Published in final edited form as:

Lipids. 2017 July ; 52(7): 587–597. doi:10.1007/s11745-017-4271-0.

Oxidation of C18 Hydroxy-Polyunsaturated Fatty Acids to Epoxide or Ketone by Catalase-Related Hemoproteins Activated with Iodosylbenzene

Tarvi Teder¹, William E. Boeglin, and Alan R. Brash²

Department of Pharmacology and the Vanderbilt Institute of Chemical Biology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232, USA

Abstract

Small catalase-related hemoproteins with a facility to react with fatty acid hydroperoxides were examined for their potential mono-oxygenase activity when activated using iodosylbenzene. The proteins tested were a *Fusarium graminearum* 41 kD catalase hemoprotein (Fg-cat, gene FGSG_02217), a *Pseudomonas fluorescens* Pfl01 catalase (37.5 kD, accession number WP_011333788.1), and a *Mycobacterium avium* ssp. *paratuberculosis* 33 kD catalase (gene MAP-2744c). 13-Hydroxy-octadecenoic acids (which are normally unreactive) were selected as substrates because these enzymes react specifically with the corresponding 13*S*-hydroperoxides (Pakhomova et al, (2009) Protein Sci. 18:2559, and Teder et al, 2017, Biochim. Biophys. Acta, 1862:706). In the presence of iodosylbenzene Fg-cat converted 13*S*-hydroxy-fatty acids to two products: the 15,16-double bond of 13*S*-hydroxy α -linolenic acid was oxidized stereospecifically to the 15*S*,16*R*-*cis*-epoxide or the 13-hydroxyl was oxidized to the 13-ketone. Products were identified by UV, HPLC, LC-MS, NMR and by comparison with authentic standards prepared for this study. The Pfl01-cat displayed similar activity. MAP-2744c oxidized 13*S*-hydroxy-linoleic acid to the 13-ketone, and epoxidized the double bonds to form the 9,10-epoxy-13-hydroxy, 11,12-epoxy-13-hydroxy, and 9,10-epoxy-13-keto derivatives; equivalent transformations occurred with 9*S*-hydroxy-linoleic acid as substrate. In parallel incubations in the presence of iodosylbenzene, human catalase displayed no activity towards 13*S*-hydroxy-linoleic acid, as expected from the highly restricted access to its active site. The results indicated that with suitable transformation to Compound I, monooxygenase activity can be demonstrated by these catalase-related hemoproteins with tyrosine as the proximal heme ligand.

Keywords

Fusarium; catalase; lipoxygenase; peroxidase; HODE; iodosylbenzene

²Corresponding author: alan.brash@vanderbilt.edu.

¹Present address: Department of Chemistry, Tallinn University of Technology, Tallinn, Estonia

Conflict of Interest: The authors declare no conflicts of interest.

Introduction

A growing number of catalase-related hemoproteins are being identified that catalyze tightly controlled transformations of fatty acid hydroperoxides; the resulting products include allene oxides, other epoxy derivatives, aldehydes formed by chain cleavage, or novel syntheses such as formation of a bicyclobutane fatty acid (reviewed in ref [1]). A fundamental difference between these fatty acid peroxide-metabolizing catalases and the classic catalase hemoproteins is the available access to their active site. The classic catalases generally have highly restricted access to the active site, which limits their reactivity to the metabolism of very small substrates, usually hydrogen peroxide [2, 3]. By contrast, the structure of the fatty acid peroxide-metabolizing catalase, while preserving the central “catalase fold”, allows access of substrates with C18 or C20 carbon chains [4, 5].

It has long been considered that the ability of the cytochromes P450 to oxidize a range of small molecule substrates is related to the special properties of the active site environment with cysteine constituting the proximal ligand to the heme [6, 7]. Certainly the thiolate ligand of P450s helps confer potent oxidative capability in comparison to the commonly occurring hemoproteins with a histidine proximal heme ligand [8]. Catalase hemoproteins have tyrosine as the proximal heme ligand, and as indicated above, on account of the exceptionally limited substrate repertoire of the classic catalases, it has not been possible to examine the facility for catalyzing oxidative transformations of “small molecules” (of molecular weights ~100 to 500) within the catalase fold. The availability of the relatively recently identified fatty acid peroxide-metabolizing catalases provides the opportunity to conduct this type of experiment.

In earlier work the catalase-related allene oxide synthase (cAOS) of the coral *Plexaura homomalla* was shown to have P450-like oxidative capabilities when activated with the oxygen donor iodosylbenzene [9]. Iodosylbenzene (PhIO) has long been employed in mechanistic studies of cytochromes P450 as it is a single oxygen donor that oxidizes the enzyme to Compound I and by-passes the need for molecular oxygen and the reducing equivalents from NADPH (e.g. refs [10–13]. When activated using PhIO, the *P. homomalla* cAOS catalyzed stereoselective epoxidations and hydroxylations of arachidonic acid and 8*R*-hydroxy-eicosatetraenoic acid (the hydroxy analog of its natural 8*R*-hydroperoxy substrate) [9]. In the current work we further explored the oxidative capacity of catalase-related hemoproteins using three enzymes: Fg-cat, a 41 kD enzyme from *Fusarium graminearum* recently shown to act as a peroxidase using exclusively the 13*S*-hydroperoxides of linoleic or α -linolenic acids [14]; a *Pseudomonas fluorescens* Pfl01 catalase (37.5 kD, accession number WP_011333788.1) with 32% sequence identity to Fg-cat; and the *Mycobacterium avium* ssp. *paratuberculosis* 33 kD catalase (gene MAB-2744c) previously shown to exhibit peroxidase activity using 13-hydroperoxy-linoleic acid (13-HPODE) as oxidizing co-substrate.

Materials and Methods

Materials

α -Linolenic, γ -linolenic and linoleic acids were purchased from Nu-Chek Prep Inc. (Elysian, MN). Iodosylbenzene was purchased from TCI America (Portland, OR, catalogue no. I0072). The 13*S*-hydroxy derivatives were synthesized from linoleic acid or α - or γ -linolenic acids using the soybean LOX [15] and reduced subsequently with sodium borohydride to the hydroxy derivatives, 13*S*-HODE, and 13*S*-HOTE- ω 3 and 13*S*-HOTE- ω 6. 9*S*-HODE was prepared using potato 9*S*-LOX activity [16]. The 9- and 13-ketones were prepared by oxidation of the 9- and 13-hydroxy derivatives using activated manganese dioxide or hematin.

