from a second pump (Surveyor MS pump). The combined effluents were introduced by ESI into the ion trap mass spectrometer (LTQ).

Bioinformatics

The ClustalW algorithm was used for sequence alignments (DNAStar, Lasergene). A hypothetical 3D homology model of 7,8-LDS of *G. graminis* was obtained by the automated mode of the