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Catalase-related allene oxide synthase, on a biosynthetic route to fatty acid cyclopentenones: expression and assay of the enzyme and preparation of the 8*R*-HPETE substrate

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Abstract

Catalase-related allene oxide synthase (cAOS) is a hemoprotein that converts a specific fatty acid hydroperoxide to an unstable allene oxide intermediate at turnover rates in the order of 1000 per second. Fatty acid allene oxides are intermediates in the formation of cyclopentenone or hydrolytic products in marine systems, most notably the prostanoïd-related clavulones. Although the key catalytic amino acid residues around the active site of cAOS are the same as in true catalases, cAOS does not react with hydrogen peroxide. cAOS occur exclusively as the N-terminal domain of a naturally occurring fusion protein with a C-terminal lipoxygenase (LOX) domain that supplies the hydroperoxide substrate. In marine invertebrates, an 8*R*-LOX domain converts arachidonic acid to 8*R*-hydroperoxy-eicosatetraenoic acid (8*R*-HPETE) and the cAOS domain forms an 8,9-epoxy allene oxide. The fusion protein from the sea-whip octocoral *Plexaura homomalla* is the prototypical model with crystal structures of the individual domains. The cAOS (43 kD) expresses exceptionally well in *E. coli*, with yields of up to 100 mg/L. This article describes in detail expression and assay of the *P. homomalla* cAOS and two methods for the preparation of its 8*R*-HPETE substrate. Another article in this volume focuses on the *P. homomalla* 8*R*-LOX (Gilbert et al, 2018).

Keywords

allene oxide; catalase; lipoxygenase; arachidonic acid; HPETE; clavulone

1. INTRODUCTION

1.1. Nomenclature and occurrence –

The catalase-related allene oxide synthase (cAOS) of corals converts the 8*R*-hydroperoxide of arachidonic acid to an 8,9-epoxy allene oxide (Fig. 1) (Koljak, Boutaud, Shieh, Samel & Brash, 1997); “catalase-related” (thus cAOS) is specified to contrast the enzyme with the cytochrome P450 AOS of plants and fungi (Brash, 2009; Hamberg, 1989; Hoffmann & Oliw, 2013; Jernerén, Hoffmann & Oliw, 2010). cAOS was discovered in the Caribbean sea whip coral *Plexaura homomalla* (Koljak et al., 1997) and so far is the only homologue with an X-ray crystal structure (Gilbert, Niebuhr, Tsuruta, Bordelon, Ridderbusch, Dassey et al., 2008; Oldham, Brash & Newcomer, 2005b). Fatty acid allene oxides are exceptionally unstable ($t_{1/2} \sim 1$ s at pH 7 and room temperature), yet they are acted upon by enzymes that

transform the allene epoxides to cyclopentenones and other derivatives (Hamberg, 1989; Mashhadi, Newcomer & Brash, 2016; Tijet & Brash, 2002). Clavulones and related cyclopentenones of corals (Kikuchi, Tsukitani, Iguchi & Yamada, 1982; Watanabe, Sekine & Iguchi, 2003) are almost certainly formed via an allene oxide intermediate (Corey, Matsuda, Nagata & Cleaver, 1988; Lansbury, 1985), although a fatty acid cyclase of corals (analogous in function to the well-characterized allene oxide cyclase of the jasmonate pathway in plants (Hofmann, Zerbe & Schaller, 2006; Neumann, Brodhun, Sauer, Herrfurth, Hamberg, Brinkmann et al., 2012)) has yet to be identified, as do subsequent enzymes involved in the transformations (top line of Figure 1). An alternative 8R-LOX-cAOS pathway to clavulones via the allene oxide-derived α -ketol hydrolysis product was proposed in 1995 and remains a theoretical option, Figure 1 (Grechkin, 1995).

1.2 cAOS-LOX fusion proteins –

P. homomalla cAOS and all variants occur naturally as the N-terminal domain of a fusion protein with a C-terminal lipoxygenase (LOX) domain that forms the specific hydroperoxy cAOS substrate (Mashhadi et al., 2016). For example, cAOS in corals are expressed with 8R-LOX which converts arachidonic acid to 8R-HPETE (8R-hydroperoxy-eicosatetraenoic acid), the coral cAOS substrate; likewise, the cAOS-LOX fusion protein in the cyanobacterium *Acaryochloris marina* converts α -linolenic acid to its 12R-hydroperoxide and on to the 12,13-epoxy allene oxide (Gao, Boeglin, Zheng, Schneider & Brash, 2009). Figure 2 illustrates the diversity of cAOS-LOX fusion proteins. The enzymes labeled “cAOS” in Figure 2 are identified by experiment, the others by close homology. Of note, there are several closely related “cAOS-LOX” genes in the primitive chordate *Branchiostoma* (amphioxus, lancelet), but most contain some unusual substitutions at key functional residues, leaving open the question of their catalytic capabilities.