Catalase-related enzymes

Three of the four catalase enzymes used in this study were cloned and expressed in *E. coli* as described previously: *Fusarium graminearum* gene FGSG_02217, encoding a 41.5 kD catalase-related hemoprotein designated here as Fg-cat [14], *Mycobacterium avium* ssp. *paratuberculosis* gene MAP_2744c, encoding a 33 kD mini-catalase [5] and human catalase was similarly expressed in *E. coli* [17]. The *Pseudomonas fluorescens* Pfl01 37.5 kD catalase (accession number WP_011333788.1) was cloned from the genomic DNA with an N-terminal His-tag (similarly to the other three catalases used in this study) and expressed in *E. coli* under the same conditions as used for Fg-cat [14]. The four catalases were purified by nickel affinity chromatography and quantified based on the absorbance of the main Soret band (at ~ 405 – 410 nm) assuming a molar extinction coefficient of 100,000 M⁻¹ cm⁻¹.

Incubations with Fg-cat and 13*S*-HOTE(ω 3) and 13*S*-HOTE(ω 6) in the presence of iodosylbenzene

Fresh 5 mM iodosylbenzene (PhIO) solution was prepared as described previously [9]. Into 3 mL 50 mM Tris pH 7.5 + 150 mM NaCl buffer containing 50 μ M iodosylbenzene and 1 μ M Fg-cat was added 50 μ M 13*S*-HOTE(ω 3) or 13*S*-HOTE(ω 6) substrate and incubated for 15 min at room temperature. The remaining PhIO was then eliminated by addition of 2 – 4 mg of solid sodium bisulfite and the reactions were acidified to pH 4.0 with 1 M KH₂PO₄ and 1 N HCl and extracted using a 1-cc HLB Oasis cartridge and eluted with MeOH. The eluted products were taken to dryness and dissolved in column solvent (methanol/water/glacial acetic acid, 80:20:0.01, by volume) prior to RP-HPLC analysis. Products were analyzed with this solvent on RP-HPLC using a Waters Symmetry C18 5- μ m column (25 $\times$ 0.46 cm) with a flow rate at 1 mL/min. The UV signals at 205, 220, 235, 280 nm were recorded on a diode array (Agilent 1200 Series). About 0.5 mg of products formed from the 13*S*-HOTE incubations were isolated on RP-HPLC for the ¹H-NMR analysis. The other enzymes studied, Pfl01-cat, MAP2744c, and human catalase were reacted with PhIO under similar conditions.

Isolation of the 15,16-epoxide of α -linolenic acid

Racemic 15,16-epoxide from α -linolenic acid was isolated from the mixture of epoxides formed by treatment of α -linolenic acid with m-chloroperoxybenzoic acid as outlined as

Scheme S1 in the Supplement. Briefly, α -linolenic acid (25 mg) was epoxidized using a 1.5-fold molar excess of m-chloroperoxybenzoic (mCPBA) in 2 ml dichloromethane at room temperature for 30 min. The sample was extracted with aqueous bicarbonate, washed with water and the DCM phase taken to dryness under a stream of nitrogen and stored in methanol. The mono-epoxides were first isolated as a group by RP-HPLC in several injections using a Waters C18 Symmetry 5 μ column (25 $\times$ 0.46 cm) with a solvent of MeOH/water/glacial acetic acid (85:15:0.01, by volume) at a flow rate of 1 ml/min (retention time of epoxides, 8.5 – 10 min). The mixture of three mono-epoxides was then resolved by SP-HPLC using an Alltech 5 μ silica column (25 $\times$ 0.46 cm) with a solvent of hexane/isopropanol/glacial acetic acid (100:0.5:0.1, by volume) at a flow rate of 1.5 ml/min; the epoxides are reported to elute in the order 12,13, 15,16, and 9,10 [18], observed at retention times of 23.3 min, 25.5 min, and 27.2 min, respectively. To confirm the middle peak is the 15,16-epoxide, small aliquots of each were tested for reaction with dilute soybean lipoxygenase (UV assay scanning, for the appearance of a conjugated diene chromophore [15]). This established that only the middle peak with intact 9Z,12Z double bonds reacted readily, in agreement with it being the 15,16-epoxide. Its structure was confirmed by ¹H-NMR (Supplement Table S1).

Resolution and absolute configuration of the enantiomers of the 15,16 epoxide

The enantiomers of 15,16-*cis*-epoxy-octadeca-9Z,12Z-dienoic acid were resolved using a semi-preparative Chiralpak AD column (25 $\times$ 1 cm) with a solvent system of hexane/MeOH/glacial acetic acid (100:2:0.05, by volume) and a flow rate of 4 ml/min with on-line UV detection at 205 nm. The two 15,16-epoxide enantiomers, designated AD1 and AD2 in order of elution (at 28 and 32 min retention times, respectively), were incompletely resolved due to some tailing when amounts in the order of 1 mg were injected. Subsequent analysis using an analytical Chiralpak AD column (25 $\times$ 0.46 cm) with the same solvent system and a flow rate of 1 ml/min showed AD1 (retention time 26.1 min) was pure while AD2 (at 29.8 min) contained 13% of AD1; this cross-contamination was removed by further purification of AD2 on the semi-preparative Chiralpak AD column.

