Biochimica et Biophysica Acta 1821 (2012) 1508–1517

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbalip

Novel insights into cyclooxygenases, linoleate diol synthases, and lipoxygenases from deuterium kinetic isotope effects and oxidation of substrate analogs

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ARTICLE INFO

Article history:
Received 25 April 2012
Received in revised form 20 August 2012
Accepted 4 September 2012
Available online 12 September 2012

Keywords:
Animal heme peroxidase
Chiral phase HPLC
Fatty acid oxygenation
Kinetic isotope effect
Mass spectrometry
Oxygenation mechanism

ABSTRACT

Cyclooxygenases (COX) and 8*R*-dioxygenase (8*R*-DOX) activities of linoleate diol synthases (LDS) are homologous heme-dependent enzymes that oxygenate fatty acids by a tyrosyl radical-mediated hydrogen abstraction and antarafacial insertion of O_2. Soybean lipoxygenase-1 (sLOX-1) contains non-heme iron and oxidizes 18:2n−6 with a large deuterium kinetic isotope effect (D-KIE). The aim of the present work was to obtain further mechanistic insight into the action of these enzymes by using a series of n−6 and n−9 fatty acids and by analysis of D-KIE. COX-1 oxidized C_{20} and C_{18} fatty acids in the following order of rates: 20:2n−6 > 20:1n−6 > 20:3n−9 > 20:1n−9 and 18:3n−3 ≥ 18:2n−6 > 18:1n−6. 18:2n−6 and its geometrical isomer (9*E*,12*Z*)18:2 were both mainly oxygenated at C-9 by COX-1, but the 9*Z*,12*E* isomer was mostly oxygenated at C-13. A *cis*-configured double bond in the n−6 position therefore seems important for substrate positioning. 8*R*-DOX oxidized (9*Z*,12*E*)18:2 at C-8 in analogy with 18:2n−6, but the 9*E*,12*Z* isomer was mainly subject to hydrogen abstraction at C-11 and oxygen insertion at C-9 by 8*R*-DOX of 5,8-LDS. sLOX-1 and 13*R*-MnLOX oxidized [11*S*-^{2}H]18:2n−6 with similar D-KIE (~53), which implies that the catalytic metals did not alter the D-KIE. Oxygenation of 18:2n−6 by COX-1 and COX-2 took place with a D-KIE of 3–5 as probed by incubations of [11,11-$^2H_2$]- and [11*S*-^{2}H]18:2n−6. In contrast, the more energetically demanding hydrogen abstractions of the allylic carbons of 20:1n−6 by COX-1 and 18:1n−9 by 8*R*-DOX were both accompanied by large D-KIE (>20).

1. Introduction

Polyunsaturated fatty acids can be oxygenated to important biological mediators. The first steps are usually catalyzed by fatty acid dioxygenases (DOX), which include heme-dependent DOX and lipoxygenases (LOX), or by monooxygenases (cytochromes P450) [1–4]. DOX contain heme and LOX non-heme iron (or rarely manganese). The DOX prototype is prostaglandin H (PGH) synthase-1, often designated cyclooxygenase (COX-1), and the most thoroughly studied LOX are soybean LOX-1 (sLOX-1), and human 5-LOX. In humans, COX and 5-LOX oxidize 20:4n−6 to prostaglandins and leukotrienes, respectively, which can activate G-protein coupled receptors in control of physiological functions, fever, inflammation, and development of cancer [3,4]. The versatile function of eicosanoids has inspired the development of enzyme inhibitors and receptor agonists and antagonists [3,4]. In plants and fungi, the products of fatty acid DOX pathways, often referred to as oxylipins, participate in plant defence and fungal development and pathogenicity [2,5–7]. These oxylipins are formed by LOX or by heme-dependent DOX, viz., α-DOX of plants, and linoleate diol synthases (LDS) and related enzymes of fungi [2,6,8].