2. PREPARATION OF *P. homomalla* cAOS ENZYME

2.1 Overview –

With the *P. homomalla* cAOS-LOX fusion protein, the separate cAOS and LOX domains express well individually (Boutaud & Brash, 1999; Koljak et al., 1997; Oldham, Brash & Newcomer, 2005a; Oldham et al., 2005b), and usually this has proved true for other homologues (Gao, Boeglin & Brash, 2010; Gao et al., 2009; Teder, Lohelaid, Boeglin, Calcutt, Brash & Samel, 2015). *P. homomalla* cAOS is expressed in *E. coli* from the pET3a plasmid using the natural DNA sequence with a C-terminal (His)₄ tag after the protein sequence PAGQN. The Asn residue at amino acid position 373 corresponds to the initiating Met in the 8R-LOX domain; this can be calculated exactly because of strict homology at the N-terminus of the 8R-LOX domain in alignment with mammalian LOX sequences.

The original construct of the *P. homomalla* cAOS had a T7 N-terminal tag (15 additional amino acids) and a C-terminal (His)₄ tag (Boutaud & Brash, 1999). Subsequently the enzyme was successfully expressed starting from the natural initiating methionine (Oldham et al., 2005b), and more recently with no His tag (unpublished). The His-tagged enzyme expresses exceptionally well in *E. coli* (up to 2 μ mole (100 mg)/L (Boutaud & Brash, 1999; Oldham et al., 2005b)), and remarkably, the construct lacking the C-terminal His tag

expresses in higher yield. These results come from culture batches of 100 ml in 500 ml flasks; upon scaling up the culture volumes to 1 L or more we obtained significant reductions in yield, and therefore use of multiple 100 ml cultures is preferred over larger volumes. Note that the expression of cAOS in *E. coli* is induced in TB medium without the addition of IPTG (cf. (Hoffman, Broadwater, Johnson, Harper, Fox & Kenealy, 1995)); in fact, addition of IPTG significantly lowers the yield.

2.2 Bacterial expression and initial isolation using NiNTA –

After transformation of *E. coli* strain BL21(DE3) with recombinant vector cAOS-(His)₄/pET-3a, the bacteria are streaked on a LB/Amp plate and grown overnight at 37 °C. Several test tubes of 1 ml LB broth (with 400 µg/ml ampicillin) are inoculated with single colonies and grown at 37 °C, 250 rpm for 3 h. Multiple 10 ml TB/400 µg/ml Amp cultures are inoculated with 200 µl of the 1 ml cultures and shaken again at 37 °C, 250 rpm for 3 h. The 10 ml cultures are then added to 100 ml TB/Amp (100 µg/ml) in 500 ml flasks and shaken at 26 °C and 250 rpm for ~30 h. The cells are spun down at 4 °C at 5,000 × g and the pellets (tinted green, showing strong expression of cAOS) are frozen at –80 °C. The frozen pellets are sonicated in Bugbuster (Novagen) supplemented with 2 mM BME, 1 mM PMSF, 0.5% Pepstatin A and 1 mM Leupeptin (alternatively, using cOmplete protease inhibitor cocktail, EDTA-free, Roche). The cell debris is removed by centrifugation at 40,000 × g for 20 min.

2.3 Nickel affinity chromatography –

The supernatant of the cell lysate from combined culture volumes of 750 ml is applied to a Ni-NTA column (7 ml, Qiagen) in a 20 ml Econocolumn (1.5 cm diameter, Biorad), washed with 50 mM Tris, pH 8 containing 500 mM NaCl and 20 mM imidazole. The cAOS protein is then eluted with the same buffer adjusted to 200 mM imidazole and six 2 ml fractions are collected. In this example, the strong green color of cAOS protein was most evident in fraction 4, containing 30.5 mg cAOS. After elution from the nickel column the enzyme purity is about 85 – 90% as judged by SDS-PAGE (not shown) and from the 406/280 nm ratio of the UV-Vis spectrum (Figure 3). For analysis of catalytic activities further purification is not required. High purity samples for crystallization or NMR are obtained by anion exchange chromatography.

2.4 Purification by anion exchange chromatography –

After elution from the nickel column the enzyme is dialyzed overnight into 10 mM Tris-HCl pH 8 (addition of 2 mM BME has been used at this stage (Oldham et al., 2005b), although not required). Approximately 50 mg of cAOS is applied to Fast-flow Q-Sepharose (13 × 1.5 cm bed volume) in 10 mM Tris, pH 8, and eluted with a gradient of 0 to 0.75 M NaCl at 2 ml/min over 60 min. Fractions of 2 ml are collected and 5 µl of the dark green fractions of cAOS analyzed by SDS-PAGE (Figure 4). In this example, the first half of the band of cAOS is pure, the second half could be re-run on Q-Sepharose or purified by HPLC using a Mono-Q column (GE Health Sciences). Following anion exchange chromatography, a final purification of cAOS and adjustment of buffer is achieved by gel filtration (Oldham et al., 2005b).

2.5 UV assay of cAOS and HPLC of products –

Figure 5A illustrates the spectrophotometric assay of cAOS activity as disappearance of the conjugated diene chromophore of the 8*R*-HPETE substrate recorded at 235 nm. The fast rate of metabolism is associated with loss of enzyme activity (trace *a*), while reaction resumes with further addition of enzyme (trace *b*). Figure 5B illustrates the typical profile of products obtained by RP-HPLC: the major α -ketol hydrolysis product of the allene oxide and the minor non-enzymatic cyclization product, preclavulone A (Figure 5C).