The absolute configuration of the 15,16-epoxy enantiomers was determined after chemical transformation to 16-HODE (methyl ester acetate derivative) using a protocol reported for conversion of epoxyeicosatrienoic acids to the corresponding hydroxy derivatives [19] and as described before in detail for characterization of epoxy-linoleates [20]. Each enantiomer of 16-HODE methyl ester (50 μ g) was dissolved in 50 μ L acetonitrile and derivatized using 1 μ L benzoyl chloride in the presence of 1 μ L 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) and a few grains of 4-dimethyl aminopyridine overnight at room temperature. A blank reaction was prepared in parallel. The benzoyl ester reactions were evaporated to dryness and applied to a 0.5 g Bond-Elut silica cartridge in hexane followed by a wash with 2 mL hexane and elution with 4 mL of 10% of EtOAc in hexane. The benzoyl derivatives were then purified by RP-HPLC using a Waters Symmetry C18 5- μ m column (25 $\times$ 0.46 cm), a solvent of MeOH/water/glacial acetic acid (80:20:0.01 by volume) at a flow rate of 1 mL/min. The collected benzoyl derivative of the 16-HODE methyl ester from each 15,16-epoxy enantiomer was taken to dryness, redissolved in acetonitrile and quantified by UV-Vis spectroscopy (ϵ ~25,000 at 235 nm) prior to circular dichroism spectroscopy (CD). The UV

absorbance of the benzoyl derivatives of 16-HODEs was adjusted to 0.8–1.0 AU at 235 nm for optimal CD analysis and the spectra recorded using a JASCO J-700 spectropolarimeter.

Preparation of the standards of 13*S*-hydroxy-15,16-epoxides

15*S*,16*R*- and 15*R*,16*S*-epoxyoctadeca-9*Z*,12*Z*-dienoic acids were individually incubated with soybean 13*S*-lipoxygenase in the presence of reducing agent SnCl₂ as described previously [21]. In addition to hydro(pero)xy-epoxy α -linolenic acid derivatives, monocyclic endoperoxide side-products formed and are reported in detail by Teder et al [21]. Enzymatically derived 15*S*,16*R*- and 15*R*,16*S*-epoxy-13*S*-hydroxy derivatives were co-analyzed with the product of Fg-cat from 13*S*-HOTE- ω 3/PhIO on SP-HPLC using a Beckman 5- μ m silica column (25 $\times$ 0.46 cm) and a solvent of hexane/isopropanol (100:5, v/v) with a flow rate at 1 mL/min (Results).

HPLC of epoxyalcohols from 9*S*-HODE and 13*S*-HODE

Epoxyalcohols and other products from the reactions of 9*S*-HODE and 13*S*-HODE with MAP-2744c/PhIO and with the epoxidizing reagent mCPBA were resolved on SP-HPLC using a Thomson 5 μ silica column (25 $\times$ 0.46 cm) with a solvent system of hexane/isopropanol/glacial acetic acid (100:2:0.01, by volume) for the free acids and hexane/isopropanol 100:1 for the methyl ester derivatives. The RP-HPLC analyses used a Waters 5 μ Symmetry C18 column (25 $\times$ 0.46 cm) with a methanol-based solvent of MeOH/water/glacial acetic acid (80:20:0.01, by volume), or an acetonitrile-based solvent of CH₃CN/water/glacial acetic acid (60:40:0.01, by volume) run at a flow rate of 0.5 ml/min or 1 ml/min with on-line detection at 205, 220, 235 and 270 nm.

LC-MS and NMR analysis

Products formed from 13*S*-HOTE by Fg-cat in the presence of PhIO were separated using a Kinetex C18 2.6- μ m column (3 $\times$ 100 mm) with acetonitrile/water/glacial acetic acid (45:55:0.01, by volume) column solvent at 0.4 mL/min, and molecular weights were established from the M-H anions measured by negative ion electrospray on a LTQ1 ion trap LC-MS instrument.

¹H-NMR and ¹H, ¹H COSY NMR spectra were obtained on a Bruker 600 MHz spectrometer at 298 K. The parts/million values are reported relative to residual nondeuterated benzene (C₆D₆; δ = 7.15 ppm). All spectra were analyzed on Bruker TopSpin 3.0 software.

Results

Reaction of Fg-cat/PhIO with 13*S*-HOTE- ω 3 and 13*S*-HOTE- ω 6

As potential substrates for PhIO-activated Fg-cat, the 13*S*-hydroxy derivatives of α -linolenic and γ -linolenic acids were selected for study because Fg-cat reacts specifically with the 13*S*-hydroperoxides of unsaturated C18 fatty acids [14]. By itself Fg-cat is completely unreactive with the hydroxy derivatives, as is their treatment with PhIO alone (data not shown). On the other hand, the two 13*S*-hydroxy substrates were converted to new products by Fg-cat in the presence of PhIO (Fig. 1). A single product was formed from 13*S*-HOTE- ω 6 and two from 13*S*-HOTE- ω 3. The product they gave in common eluted just before the

unreacted hydroxy substrate on RP-HPLC and displayed the typical smooth absorbance chromophore of a conjugated dienone (λ_{max} 282 nm in the RP column solvent for the product from the ω 6 13-hydroxy substrate and 284 nm for the product from ω 3 13-hydroxy). Based on the HPLC retention times, the UV spectra and in the case of the product from 13*S*-HOTE- ω 6, on the ^1H -NMR spectrum (Supplement, Table S1), the ~280 nm-absorbing products eluting near the hydroxy starting materials were identified as the corresponding 13-ketone derivatives (13-keto-octadeca-6*Z*,9*Z*,11*E*-trienoic acid and 13-keto-octadeca-9*Z*,11*E*,15*Z*-trienoic acid, respectively).

From 13*S*-HOTE- ω 3, RP-HPLC analysis also indicated the presence of an early-eluting peak, product **1**, with a retention time at 6.5 min, with 235 nm absorbance and the UV spectrum typical of a conjugated diene. No corresponding peak was observed from the incubation with 13*S*-HOTE- ω 6. The [M-1] value of product **1** was determined by LC-MS as 309, corresponding to a molecular weight of 310 and the addition of 16 a.m.u compared to the 13*S*-HOTE starting material (MW = 294). This fits with the addition of oxygen across the 15,16-double bond of 13*S*-HOTE with the retention of the 9*Z*,11*E*-conjugated diene, and also would explain the lack of a related product from the γ -linolenic acid derivative 13*S*-HOTE- ω 6. ^1H -NMR analysis proved the structure to be 13*S*-hydroxy-15,16-*cis*-epoxy-octadeca-9*Z*,11*E*-dienoic acid (Supplement Fig. S1 and Table S2). The COSY spectrum clearly established the covalent linkage of all functionalities between H7 through C18. The chemical shifts and coupling constants defined the configurations of the 9,10-*cis* and 11,12-*trans* double bonds and location of the hydroxyl at C13. The COSY cross peaks and characteristic chemical shifts confirmed the existence of a 15,16-epoxide, with the coupling constant $J_{15,16} = 4.2$ Hz characteristic of the *cis*-epoxy configuration (cf. *trans*-epoxy ~ 2.5 Hz [22]).