The LOX family constitutes homologous enzymes with a single peptide chain, folded into a small N-terminal β-barrel domain and a C-terminal domain with the catalytic metal [1,9]. All LOX contain catalytic iron except two enzymes with manganese [10,11]. The metal center redox cycles in a process of proton-coupled electron transfer from the C–H bond of a *bis*-allylic carbon to the catalytic base of the metal center ($Fe^{3+}OH^-$), followed by antarafacial insertion of molecular oxygen [1]. The hydrogen abstraction is characterized by a large deuterium kinetic isotope effect (D-KIE) of ~80 [12]. Whether the D-KIE differs between Fe- and MnLOX is unknown. Differences in zero point energies and tunneling contribute to the D-KIE [13]. The zero point energy accounts for the 5- to 10-fold reduction of the reaction rate, which is due to the difference of activation energy needed for breaking C–H and C–D bonds (~1 kcal/mol). Larger D-KIE (>20) can be explained by hydrogen tunneling through the energy barrier, and this has been demonstrated for sLOX-1 by Klinman and co-workers [12,14]. The

Abbreviations: CP, chiral phase; COX, cyclooxygenase; DOX, dioxygenase; HODE, hydroxyoctadecadienoic acid; HETE, hydroxyeicosatetraenoic acid; HPEME, hydroperoxyeicosenoic acid; HPOME, hydroperoxyoctadecenoic acid; HEME, hydroxyeicosenoic acid; HOME, hydroxyoctadecenoic acid; D-KIE, deuterium kinetic isotope effect; LC, liquid chromatography; LDS, linoleate diol synthase; LOX, lipoxygenase; MS, mass spectrometry; NP, normal phase; [11*R*-^{2}H] (9*Z*,12*E*)18:2, [11*R*-^{2}H]-9*Z*,12*E*-octadecadienoic acid; PGH, prostaglandin H; RP, reversed phase; RSV, ram seminal vesicles; sLOX, soybean lipoxygenase; TIC, total ion current

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http://dx.doi.org/10.1016/j.bbalip.2012.09.001

extent of tunneling during sLOX-1 catalysis is influenced by structural fluctuations and dynamic effects, as shown by these authors [14].

The heme-dependent DOX are homologous to animal heme peroxidases, and share fundamental catalytic properties [5,8,15]. Oxidation of the heme group of COX, α-DOX, and LDS generates in a peroxidase cycle a tyrosyl radical (Tyr•), which catalyzes hydrogen removal. Tyr• of COX-1 abstracts the *proS* hydrogen at C-13 of 20:4n−6, and this occurs with a D-KIE of ~2–3 at 20–30 °C [1,4,16], whereas Tyr• of α-DOX abstracts hydrogen of palmitate with a D-KIE of ~54 [17]. A large D-KIE (>20) was also reported for COX-2 with perdeuterated 18:2n−6 as a substrate [18]. 7,8- And 5,8-LDS are fusion proteins of N-terminal domains with 8*R*-DOX activities and C-terminal P450 domains with diol synthase activities [19,20]. The Tyr• of 8*R*-DOX of 7,8- and 5,8-LDS abstracts the *proS* hydrogen from the allylic position at C-8 of 18:2n−6 and 18:1n−9 [5,15,21], whereas the heme-thiolate abstracts the *proS* hydrogens at C-7 and C-5, respectively [5,21]. Recently, 5,8-LDS of *Aspergillus nidulans*[1] was found to catalyze these oxidations at C-8 and at C-5 of 18:1n−9 with D-KIE ~33 and ~1.1, respectively [22].

sLOX-1 and COX-1 oxidize 20:4n−6 with more than a 10-fold difference in D-KIE. This large difference is enigmatic, but could be due to kinetic factors, variable transitional complexes, and hydrogen donor–acceptor distances [16]. The C–H bond dissociation enthalpies of *bis*-allylic and allylic carbons are ~73 and ~87 kcal/mol, respectively [23], and this difference may influence the magnitude of the D-KIE. Suboptimal substrates may have higher D-KIE than native substrates due to steric factors.