3. PREPARATION OF 8*R*-HPETE: Method 1, via Autoxidation of Arachidonate

3.1 Overview, vitamin E-controlled autoxidation –

Currently, the substrate of cAOS, 8*R*-HPETE, is not available commercially. A few milligrams can be prepared by vitamin E-controlled non-enzymatic oxidation of arachidonate (Peers & Coxon, 1983), although the subsequent isolation of the desired 8*R*-HPETE is expensive in terms of time, solvents, and HPLC supplies. Major advantages are the simplicity and effectiveness of the vitamin E-controlled autoxidation, which is applicable to any polyunsaturated fatty acid ester (Peers & Coxon, 1983; Peers, Coxon & Chan, 1981; Schneider, Yu, Boeglin, Zheng & Brash, 2007b). By maintaining the fatty acid ester in the presence of vitamin E, (α -tocopherol, 5% by weight), under an atmosphere of oxygen at 37 °C or 40 °C for 3 – 5 days, a yield of 30% of an equal abundance of all the primary hydroperoxy isomers is obtained. The vitamin E (α -tocopherol) stabilizes the fatty acid hydroperoxides as they are formed, preventing secondary reactions that otherwise drastically reduce the yield. For example, from arachidonate methyl ester, the final mixture contains ~30% of total hydroperoxides (equal amounts of 5-, 8-, 9-, 11-, 12-, and 15-HPETE methyl esters).

Complications of this non-enzymatic approach include (i) the preferred start with arachidonate methyl ester (otherwise, using the free acid, the vitamin E procedure forms a mixture of HPETEs and HETEs (~3:1 ratio), adding complexity to the HPLC profile (with twelve products on the chromatogram!) and reducing the yield); (ii) the closely chromatographing HPETE isomers are difficult to separate on a preparative scale; (iii) the methyl ester has to be hydrolyzed to the free acid (potentially with significant losses due to instability of the fatty acid hydroperoxide in alkali (Gardner, Simpson & Hamberg, 1996)); (iv) and finally the non-enzymatic HPETEs are racemic and the enantiomeric 8*S*-HPETE strongly inhibits the activity of cAOS (Boutaud & Brash, 1999), and therefore chiral resolution of the hydroperoxide is also required to produce 8*R*-HPETE (Schneider et al., 2007b).

3.2 Vitamin E-controlled autoxidation, example –

Starting with 0.5 g arachidonate methyl ester and having suitable preparative and chiral HPLC columns available, and using liters of HPLC solvent, the best expected yield of 8*R*-HPETE might be in the order of 5 mg (1%). Smaller scale reactions should give improved

yields (2%) due to fewer problems with overloading of HPLC columns and improved resolution of HPETE isomers.

Arachidonate methyl ester (0.5 g) is mixed with 25 mg (5%) or 50 mg (10%) α -tocopherol (Sigma) in any mutually soluble solvent (e.g. ethanol, chloroform, dichloromethane), taken to dryness in a 0.5 or 1L flask, filled with oxygen, stoppered and left at 37 °C overnight. Each day, the flask is flushed again with oxygen. After 5 days at 37 °C there should be accumulation of about 30% total mono-hydroperoxides; formation of dihydroperoxides will eventually overtake the reaction and lower the yield, so stopping at 30% mono-hydroperoxides is about optimal.

3.3 Chromatographic separation of HPETE isomers –

Prior to HPLC purification, an open-bed silica column (e.g. Bond-Elut silica 10 g, Agilent) is run to remove unreacted methyl arachidonate and α -tocopherol (both early eluting), and to partially resolve the different HPETE isomers, leaving the polar dihydroperoxides on the column. The sample is loaded in 2% ethyl acetate in hexane, the arachidonate and α -tocopherol eluted with 5% ethyl acetate, and the mixed HPETE methyl esters with 10% or 15% ethyl acetate in hexane. The eluant is best assayed by scanning UV spectroscopy of small aliquots (e.g. 50 μ l out of 10 ml fractions, scanned over 200 – 350 nm), which, after evaporation to remove the ethyl acetate and dissolving in ethanol, will distinguish arachidonate (strong end absorbance at ~210 nm), α -tocopherol (a characteristic absorbance at 292 nm in ethanol), and the conjugated diene of HPETEs (λ_{max} 235 – 237 nm) (Figure 6). The less polar HPETE methyl esters (15-, 12-, and 11-HPETE-Me) will be partially resolved from the intermediate polarity of 8- and 9-HPETE-Me, leaving the most polar 5-HPETE-Me to elute last.

Silica-based SP-HPLC will resolve all HPETE-Me isomers, except that 8- and 9-HPETE-Me will almost co-chromatograph (Peers & Coxon, 1983). The two can be separated after alkaline hydrolysis of the methyl ester because 8- and 9-HPETE are well separated as the free acids (Kühn, Wiesner, Lankin, Nekrasov, Alder & Schewe, 1987). RP-HPLC will achieve baseline separation of 8-HPETE and 9-HPETE using a C18 column and solvent of MeOH/water (80:20, v/v) but is not recommended prior to SP-HPLC separation of the other HPETE isomers.