Stereochemical analysis of product **1**

Product **1** gave a single peak on both RP-HPLC and SP-HPLC, suggesting that it represents a single diastereomer. Given that the 13*S*-hydroxy structure is not directly involved in the enzymatic reaction and can be assumed to be unchanged, the question arises as to the configuration of the 15,16-epoxide? To address this issue, we prepared the *cis*-mono-epoxides of α -linolenic acid, resolved the mixture by RP-HPLC and SP-HPLC and identified the positional isomers (Supplement, Scheme S1) [18]. The ^1H -NMR spectra of these products are reported, although not with assignments of specific protons to the NMR signals [18]; in the Supplement we include the full NMR spectrum and COSY spectrum of the 15,16-epoxide with full assignments (Supplement Fig. S2, Table S3). The enantiomers of the 15,16-epoxide were then resolved using a Chiralpak AD column (Methods) and designated as AD1 and AD2 in order of elution.

The AD1 and AD2 enantiomers of 15,16-epoxy-C18:2 were converted by chemical transformations [19] to the corresponding 16-hydroxy-octadeca-9*Z*,12*Z*,14*E*-trienoates (Methods). The 16-hydroxy enantiomers were further converted to the 16-O-benzoate esters and the circular dichroism (CD) spectra recorded [23–25]. This allows assignment of the 16-HOTEs as the 16*R*-hydroxy enantiomer from AD1 and 16*S*-hydroxy from AD2, Fig. 2.

Thus, the original *cis*-epoxides are assigned as 15*S*,16*R*-epoxy (AD1) and 15*R*,16*S*-epoxy (AD2).

From this point, the AD1 and AD2 epoxides were reacted with soybean LOX-1 and the resulting 13*S*-hydroperoxides reduced to 13*S*-hydroxy. These 13*S*-hydroxy-15,16-epoxides were then compared on SP-HPLC with product **1** of Fg-cat, Fig. 3. (Note that the early-eluting peaks in Fig. 3 are irrelevant to this analysis: they are fatty acid endoperoxides resulting from the further transformation of 13*S*-hydroperoxy-15,16-epoxides as characterized previously [21]). The results in Fig. 3 show that Fg-cat gives a single peak on SP-HPLC (Fig. 3A), the two 13*S*-hydroxy-15,16-epoxide standards give separate peaks at 7.4 min (15*R*,16*S*, AD2) and 7.65 min (15*S*,16*R*, AD1) (Fig. 3B), and that, by co-injection, the Fg-cat product co-chromatographs with the second of the two peaks (Fig. 3C). Thus, the early-eluting Fg-cat Product 1 from 13*S*-HOTE- ω 3 is identified as 13*S*-hydroxy-15*S*,16*R*-epoxy-octadeca-9*Z*,11*E*-dienoic acid, Scheme 1.

Reaction of Pfl01-cat/PhIO with 13*S*-HOTE ω 3 and 13*S*-HOTE ω 6

We selected the 41.5 kD *P. fluorescens* catalase (Pfl01-cat) for study because the histidine residue on the distal heme is in the sequence context RATH, the Thr residue adjacent to the distal His being characteristic of catalase hemoproteins that can metabolize polyunsaturated fatty acid hydroperoxides [1]. This *P. fluorescens* catalase (Pfl01-cat) has 32% sequence identity with Fg-cat and we have unpublished work establishing that it exhibits peroxidase activity using fatty acid 13*S*-hydroperoxides as the oxidizing co-substrate (similar to Fg-cat [14]). When we tested the corresponding 13*S*-hydroxy analogues as substrate for the Pfl01-cat in the presence of PhIO, the 18:3 ω 3 analogue was completely transformed to the 15,16-epoxide with only a trace of 13-ketone formation (Fig. 4A and Scheme 1). This incubation was conducted side-by-side with the Fg-cat reaction shown in Fig. 1, indicating preferential transformation to the 15,16-epoxy derivative by the Pfl01 catalase. On the other hand, metabolism of the 13-hydroxy 18:3 ω 6 substrate was less extensive than with Fg-cat (compare Fig. 4B with Fig. 1B). Structures of the 15,16-epoxy derivative and the 13-ketone were confirmed by ¹H-NMR.

Reaction of MAP_2744c/PhIO and human catalase/PhIO with HODE substrates

The *Mycobacterium avium* ssp. *paratuberculosis* 33 kD catalase was shown previously to exhibit peroxidase activity using 13*S*-HPODE as oxidizing co-substrate [5]. When incubated with the 13*S*-hydroxy derivative of linoleic acid (13-HODE) the enzyme formed four products, in order of elution from the RP-HPLC column assigned as the 9,10-epoxy-13-hydroxy derivative (product **1** in Fig. 5A), predominantly (10:1) the 9*R*,10*S*,13*S* diastereomer as established in comparison to authentic standards [16], the 9,10-*cis*-epoxy-13-ketone (product **2** in Fig. 5A), the minor 11,12-epoxy-9-hydroxy derivative (product **3** in Fig. 5A), and the 13-ketone (product **4**), Scheme 2. The equivalent reactions occurred also using 9*S*-HODE as substrate (Fig. 5B), forming the 9-hydroxy-12,13-*cis*-epoxide (product **5**), the 9-keto-12,13-*cis*-epoxide (product **6**, identical to the reported product of Fg-cat with 13*S*-HPODE [14]), a minor 9-hydroxy-10,11-epoxy product **7**, and the 9-ketone (product **8**). As a (hopefully!) negative control, a parallel incubation was conducted with human catalase in the presence of PhIO and 13*S*-HODE. Analysis by RP-

HPLC indicated no transformation of the 13-HODE substrate, as expected for a conventional catalase enzyme with highly restricted access to the heme active site (Fig. 5C).