The catalytic base of sLOX-1, $Fe^{3+}OH^{-}$, is restricted to hydrogen abstraction from *bis*-allylic carbons [24], whereas Tyr• of both COX-1 and LDS can oxidize allylic carbons [25]. Based on the large D-KIE of LOX, we hypothesized that a larger D-KIE of COX-1 and LDS might be observed during activation of C–H at singly allylic than *bis*-allylic positions since the contribution of hydrogen tunneling to the reaction rate is likely larger in reactions, which require more energy.

The first goal was to investigate mono- and polyunsaturated fatty acids and stereoisomers of 18:2n−6 as substrates of COX-1, 8*R*-DOX, and sLOX-1. The second goal was to determine the D-KIE during hydrogen abstraction and oxidation of *bis*-allylic carbons by COX-1 and COX-2, and allylic carbons by COX-1 and 8*R*-DOX. The third goal was to compare the D-KIE of LOX with $Fe^{3+}OH^{-}$ and $Mn^{3+}OH^{-}$ as catalytic bases.

2. Materials and methods

2.1. Materials

HPLC solvents (Lichrosolve) and routine chemicals were from Merck. 18:1n−9 (99%), 20:1n−6 (99%), 20:1n−9 (99%), 20:4n−6 (99%), and [$^{13}C_{18}$]18:2n−6 were from Larodan. 18:1n−6 (99%), (9*E*,12*Z*)- and (9*Z*,12*E*)-18:2, 9*R*,*S*-HODE(10*E*,12*E*) and 9*S*-HODE(10*E*,12*E*) were from Lipidox. [11*S*-^{2}H]18:2n−6 was prepared as described [5,26]. 20:3n−9 (99%) and purified ovine COX-2 (4.1 kU/mg) were from Cayman. 18:3n−6, 18:2n−6, (9*E*)-18:1, hematin, N-hydroxyphthalimide, ceric ammonium nitrate, lipoxidase type 5 (sLOX-1), and α-tocopherol were from Sigma-Aldrich. Fatty acids were dissolved in ethanol and stored in stock solutions (30–100 mM) at −20 °C. Photooxidation of [$^{13}C_{18}$] 18:2n−6 was performed with methylene blue in methanol. Microsomes of ram seminal vesicles (RSV) were prepared as described [27]. Recombinant 8*R*-DOX domains (5,8-LDS, residues 1–674 of *Aspergillus fumigatus*; 7,8-LDS, residues 1–673 of *Gaeumannomyces graminis*) were expressed in *Escherichia coli* [20], and referred to as 8*R*-DOX of 5,8- and 7,8-LDS. Full-length 5,8- and 7,8-LDS were expressed in *E. coli* [20]. Chiralcel OB-H (250×4.6 mm; Daicel) was purchased locally (Dalco Chromtech). Recombinant 13*R*-MnLOX was prepared as described [24].

[1] 5,8-LDS is also designated psi producing oxygenase A (ppoA) [19].