3.4 Hydrolysis of HPETE-Me to the free acid –

Hydrolysis of 8-HPETE-Me to the free acid is potentially a low-yield procedure unless precautions are taken:

- i. Alkaline hydrolysis is achieved in dilute solution to counteract the inherent solubility issue with the methyl ester in an aqueous environment; dilution reduces the required reaction time to 30 min at room temperature.
- ii. A small percentage of dichloromethane is included to enhance solubility: the solvent proportions are MeOH/dichloromethane/1M aqueous KOH (50:5:50, by volume), using about 1 ml of solution per mg HPETE-Me. Reaction is conducted under nitrogen or argon. The sample is dissolved in the organic solvents before

addition of the aqueous KOH, then agitated occasionally in an ultrasonic bath to break up micelles and facilitate contact of the methyl ester with the alkali.

- iii. After 30 min at room temperature the sample is placed on ice and the following extraction uses ice-cold solvents. In this order add 3 volumes dichloromethane (do not mix), 2 volumes of water, 0.25 volume of 1M KH₂PO₄ (to help stabilize the final pH), and a predetermined quantity of 1N HCl that will acidify to a final pH 4 – 5, and immediately mix the phases vigorously. After allowing the phases to settle or a brief centrifugation, check that the upper aqueous/methanol phase is slightly acidic before discarding; assuming the phases were very thoroughly mixed, UV scanning (200 – 300 nm) will confirm there is negligible conjugated diene in the upper phase. The lower phase is washed twice with ~ 20% volume of water, and the dichloromethane transferred to a clean dry vial and evaporated to dryness. The HPETE product is immediately redissolved in a small volume of ethanol and stored at –20 °C under nitrogen or argon until the final purification. The sample may contain a small percentage of methyl ester and traces of the hydroxy analog, 8-HETE.

3.5 Chiral resolution of 8RS-HPETE –

Satisfactory purification of 8-HPETE with resolution of the R and S enantiomers can be completed by chiral-phase HPLC (Schneider et al., 2007b). Although Chiralpak AD columns in hexane-based solvents give excellent resolution of HETE (hydroxy) enantiomers, the corresponding hydroperoxides are poorly resolved (Schneider et al., 2007b). Chiral separation of 8RS-HPETE is achieved in a reversed-phase system using a Chiralpak AD-RH column (15 × 0.46 cm) with a solvent system of methanol/water/glacial acetic acid (88:12:0.01, v/v/v) run at a flow rate of 1 ml/min (Schneider et al., 2007b).

4. PREPARATION OF 8R-HPETE: Method 2, using 8R-LOX

4.1. Substrate solubility issues:

The 8R-LOX domain of the *P. homomalla* AOS-LOX fusion protein is prepared as described (Boutaud & Brash, 1999). An alternative approach is to prepare the pseudo wild-type enzyme (psWT-8R-LOX) as outlined in an accompanying chapter (Gilbert, Neau & Newcomer, 2018). psWT-8R-LOX lacks a hydrophobic membrane insertion loop and Ca²⁺ binding residues and is soluble at 10 mg/ml without the addition of detergent (Neau, Gilbert, Bartlett, Boeglin, Brash & Newcomer, 2009; Neau, Gilbert, Bartlett, Dassey & Newcomer, 2007). The 8R-LOX enzyme gives higher yields of product in the presence of calcium (e.g. 5 mM) (Boutaud & Brash, 1999), which complicates the usual LOX reaction procedure because fatty acids precipitate if left in the presence of mM calcium. Hence, to get the best yields with this enzyme it is imperative to work quickly as detailed below. (Use of the psWT-8R-LOX construct may circumvent the need for Ca²⁺ and the precipitation issues with arachidonic acid).

The precipitation of substrate can seriously confound the typical UV assay for LOX activity. With mM calcium in the buffer, if absorbance is measured only at 235 nm, large increases in apparent absorbance will be found upon adding substrate to the reaction buffer, *even in the*

absence of enzyme. The only reliable UV assay is to scan 200 – 300 nm and observe the typical conjugated diene chromophore. This allows real enzyme activity to be easily distinguished from the elevated background due to the cloudiness that occurs across all wavelengths as the fatty acid precipitates in the presence of mM calcium.

For routine 8*R*-LOX assays the precipitation problem can be counteracted by including a detergent such as Emulphogene (Sigma) in the buffer (0.01%, see (Boutaud & Brash, 1999)). But for preparative work, because the detergent is extractable, its presence complicates the purification, so it is omitted. Also, the detergent slows the rate of reaction, so it is best to leave it out of the preparative reaction. Again, the use of the psWT-8*R*-LOX construct, which has wild-type activity, is likely to minimize this problem.

4.2 Pretesting 8*R*-LOX for activity:

In this example, prior tests by scanning spectrophotometry (200 – 350 nm) showed that 1 μ l of 8*R*-LOX enzyme prep (Ni column purified, ~3 mg protein/ml, stored for 1 year at –15 °C) will convert 30 μ g of arachidonic acid (of 40 μ g added) to 8*R*-HPETE within 3 min (by which time reaction has stopped). Reaction is carried out as follows:

In a 1 ml quartz glass cuvette: 1 ml 50 mM Tris pH 7.5 containing 500 mM NaCl and 5 mM CaCl₂. Add 40 μ g arachidonic acid in 4 μ l EtOH, immediately cap cuvette with Parafilm and mix for a few seconds by vigorous shaking. Rapid scanning in the UV (350 – 200 nm) will reveal an elevated baseline (to 0.2 – 0.5 AU at 350 nm and rising further at lower wavelengths) due to precipitation of the arachidonate calcium salt. Ignore this. After adding arachidonic acid and vigorously mixing, promptly add 1 μ l of enzyme prep, briefly mix again by a few quick inversions of the cuvette and re-scan until reaction is complete (less than 5 min). The solution clarifies as the more polar 8*R*-HPETE product is formed (2.5 AU final at 238 nm). Note: it is important to add the enzyme after the substrate because it rapidly loses activity upon dilution into the reaction buffer.