The products from 9*S*-HODE and MAP-2744c/PhIO were also separated using a different RP-HPLC system (acetonitrile/water-based solvent as opposed to the MeOH/water systems used in the other chromatograms) and compared to the epoxyalcohols prepared by treatment of 9*S*-HODE with the epoxidizing reagent mCPBA, Fig. 6. The purely chemical transformation using mCPBA gave four epoxyalcohols separated on SP-HPLC as two 9-hydroxy-10,11-epoxides (eluting at 10.2 and 15.5 min, and designated as SP1 and SP2, respectively), followed by two 9-hydroxy-12,13-epoxides (eluting at 21.4 and 23.0 min, and designated as SP3 and SP4, respectively, Methods, and data not shown). As illustrated in Fig. 6A, on RP-HPLC the mCPBA products gave only three peaks, the first containing a 1:1 mixture of SP3 and SP4, the late-eluting 9-hydroxy-12,13-epoxides on SP-HPLC.

From the MAP-2744c/PhIO reaction with 9*S*-HODE the prominent product **5** (Fig. 6B) resolved on SP-HPLC analysis as a 1:9 mixture of the SP3 and SP4 diastereomers respectively (data not shown), the absolute configurations of which were not determined. In the enzyme reaction there is no product corresponding to the mCPBA RP2, while the minor product **7** corresponds to mCPBA RP3. Based on published data on the relative polarity of 9*S*-hydroxy-10,11-*trans*-epoxy-octadecenoates in which the more polar isomer is of the 9,10-*threo* configuration and 9*S*,10*R*,11*R* [26], the MAP-2744c epoxyalcohol **7** co-chromatographing with mCPBA peak RP3 is the less polar diastereomer and can be assigned as the 9,10-*erythro* isomer and 9*S*-hydroxy-10*S*,11*S*-*trans*-epoxy in configuration. This stereochemical assignment was confirmed by ¹H-NMR of the SP1/RP3 and SP2/RP2 products of the 9*S*-HODE/mCPBA reaction (Supplement Fig. S3); the epoxide proton at the 10-carbon gives a diagnostic J_{9,10} coupling constant of 3.1 Hz (*erythro*) in the less polar peak SP1/RP3 and 4.6 Hz (*threo*) in SP2/RP2, and in each case confirming the 10,11-*trans*-epoxide (J_{10,11} = 2.3 Hz) [27, 28]. The results show that in regard to the stereochemistry of epoxidation α,β to the 9-hydroxyl, the MAP-2744c enzyme exhibits opposite stereoselectivity to a *Saprolegnia parasitica* hydroperoxide isomerase that forms exclusively the 9,10-*threo* diastereomer [26, 28]). This difference in stereochemistry is true also for the main epoxyalcohol product **1** formed from 13*S*-HODE, 9*R*,10*S*-epoxy-13*S*-hydroxy in our experiments compared to 9*S*,10*R*-epoxy-13*S*-hydroxy when formed from 13*S*-HODE by the *S. parasitica* isomerase [26]. Formation of the products from 9*S*-HODE and MAP-2744c/PhIO are summarized in Scheme 3.

Discussion

When activated with PhIO, the 41 kD Fg-cat and the 37.5 kD Pfl01-cat catalyzed a positional- and stereo-specific epoxidation of the 15,16 double bond of α-linoleic acid-derived 13*S*-HOTE. The stereospecific epoxidation of 13*S*-HOTE by Fg-cat matches the catalytic activity of the PhIO-activated *P. homomalla* cAOS with 8*R*-HETE [9]. In the current work we also found that the 33-kD mini-catalase of *Mycobacterium avium* ssp. *paratuberculosis* [5] will epoxidize the *cis* double bond of 9*S*- and 13*S*-HODE. Regarding olefin epoxidation, there is some debate in the P450 literature as to the involvement of Compound I or Compound 0 (the Fe-peroxy precursor of Cmpd I in the P450 catalytic

cycle) as the active enzyme species [29]. In our case the use of the single oxygen donor PhIO is compatible only with oxidation via Compound I. Compared to oxidation of alkanes, olefin epoxidation is a relatively “easy” transformation and is common not only among P450s [29], but also for the heme-thiolate unspecific fungal peroxygenases (EC 1.11.2.1) [30], and the plant heme peroxygenases [31]. Dioxygenation and monooxygenation activity in the presence of hydrogen peroxide or cumene peroxide is also exhibited by hemoproteins such as myoglobin with an accessible active site, forming H(P)ODE or epoxides from linoleic acid [32, 33]. Notably, in our experiments no reaction whatsoever occurred using human catalase/PhIO/13-HODE in accord with the narrow channel limiting access to the distal heme of conventional catalases [3].

A second product from 13*S*-HOTES and 9- and 13-HODEs, also of mechanistic interest, is oxidation of the hydroxyl to the corresponding ketone. (By contrast, the PhIO-activated *P. homomalla* cAOS with 8*R*-HETE did not form any ketones [9]). The enzymatic oxidation of substrate hydroxyl to ketone and not involving dehydrogenases is the topic of several mechanistic studies (e.g. [34–36]). Two main routes to the ketone are usually considered, one involving mono-oxygenation of the alcohol to the gem-diol followed by loss of H₂O, and alternatively a double hydrogen abstraction mechanism, from the OH and the geminal H, leaving the ketone as product. Some of the lines of evidence in favor of the gem diol mechanism use initial ¹⁸O-labeling of the alcohol and interpreting a loss of ¹⁸O in the product as evidence of the 50:50 loss of oxygen expected from the gem diol intermediate [34]; nonetheless, interpretation of such studies may be compromised by facile oxygen exchange from the resulting ketone. For the ketone formation by Fg-cat, an attractive feature of the dual H-abstraction mechanism is its parallel to the mechanism we proposed in the transformation of 13*S*-hydroperoxides [14]; Fg-cat will rearrange 13-hydroperoxy-linoleic acid to the 9-oxo-12,13-epoxide derivative and explaining how the 9-oxo moiety arises most likely involves transformation of a 9-hydroperoxide intermediate to the 9-ketone via dual H-abstraction [14].