2.2. Synthesis of mono- and dideuterated fatty acids

2.2.1. [11,11-$^{2}H_2$]18:2n−6

2-Octynoic acid (9.2 g; Sigma-Aldrich) was refluxed for 1 h with 200 ml of methanol containing 2 ml conc. hydrochloric acid. The resulting methyl ester (8.9 g) was dissolved in 30 ml of diethyl ether and slowly added at 0 °C under magnetic stirring to a suspension of 1.8 g $LiAlD_4$ in 50 ml of diethyl ether. After stirring at 0 °C for 1 h, the reaction was quenched by slow addition of tetrahydrofuran/water and the alcohol (7.8 g) was isolated by extraction with diethyl ether. The material was dissolved in 50 ml of diethyl ether containing 1 ml of dry pyridine. PBr_3 (8.1 g) was slowly added at 0 °C under magnetic stirring, and the solution was subsequently refluxed for 1.5 h. [1,1-$^{2}H_2$]1-bromo-2-octyne (7.9 g; yield, 63%) was obtained following purification on a silica gel column (elution with hexane). [1,1-$^{2}H_2$]1-bromo-2-octyne (1.91 g) and methyl 9-decynoate (1.82 g) were stirred at 23 °C for 3 h with 1 eq. of CuI, 2 eq. of NaI, and 2 eq. of Cs_2CO_3, suspended in 30 ml of dry *N*,*N*-dimethylformamide (cf. [28]). After quenching with ammonium chloride, extraction with diethyl ether, drying over $MgSO_4$ and chromatography on silica gel, the methyl ester of the title compound was obtained in >90% yield. Part of this material (584 mg) was subjected to semihydrogenation using P-2 nickel [29] and purified by RP-HPLC. An aliquot of the deuterated methyl 9*Z*,12*Z*-octadecadienoate (184 mg) was saponified, and the free acid purified by RP-HPLC. This afforded the title compound (89 mg) as a colorless oil (purity >98%). The isotope composition as determined by GC–MS was 94.3% dideuterated, 5.1% monodeuterated, and 0.6% undeuterated molecules.

2.2.2. [8,8-$^{2}H_2$]18:1n−9

8-Hydroxyoctanoic acid, prepared by alkali treatment of 8-bromooctanoic acid (Sigma-Aldrich), was treated with dihydro pyran/*p*-toluenesulfonic acid to provide the tetrahydropyranyl ester/ether derivative. This material (5.99 g) in 100 ml of THF was refluxed with $LiAlD_4$ (3 g) for 2 h, and the deuterated 8-tetrahydropyranyloxy-1-octanol (4.47 g) was sequentially treated with methanesulfonyl chloride/triethylamine and sodium iodide to afford 8-tetrahydropyranyloxy-1-iodooctane (3.63 g; yield, 58%). An aliquot of this material (1 g; 2.9 mmol) in 5 ml of THF was added to the lithio derivative of 1-decyne (9 mmol) in 15 ml of THF and 5 ml of DMPU at 0 °C. The mixture was stirred at 0 °C for 2.5 h, quenched with ammonium chloride and extracted with diethyl ether. The resulting deuterated 1-tetrahydropyranyloxy-9-octadecyne was deprotected by treatment with *p*-toluenesulfonic acid, and the deuterated acetylenic alcohol (0.65 g; 2.43 mmol) was oxidized with pyridinium dichromate in *N*,*N*-dimethylformamide [30]. Semihydrogenation of the methyl ester using P-2 nickel [29], saponification, and purification by RP-HPLC afforded the title compound as a colorless oil (0.26 g; purity, >98%; >96% deuterated molecules; yield from the deuterated acetylenic alcohol, 38%).

2.2.3. [13,13-$^{2}H_2$]20:1n−6

12-Bromo-1-dodecanol (1 g; Sigma-Aldrich) was treated with dihydropyran/*p*-toluenesulfonic acid to afford 1-bromo-12-tetrahydropyranyloxy-dodecane (1.3 g; yield, 99%). The Grignard derivative of this material (3.7 mmol) in 5 ml of THF was stirred at 23 °C for 5 min with 60 μmol of dilithium tetrachlorocuprate(II) and then stirred for further 3 h following addition of 764 mg (4 mmol) of [1,1-$^{2}H_2$]1-bromo-2-octyne. Extractive isolation with diethyl ether, removal of the tetrahydropyranyl blocking group by treatment with *p*-toluenesulfonic acid and chromatography on a silica gel column afforded material (0.54 g) consisting of the desired deuterated 14-eicosyn-1-ol admixed with a branched chain rearrangement product, ratio ~1:2. An aliquot of the mixture was oxidized by treatment with pyridinium dichromate in *N*,*N*-dimethylformamide [30], and pure deuterated 14-eicosynoic acid (70 mg) was obtained following RP-HPLC. Semihydrogenation of the methyl ester using P-2 nickel [29], saponification to the free acid, and