4.3. Preparative reaction:

In this example we used the same concentrations of reactants as in the “test” reaction above. For preparative purposes the solutions can be made more concentrated, with the critical proviso that the dissolved oxygen in the solution is not completely consumed, otherwise LOX-catalyzed secondary reactions in the anaerobic environment will destroy the primary hydroperoxide product (Garssen, Vliegenthart & Boldingh, 1971). Buffer equilibrated with air at room temperature contains ~ 250 μ M O₂ and this can be increased 5-fold to ~ 1.25 mM by prior bubbling with O₂ gas; the available oxygen concentration sets a limit to the maximal conversion of substrate while preserving the HPETE product.

In a 250 ml beaker place 90 ml of 50 mM Tris pH 7.5 containing 500 mM NaCl + 5 mM CaCl₂. Stir vigorously at room temperature so the solution is frothing. Add 3 mg of arachidonic acid in 300 μ l of ethanol (shoot the EtOH solution into the buffer using a Hamilton syringe). [The solution will appear cloudy but will become clear as the HPETE is formed.] Ignore the cloudiness and *immediately* add 100 μ l of *P. homomalla* 8*R*-LOX (the same preparation used for pretesting). The reaction is monitored by rapid scanning of

aliquots in the UV, 200 – 350 nm, returning the solution to the main batch after scanning. The reaction is complete in 10 minutes.

4.4 Extraction and purification:

The solution is placed on ice and acidified by addition of 10 ml (cold) 1M KH_2PO_4 and sufficient (cold) 1N HCl to achieve pH 4.5. (The required amounts can be predetermined using “blank” buffer). The cold solution is extracted by addition of 1/3 volume of cold methylene chloride and shaken vigorously. The phases should split well and can be clarified by brief centrifugation. UV scanning of the upper aqueous phase should reveal virtually no conjugated diene, and it is discarded. The cold methylene chloride extract is then washed twice with 0.2 volume of cold water, transferred to a 50 ml glass conical tube (leaving any spots of water behind) and taken to dryness under a strong stream of nitrogen. As soon as the sample is dry (watch carefully), the 8*R*-HPETE is dissolved in 1 ml MeOH or EtOH and stored under argon at -20°C .

For analytical SP-HPLC, a small aliquot (1/300th) of the extract is run on an analytical silica HPLC system, for example using a Whatman 5 μ Partisil column (25 $\times$ 0.46 cm), with a solvent of hexane/isopropanol/glacial acetic acid (100:3:0.1, by volume), a flow rate of 1 ml/min, and with UV detection at 235 nm. The retention time of 8*R*-HPETE in this system is ~8 min. This analytical system can purify up to 0.5 mg 8*R*-HPETE per injection. Alternatively, use of a semi-preparative 5 μ silica column (25 $\times$ 1 cm) allows 1 – 3 mg 8*R*-HPETE to be purified per injection using the same solvent system with a flow rate of 4 ml/min (Figure 7).

5. KEY DIFFERENCES BETWEEN HPETE-METABOLIZING cAOS AND CLASSIC CATALASES

5.1 Substrate access to the distal heme:

The crystal structures of classic catalases reveal a long narrow channel leading from the exterior of the protein down to the distal face of the heme where hydrogen peroxide is converted to molecular oxygen and water, well illustrated for human catalase (Putnam, Arvai, Bourne & Tainer, 2000). This narrow pathway severely restricts substrate access and limits reaction to the “catalatic” dismutation of H_2O_2 and the “peroxidatic” co-oxidation of small substrates such as ethanol. By contrast, cAOS has open pathways to the distal heme, allowing access to the 8*R*-hydroperoxide of long chain polyunsaturated fatty acids, Figure 8. With this open access, cAOS and other catalase-related enzymes can also function as monooxygenases when supplied with an oxygen donor to the heme iron (Boeglin & Brash, 2012; Teder, Boeglin & Brash, 2017).

5.2 Val/Thr substitution on the distal heme:

Remarkably, given this open access, cAOS shows no reaction whatsoever with hydrogen peroxide. This lack of reaction is partly attributed to a key amino acid substitution immediately adjacent to the catalytic His67 residue on the distal heme (Tosha, Uchida, Brash & Kitagawa, 2006): the pairing is conserved as Val-His in true catalase and changed to Thr-His in all cAOS-related enzymes that metabolize fatty acid hydroperoxides (Mashhadi et al., 2016). Mutation of the Thr66 to Val in *P. homomalla* cAOS induces a short

burst of reaction with H₂O₂ associated with degradation of the heme (Tosha et al., 2006). The crystal structure of *P. homomalla* cAOS suggests the distal heme His is in a flipped conformation compared to the His in true catalases and hydrogen bonded to the adjacent Thr residue, and it is speculated that this will inhibit reaction with H₂O₂. By contrast, computational (molecular dynamics) studies invoke a role for the cAOS distal heme Thr residue in hydrogen bonding with H₂O₂, preventing its reaction with the heme iron (De Luna, Bushnell & Gauld, 2013). As the interaction with peroxides and hydrogen peroxide is a common issue among heme proteins in general, further attention to this striking feature of cAOS is well warranted.