Also in favor of the dual hydrogen abstraction one can recall the uncommon, although well documented alkane desaturase activities of certain cytochrome P450s [37–39]. The P450-catalyzed desaturation of alkanes entails initial H-abstraction by Compound I as in the conventional hydroxylation pathway. However, instead of being followed by oxygen rebound and thus hydroxylation of the substrate, a second H-abstraction is achieved by the Compound II intermediate, producing the alkene as product, the latter step having mechanistic parallels to the mechanism of fatty acid AOS enzymes [40]. Thus, we propose the mechanisms of epoxidation and ketone formation as illustrated in Scheme 4. For the sake of simplicity not all potential intermediates in these transformations are shown. For example, in the ketone pathway the order of hydrogen abstractions could be reversed, and in the epoxide pathway there are several ways to represent the interaction of the ferryl oxygen with the double bond [29]. The pathways illustrated suggest a similar steric relationship of the enzyme (Fe=O) to the reacting substrate, removing the geminal H13 in the ketone pathway and bonding with the reacting substrate on the same side of the fatty acid molecule (Scheme 3).

As noted in the Introduction, the possibilities for catalase-related hemoproteins to catalyze fatty acid oxygenations are restricted to the enzymes such as Fg-cat that can allow access of medium sized substrates to the active site. We show here that under suitable circumstances alkene epoxidation is a possibility. In our experiments the heme iron was activated artificially using PhIO and whether there is a physiological oxidant that could perform a similar function is perhaps doubtful. Fg-cat shows minimal reaction with hydrogen peroxide and the fatty acid 13*S*-hydroperoxides that are substrates and potential oxidants induce rapid turnover with formation of new fatty acid derivatives and enzyme inactivation [14]. Nonetheless, our findings help consolidate the view that the heme center of catalases with its tyrosine proximal heme ligand has the potential to catalyze mono-oxygenation of alkenes, and in some cases fatty acid hydroxylation too [9].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported in part by National Institutes of Health Grants GM-074888 and GM-15431 (to A. R. B.) and by the Institutional Research Funding IUT19-9 of the Estonian Ministry of Education and Research and the Estonian Science Foundation Grant 9410 (to Professor Nigulas Samel).

Abbreviations

PhIO	iodosylbenzene
cAOS	catalase-related allene oxide synthase
COSY	correlation spectroscopy
Fg-cat	<i>Fusarium graminearum</i> catalase of the gene FGSG_02217
H(P)ODE	hydro(pero)xyoctadecadienoic acid
H(P)OTE	hydro(pero)xyoctadecatrienoic acid
HODE	hydroxyoctadecadienoic acid
LOX	lipoxygenase
MAP-2744c	<i>Mycobacterium avium</i> ssp. <i>paratuberculosis</i> catalase of gene MAP-2744c
mCPBA	meta-chloroperoxybenzoic acid
Pfl01-cat	<i>Pseudomonas fluorescens</i> Pf01 catalase from the gene, accession number WP_011333788.1
RP-HPLC	reversed phase high pressure liquid chromatography
SP-HPLC	straight phase high pressure liquid chromatography

References

1. Mashhadi Z, Newcomer ME, Brash AR. The Thr-His connection on the distal heme of catalase-related hemoproteins: a hallmark of reaction with fatty acid hydroperoxides. *Chembiochem*. 2016; 17:2000–2006. [PubMed: 27653176]
2. Fita I, Rossman MG. The active center of catalase. *J Mol Biol*. 1985; 185:21–37. [PubMed: 4046038]
3. Putnam CD, Arvai AS, Bourne Y, Tainer JA. Active and inhibited human catalase structures: ligand and NADPH binding and catalytic mechanism. *J Mol Biol*. 2000; 296:295–309. [PubMed: 10656833]
4. Oldham ML, Brash AR, Newcomer ME. The structure of coral allene oxide synthase reveals a catalase adapted for metabolism of a fatty acid hydroperoxide. *Proc Natl Acad Sci USA*. 2005; 102:297–302. [PubMed: 15625113]
5. Pakhomova S, Gao B, Boeglin WE, Brash AR, Newcomer ME. The structure and peroxidase activity of a 33-kDa catalase-related protein from *Mycobacterium avium* ssp. *paratuberculosis*. *Protein Sci*. 2009; 18:2559–2568. [PubMed: 19827095]
6. Ortiz de Montellano PR, De Voss JJ. Oxidizing species in the mechanism of cytochrome P450. *Nat Prod Rep*. 2002; 19:477–493. [PubMed: 12195813]
7. Denisov IG, Makris TM, Sligar SG, Schlichting I. Structure and chemistry of cytochrome P450. *Chem Rev*. 2005; 105:2253–2277. [PubMed: 15941214]
8. Ortiz de Montellano P. Catalytic sites of hemoprotein peroxidases. *Ann Rev Pharmacol Toxicol*. 1992; 32:89–107. [PubMed: 1605582]
9. Boeglin WE, Brash AR. Cytochrome P450-type hydroxylation and epoxidation in a tyrosine-liganded hemoprotein, catalase-related allene oxide synthase. *J Biol Chem*. 2012; 287:24139–24147. [PubMed: 22628547]
10. Lichtenberger F, Nastainczyk W, Ullrich V. Cytochrome P450 as an oxene transferase. *Biochem Biophys Res Commun*. 1976; 70:939–946. [PubMed: 7255]
11. Gelb MH, Toscano WA Jr, Sligar SG. Chemical mechanisms for cytochrome P-450 oxidation: spectral and catalytic properties of a manganese-substituted protein. *Proc Natl Acad Sci USA*. 1982; 79:5758–5762. [PubMed: 6964386]
12. Macdonald TL, Burka LT, Wright ST, Guengerich FP. Mechanisms of hydroxylation by cytochrome P-450: Exchange of iron-oxygen intermediates with water. *Biochem Biophys Res Commun*. 1982; 104:620–625. [PubMed: 6803786]
13. Ortiz de Montellano PR. Hydrocarbon hydroxylation by cytochrome P450 enzymes. *Chem Rev*. 2010; 110:932–948. [PubMed: 19769330]
14. Teder T, Boeglin WE, Schneider C, Brash AR. A fungal catalase reacts selectively with the 13*S* fatty acid hydroperoxide products of the adjacent lipoxygenase gene and exhibits 13*S*-hydroperoxide-dependent peroxidase activity. *Biochim Biophys Acta*. 2017; 1862:706–715. [PubMed: 28363790]
15. Brash AR, Song W-C. Detection, assay, and isolation of allene oxide synthase. *Methods Enzymol*. 1996; 272:250–259. [PubMed: 8791784]
16. Thomas CP, Boeglin WE, Garcia-Diaz Y, O'Donnell VB, Brash AR. Steric analysis of epoxyalcohol and trihydroxy derivatives of 9-hydroperoxy-linoleic acid from hematin and enzymatic synthesis. *Chem Phys Lipids*. 2013; 167–168:21–32.
17. Gao B, Boeglin WE, Brash AR. Role of the conserved distal heme asparagine of coral allene oxide synthase (Asn137) and human catalase (Asn148): mutations affect the rate but not the essential chemistry of the enzymatic transformations. *Arch Biochem Biophys*. 2008; 477:285–290. [PubMed: 18652800]
18. Cui PH, Duke RK, Duke CC. Monoepoxy octadecadienoates and monoepoxy octadecatrienoates 1: NMR spectral characterization. *Chem Phys Lipids*. 2008; 152:122–130. [PubMed: 18339314]
19. Falck JR, Manna S, Jacobson HR, Estabrook RW, Chacos N, Capdevila J. Absolute configuration of epoxyeicosatrienoic acids (EETs) formed during catalytic oxygenation of arachidonic acid by purified rat liver microsomal cytochrome P-450. *J Am Chem Soc*. 1984; 106:3334–3336.