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Abbreviations:

BME	beta-mercaptoethanol
cAOS	catalase-related allene oxide synthase
HPETE	hydroperoxy-eicosatetraenoic acid
IPTG	isopropyl β-D-1-thiogalactopyranoside
LB	Luria broth
LOX	lipoxygenase
-Me	methyl ester
Ni-NTA	nickel-nitrilotriacetic acid
PMSF	phenylmethanesulfonyl fluoride
RP-HPLC	reversed-phase high pressure liquid chromatography
SP-HPLC	straight-phase or normal-phase HPLC
TB	terrific broth

References

- Boeglin WE, & Brash AR (2012). Cytochrome P450-type hydroxylation and epoxidation in a tyrosine-liganded hemoprotein, catalase-related allene oxide synthase. *J. Biol. Chem* 287, 24139–24147. [PubMed: 22628547]
- Boutaud O, & Brash AR (1999). Purification and catalytic activities of the two domains of the allene oxide synthase-lipoxygenase fusion protein of the coral *Plexaura homomalla*. *J. Biol. Chem* 274, 33764–33770. [PubMed: 10559269]
- Brash AR (2009). Mechanistic aspects of CYP74 allene oxide synthases and related cytochrome P450 enzymes. *Phytochemistry* 70, 1522–1531. [PubMed: 19747698]
- Corey EJ, Matsuda SPT, Nagata R, & Cleaver MB (1988). Biosynthesis of 8-*R*-HPETE and preclavulone A from arachidonate in several species of Caribbean coral. A widespread route to marine prostanoids. *Tetrahedron Lett* 29, 2555–2558.

- De Luna P, Bushnell EA, & Gauld JW (2013). A molecular dynamics examination on mutation-induced catalase activity in coral allene oxide synthase. *J. Phys. Chem. B* 117, 14635–14641. [PubMed: 24164352]
- Gao B, Boeglin WE, & Brash AR (2010). Omega-3 fatty acids are oxygenated at the n-7 carbon by the lipoxygenase domain of a fusion protein in the cyanobacterium *Acaryochloris marina*. *Biochim. Biophys. Acta* 1801, 58–63. [PubMed: 19786119]
- Gao B, Boeglin WE, Zheng Y, Schneider C, & Brash AR (2009). 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* 284, 22087–22098. [PubMed: 19531485]
- Gardner HW, Simpson TD, & Hamberg M (1996). Mechanism of linoleic acid hydroperoxide reaction with alkali. *Lipids* 31, 1023–8. [PubMed: 8898300]
- Garssen GJ, Vliegthart JFG, & Boldingh J (1971). An anaerobic reaction between lipoxygenase, linoleic acid and its hydroperoxides. *Biochem. J* 122, 327–332. [PubMed: 5165730]
- Gilbert NC, Neau DB, & Newcomer ME (2018). Expression of 8R-lipoxygenase from *P. homomalla*. *Methods Enzymol.* this volume
- Gilbert NC, Niebuhr M, Tsuruta H, Bordelon T, Ridderbusch O, Dassey A, et al. (2008). A covalent linker allows for membrane targeting of an oxylipin biosynthetic complex. *Biochemistry* 47, 10665–10676. [PubMed: 18785758]
- Grechkin AN (1995). Clavulones and related tert-hydroxycyclopentenone fatty acids: occurrence, physiological activity and problem of biogenetic origin. *J. Lipid Mediator Cell Signal* 11, 205–218.
- Hamberg M (1989). Fatty acid allene oxides. *J. Am. Oil Chem. Soc* 66, 1445–1449.
- Hoffman BJ, Broadwater JA, Johnson P, Harper J, Fox BG, & Kenealy WR (1995). Lactose fed-batch overexpression of recombinant metalloproteins in *Escherichia coli* BL21 (DE3): Process control yielding high levels of metal-incorporated soluble protein. *Prot. Express. Purific* 6, 646–654.
- Hoffmann I, & Oliw EH (2013). Discovery of a linoleate 9S-dioxygenase and an allene oxide synthase in a fusion protein of *Fusarium oxysporum*. *J. Lipid Res* 54, 3471–3480. [PubMed: 24082064]
- Hofmann E, Zerbe P, & Schaller F (2006). The crystal structure of *Arabidopsis thaliana* allene oxide cyclase: Insights into the oxylipin cyclization reaction. *Plant Cell* 18, 3201–3217. [PubMed: 17085685]
- Jernerén F, Hoffmann I, & Oliw EH (2010). Linoleate 9R-dioxygenase and allene oxide synthase activities of *Aspergillus terreus*. *Arch. Biochem. Biophys* 495, 67–73. Erratum [H-abstraction/oxygenation is suprafacial]: *Arch Biochem Biophys*.2010 Aug 15;500(2):210. . [PubMed: 20043865]
- Kikuchi H, Tsukitani Y, Iguchi K, & Yamada Y (1982). Clavulones, new type of prostanoids from the stolonifer *Clavularia viridis* Quoy and Gaimard. *Tetrahedron Lett* 23, 5171–5174.
- Koljak R, Boutaud O, Shieh B-H, Samel N, & Brash AR (1997). Identification of a naturally occurring peroxidase-lipoxygenase fusion protein. *Science* 277, 1994–1996. [PubMed: 9302294]
- Kühn H, Wiesner R, Lankin VZ, Nekrasov A, Alder L, & Schewe T (1987). Analysis of the stereochemistry of lipoxygenase-derived hydroxypolyenoic fatty acids by means of chiral phase high-pressure liquid chromatography. *Anal. Biochem* 160, 24–34. [PubMed: 3105349]
- Lansbury PT Jr. (1985). The oxidative metabolism of arachidonic acid (lipoxygenase, coral, clavulone) Ph.D. dissertation, Harvard University, Boston, Massachusetts.
- Mashhadi Z, Newcomer ME, & Brash AR (2016). The Thr-His connection on the distal heme of catalase-related hemoproteins: a hallmark of reaction with fatty acid hydroperoxides. *ChemBioChem* 17, 2000–2006. [PubMed: 27653176]
- Neau DB, Gilbert NC, Bartlett SG, Boeglin W, Brash AR, & Newcomer ME (2009). The 1.85 Å structure of an 8R-lipoxygenase suggests a general model for lipoxygenase product specificity. *Biochemistry* 48, 7906–7915. [PubMed: 19594169]
- Neau DB, Gilbert NC, Bartlett SG, Dassey A, & Newcomer ME (2007). Improving protein crystal quality by selective removal of a Ca²⁺-dependent membrane-insertion loop. *Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun* 63, 972–975.