20. Gao B, Boeglin WE, Zheng Y, Schneider C, Brash AR. Evidence for an ionic intermediate in the transformation of fatty acid hydroperoxide by a catalase-related allene oxide synthase from the cyanobacterium *Acaryochloris marina*. *J Biol Chem*. 2009; 284:22087–22098. [PubMed: 19531485]
21. Teder T, Boeglin WE, Brash AR. Lipoyxygenase-catalyzed transformation of epoxy fatty acids to hydroxy-endoperoxides: a potential P450 and lipoyxygenase interaction. *J Lipid Res*. 2014; 55:2587–2596. [PubMed: 25293588]
22. Silverstein, RM., Webster, FX., Kiemle, DJ. Spectrometric identification of organic compounds. John Wiley & Sons; New York: 2005.
23. Schneider C, Schreier P, Humpf H-U. Exciton-coupled circular dichroism (ECCD) in acyclic hydroxylated dienes: a sensitive method for the direct stereochemical assignment of lipoyxygenase products. *Chirality*. 1997; 9:563–567.
24. Gonnella NC, Nakanishi K, Martin VS, Sharpless BK. General method for determining absolute configurations of acyclic allylic alcohols. *J Am Chem Soc*. 1982; 104:3775–3776.
25. Humpf H-U, Berova N, Nakanishi K, Jarstfer MB, Poulter CD. Allylic and homoallylic exciton coupled CD: A sensitive method for determining the absolute stereochemistry of natural products. *J Org Chem*. 1995; 60:3539–3542.
26. Hamberg M. Fatty acid hydroperoxide isomerase in *Saprolegnia parasitica* - Structural studies of epoxy alcohols formed from isomeric hydroperoxyoctadecadienoates. *Lipids*. 1989; 24:249–255.
27. Mercier J, Agoh B. Comportement d'hydroperoxydes allyliques a longue chaine en presence de complexes de certains metaux de transition. II. Structure des époxy-alcools formés à partir d'hydroperoxydes d'octadécène-9 oates de méthyle cis et trans en présence d'acétylacétionate de vanadyle. *Chem Phys Lipids*. 1974; 12:239–248.
28. Hamberg M, Herman RP, Jacobsson U. Stereochemistry of two epoxy alcohols from *Saprolegnia parasitica*. *Biochim Biophys Acta*. 1986; 879:410–418.
29. Guengerich FP. Cytochrome P450 oxidations in the generation of reactive electrophiles: epoxidation and related reactions. *Arch Biochem Biophys*. 2003; 409:59–71. [PubMed: 12464245]
30. Hofrichter M, Ullrich R. Oxidations catalyzed by fungal peroxygenases. *Curr Opin Chem Biol*. 2014; 19:116–125. [PubMed: 24607599]
31. Blée E, Flenet M, Boachon B, Fauconnier ML. A non-canonical caleosin from *Arabidopsis* efficiently epoxidizes physiological unsaturated fatty acids with complete stereoselectivity. *FEBS J*. 2012; 279:3981–3995. [PubMed: 22913587]
32. Rao SI, Wilks A, Hamberg M, Ortiz de Montellano PR. The lipoyxygenase activity of myoglobin. Oxidation of linoleic acid by the ferryl oxygen rather than protein radical. *J Biol Chem*. 1994; 269:7210–7216. [PubMed: 8125933]
33. Hamberg M. Myoglobin-catalyzed bis-allylic hydroxylation and epoxidation of linoleic acid. *Arch Biochem Biophys*. 1997; 344:194–199. [PubMed: 9244397]
34. Bellucci G, Chiappe C, Pucci L, Gervasi PG. The mechanism of oxidation of allylic alcohols to alpha, beta-unsaturated ketones by cytochrome P450. *Chem Res Toxicol*. 1996; 9:871–874. [PubMed: 8828923]
35. Matsunaga T, Tanaka H, Higuchi S, Shibayama K, Kishi N, Watanabe K, Yamamoto I. Oxidation mechanism of 7-hydroxy-delta 8-tetrahydrocannabinol and 8-hydroxy-delta 9-tetrahydrocannabinol to the corresponding ketones by CYP3A11. *Drug Metab Dispos*. 2001; 29:1485–1491. [PubMed: 11602526]
36. Wang Y, Yang C, Wang H, Han K, Shaik S. A new mechanism for ethanol oxidation mediated by cytochrome P450 2E1: bulk polarity of the active site makes a difference. *Chembiochem*. 2007; 8:277–281. [PubMed: 17219453]
37. Rettie AE, Rettenmeier AW, Howald WN, Baillie TA. Cytochrome P-450-catalyzed formation of D4-VPA, a toxic metabolite of valproic acid. *Science*. 1987; 235:890–893. [PubMed: 3101178]
38. Rettie AE, Sheffels PR, Korzekwa KR, Gonzalez FJ, Philpot RM, Baillie TA. CYP4 isozyme specificity and the relationship between omega-hydroxylation and terminal desaturation of valproic acid. *Biochemistry*. 1995; 34:7889–7895. [PubMed: 7794900]