- Neumann P, Brodhun F, Sauer K, Herrfurth C, Hamberg M, Brinkmann J, et al. (2012). Crystal structures of *Physcomitrella patens* AOC1 and AOC2: Insights into the enzyme mechanism and differences in substrate specificity. *Plant Physiol* 160, 1251–1266. [PubMed: 22987885]
- Niisuke K, Boeglin WE, Murray JJ, Schneider C, & Brash AR (2009). Biosynthesis of a linoleic acid allylic epoxide: mechanistic comparison with its chemical synthesis and leukotriene A biosynthesis. *J. Lipid Res* 50, 1448–1455. [PubMed: 19244216]
- Oldham ML, Brash AR, & Newcomer ME (2005a). Insights from the X-ray crystal structure of coral 8*R*-lipoxygenase: Calcium activation via a C2-like domain and a structural basis of product chirality. *J. Biol. Chem* 280, 39545–39552.
- Oldham ML, Brash AR, & Newcomer ME (2005b). The structure of coral allene oxide synthase reveals a catalase adapted for metabolism of a fatty acid hydroperoxide. *Proc. Natl. Acad. Sci. USA* 102, 297–302. [PubMed: 15625113]
- Peers KE, & Coxon DT (1983). Controlled synthesis of monohydroperoxides by α -tocopherol inhibited autoxidation of polyunsaturated lipids. *Chem. Phys. Lipids* 32, 49–56.
- Peers KE, Coxon DT, & Chan HW-S (1981). Autoxidation of methyl linolenate and methyl linoleate: the effect of α -tocopherol. *J. Sci. Food Agric* 32, 898–904.
- Putnam CD, Arvai AS, Bourne Y, & Tainer JA (2000). Active and inhibited human catalase structures: ligand and NADPH binding and catalytic mechanism. *J. Mol. Biol* 296, 295–309. [PubMed: 10656833]
- Schneider C, Niisuke K, Boeglin WE, Voehler M, Stec DF, Porter NA, et al. (2007a). Enzymatic synthesis of a bicyclobutane fatty acid by a hemoprotein-lipoxygenase fusion protein from the cyanobacterium *Anabaena* PCC 7120. *Proc. Natl. Acad. Sci. USA* 104, 18941–18945. [PubMed: 18025466]
- Schneider C, Yu Z, Boeglin WE, Zheng Y, & Brash AR (2007b). Enantiomeric separation of hydroxy and hydroperoxy eicosanoids by chiral column chromatography. *Methods Enzymol* 433, 145–157. [PubMed: 17954233]
- Teder T, Boeglin WE, & Brash AR (2017). Oxidation of C18 hydroxy-polyunsaturated fatty acids to epoxide or ketone by catalase-related hemoproteins activated with iodosylbenzene. *Lipids* 52, 587–597. [PubMed: 28631071]
- Teder T, Lohelaid H, Boeglin WE, Calcutt WM, Brash AR, & Samel N (2015). A catalase-related hemoprotein in coral is specialized for synthesis of short-chain aldehydes: DISCOVERY OF P450-TYPE HYDROPEROXIDE LYASE ACTIVITY IN A CATALASE. *J. Biol. Chem* 290, 19823–19832. [PubMed: 26100625]
- Tijet N, & Brash AR (2002). Allene oxide synthases and allene oxides. *Prostaglandins Other Lipid Mediators* 68–69, 423–431.
- Tosha T, Uchida T, Brash AR, & Kitagawa T (2006). On the relationship of coral allene oxide synthase to catalase. A single active site mutation that induces catalase activity in coral allene oxide synthase. *J. Biol. Chem* 281, 12610–12617. [PubMed: 16513636]
- Watanabe K, Sekine M, & Iguchi K (2003). Isolation of three marine prostanoids, possible biosynthetic intermediates for clavulones, from the Okinawan soft coral *Clavularia viridis*. *Chem. Pharm. Bull. (Tokyo)* 51, 909–913. [PubMed: 12913227]

Figure 1.

The 8*R*-LOX-cAOS pathway in corals and potential transformations to clavulones

Figure 2. Phylogenetic tree and percent identities of representative cAOS-related enzymes.

The top group of octocoral cAOS domains share >80% sequence amino acid identity, and ~80% with the catalase-related fatty acid hydroperoxide lyase (cHPL) (Teder et al., 2015); the middle sequences include from *Acropora digitifera* (staghorn coral), *Stylophora pistillata* (smooth cauliflower coral), and *Exaoptasia pallida* (brown or pale anemone); marine worm *Capitella capitata* and the Red Sea coral *Primnoa pacifica* are 30 – 33% identical to the top group; the cyanobacterium *A. marina* has an authenticated cAOS-LOX gene (Gao et al., 2009), while the *Anabaena* PCC7120 enzyme has linoleate diol synthase (LDS), leukotriene synthase, and epoxy-bicyclobutane fatty acid synthase activities (Niisuke, Boeglin, Murray, Schneider & Brash, 2009; Schneider, Niisuke, Boeglin, Voehler, Stec, Porter et al., 2007a). The phylogenetic tree was constructed using the Clustal-W alignment of DNASTar.

Figure 3. UV-Vis spectrum of *P. homomalla* cAOS domain

The main Soret band of the cAOS heme protein shows a λ_{max} at 406 nm. This spectrum, recorded after expression in *E. coli* and purification by nickel affinity chromatography, shows a 406/280 absorbance ratio of 1.60, indicating ~90% purity. Further purification by ion exchange chromatography and gel filtration can achieve a 406/280 ratio of up to 1.8 (Oldham et al., 2005b).

Figure 4. SDS-PAGE analysis of cAOS purification on open-bed Q-Sepharose

Approximately 50 mg of cAOS was applied to Fast-flow Q-Sepharose (13 × 1.5 cm bed volume) in 10 mM Tris, pH 8, and eluted with a gradient of 0 to 0.75 M NaCl at 2 ml/min over 60 min. Fractions of 2 ml were collected and 5 µl of the bright green fractions of cAOS analyzed by SDS-PAGE.

Figure 5. UV assay and RP-HPLC analysis of 8R-HPETE reaction with *P. homomalla* cAOS
 A: Rates of reaction recorded as the disappearance of UV absorbance of the substrate at 235 nm. Trace *a*: *P. homomalla* cAOS domain reacting with 50 μ M 8R-HPETE; trace *b*, same as *a*, followed by a second addition of cAOS enzyme. AU, absorbance units. B: RP-HPLC analysis of the products of reaction of [14 C]8R-HPETE with cAOS using a Beckman Ultrasphere ODS 5 μ or Waters Symmetry C18 5 μ column (25 $\times$ 0.46 cm) with a solvent of methanol/water/acetic acid (85/15/0.01 by volume) and flow rate of 1 ml/min. C: Partial structures showing conversion of 8R-HPETE to the unstable 8,9-epoxy allene oxide followed by its non-enzymatic hydrolysis or cyclization to α -ketol and cyclopentenone, respectively.

Figure 6. UV spectra of arachidonate, HPETE and α -tocopherol

Spectra were recorded in ethanol. The concentrations illustrated are approximately 10 $\mu\text{g/ml}$ for arachidonate and HPETE (for HPETE, $\epsilon = 25,000 \text{ M}^{-1} \text{ cm}^{-1}$) and 25 $\mu\text{g/ml}$ of α -tocopherol (100 $\mu\text{g/ml} = 0.76 \text{ AU}$). AU = absorbance unit.

Figure 7. SP-HPLC purification of 8R-HPETE from 8R-LOX reaction

A: A small aliquot of the preparation from 3 mg of arachidonic acid is analyzed on a semi-preparative Beckman Ultrasphere 5 μ silica column (25 $\times$ 1 cm) using a solvent system of hexane/isopropanol/glacial acetic acid (100:2:0.1, by volume) at a flow rate of 4 ml/min with UV detection at 235 nm. B: The main sample is then injected under the same conditions (three injections of one third of the extract) and the peak of 8R-HPETE collected as shown. The early-eluting peak at ~ 6.5 min is probably 11R-HPETE, a minor product of the reaction (Boutaud & Brash, 1999). AU = absorbance units.

Figure 8. A slice of surface renderings of catalase and cAOS to reveal the solvent accessible heme.

Left: The narrow channel in catalase can help direct H_2O_2 towards a productive orientation with the heme (shown in green with position of the heme iron in red). The distal heme His75 of catalase shows hydrogen bonding to Ser114. Right: in cAOS the channel is larger and much more open, and the heme is accessible from various angles. Notice also the “flipped” His67 in cAOS, which is hydrogen bonded to Asn137 and Thr66. The black regions represent solvent inaccessible volumes. Reproduced from ChemBioChem with permission (Mashhadi et al., 2016).