39. Korzekwa KR, Trager WF, Nagata K, Parkinson A, Gillette JR. Isotope effect studies on the mechanism of the cytochrome P-450IIA1-catalyzed formation of delta 6-testosterone from testosterone. *Drug Metab Dispos.* 1990; 18:974–979. [PubMed: 1981547]
40. Brash AR. Mechanistic aspects of CYP74 allene oxide synthases and related cytochrome P450 enzymes. *Phytochemistry.* 2009; 70:1522–1531. [PubMed: 19747698]

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 1. RP-HPLC analysis of products formed from (A) 13S-HOTE- ω 3 and (B) 13S-HOTE- ω 6 by Fg-cat in the presence of iodosylbenzene. The reactions were analyzed using a Waters C18 Symmetry column (25 $\times$ 0.46 cm), a solvent of methanol/water/glacial acetic acid (80:20:0.01 by volume) and a flow rate of 1 ml/min.

Figure 2.

CD spectra of 16*R*- and 16*S*-HODE benzoyl derivatives chemically derived from corresponding 15*S*,16*R*- (AD1) and 15*R*,16*S*-epoxy- α -linolenic acid (AD2) as described in Materials and Methods.

Figure 3.

Assignment of the absolute configuration of 13*S*-hydroxy-15,16-epoxy-octadeca-9*Z*,11*E*-dienoic acid synthesized by Fg-cat/PhIO. **A** – the SP-HPLC chromatogram of 15,16-epoxy-13*S*-hydroxy-octadeca-9*Z*,11*E*-dienoic acid by Fg-cat/PhIO; **B** – the co-injection of 13*S*-hydroxy derivatives formed from 15*S*,16*R*-epoxy- (AD1) or 15*R*,16*S*-epoxy-octadeca-9*Z*,11*E*-dienoic acid (AD2) by soybean 13*S*-lipoxygenase; **C** – the co-injection of 15,16-epoxy-13*S*-hydroxy-octadeca-9*Z*,11*E*-dienoic acid synthesized by Fg-cat/PhIO with both diastereomers of 15,16-epoxy-13*S*-hydroxy-octadeca-9*Z*,11*E*-dienoic acid. The

products were analyzed as methyl esters and chromatographed on a Beckman silica column (0.46×25 cm) using a hexane/isopropanol (100:5; v/v) at a flow rate of 1 mL/min with the UV detection at 235 nm. Early-eluting products on chromatograms **B** and **C** are endoperoxide derivatives characterized by Teder *et al* [21].

Figure 4. RP-HPLC analysis of products formed from (A) 13S-HOTE- ω 3 and (B) 13S-HOTE- ω 6 by the *P. fluorescens* enzyme Pfl01-cat in the presence of iodosylbenzene. The HPLC conditions were identical to those in Fig. 1.

Figure 5.

RP-HPLC analysis of products formed from (A) 13*S*-HODE ($\omega 6$) with MAP_2744c and (B) 9*S*-HODE with MAP_2744c, and (C) 13*S*-HODE with human catalase, each in the presence of iodosylbenzene. The HPLC conditions were the same as in Fig. 1. The four products from 13*S*-HODE were identified as (1) the 9,10-epoxy-13-hydroxy derivative, (2) the 9,10-epoxy-13-keto derivative, (3) the 11,12-epoxy-13-hydroxy derivative, and (4) the 13-ketone. From 9*S*-HODE the products are (5) the 9-hydroxy-12,13-epoxide, (6) the 9-keto-12,13-epoxide, (7) the 9-hydroxy-10,11-epoxide, and (8) the 13-ketone. The peaks marked "S" are UV artefacts related to injection of solvent.

Figure 6. RP-HPLC analysis of 9S-HODE products formed by mCPBA or MAP-2744c/PhIO
 The chromatograms were run using a Waters Symmetry 5 μ C18 column (25 $\times$ 0.46 cm) with acetonitrile/water/glacial acetic acid (60:40:0.01, by volume) at 1 ml/min. **(A)** RP-HPLC analysis of products formed by treatment of 9S-HODE with the epoxidizing reagent mCPBA, producing three peaks of epoxy products on RP-HPLC (detected at 205 nm) and four peaks on SP-HPLC (the latter not illustrated, see main text). **(B)** Oxidation of 9S-HODE by MAP_2744c/PhIO. The products in order of elution were identified as **(5)** a 1:9 mixture of 9S-hydroxy-12,13-*cis*-epoxides corresponding on SP-HPLC to the mCPBA

products SP3 and SP4, respectively, **(7)** 9*S*-hydroxy-10*S*,11*S*-*trans*-epoxy-octadeca-12*Z*-enoic acid, corresponding to mCPBA product RP3 (RP2 being absent in the enzymatic reaction), **(6)** 9-keto-12,13-*cis*-epoxy-octadeca-10*E*-enoic acid, and **(8)** 9-keto-octadeca-10*E*, 12*Z*-dienoic acid. A Waters Symmetry 5 μ C18 column (25 $\times$ 0.46 cm) was eluted with acetonitrile/water/glacial acetic acid (60:40:0.01, by volume) at 1 ml/min.

Scheme 1.

Transformation of 13S-HOTE- ω 3 by PhIO-activated Fg-cat. The γ -linolenic acid analog, 13S-HOTE- ω 6, lacks the 15,16-double bond and is transformed only to the 13-ketone.

Scheme 2.

Products formed from 13*S*-HODE by the *M. avium* ssp. *paratuberculosis* 33 kD catalase MAP-2744c. R = HO₂C(CH₂)₆ and R' = C₅H₁₁

Scheme 3.

Products formed from 9S-HODE by the *M. avium* ssp. *paratuberculosis* 33 kD catalase MAP-2744c. R = HO₂C(CH₂)₆ and R' = C₅H₁₁

Scheme 4.

Mechanisms of PhIO-activated Fg-cat transformation of 13*S*-HOTE- ω 3. (13*S*-HOTE- ω 6 is transformed through the ketone pathway only).