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Omega-3 fatty acids are oxygenated at the n-7 carbon by the lipoxygenase domain of a fusion protein in the cyanobacterium *Acaryochloris marina*

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Abstract

Lipoxygenases (LOX) are found in most organisms that contain polyunsaturated fatty acids, usually existing as individual genes although occasionally encoded as a fusion protein with a catalase-related hemoprotein. Such a fusion protein occurs in the cyanobacterium *Acaryochloris marina* and herein we report the novel catalytic activity of its LOX domain. The full-length protein and the C-terminal LOX domain were expressed in *Escherichia coli*, and the catalytic activities characterized by UV, HPLC, GC-MS, and CD. All omega-3 polyunsaturates were oxygenated by the LOX domain at the n-7 position and with *R* stereospecificity: α -linolenic and the most abundant fatty acid in *A. marina*, stearidonic acid (C18.4 ω 3), are converted to the corresponding 12*R*-hydroperoxides, eicosapentaenoic acid to its 14*R*-hydroperoxide, and docosahexaenoic acid to its 16*R*-hydroperoxide. Omega-6 polyunsaturates were oxygenated at the n-10 position, forming 9*R*-hydroperoxy-octadecadienoic acid from linoleic acid and 11*R*-hydroperoxy-eicosatetraenoic acid from arachidonic acid. The metabolic transformation of stearidonic acid by the full-length fusion protein entails its 12*R* oxygenation with subsequent conversion by the catalase-related domain to a novel allene epoxide, a likely precursor of cyclopentenone fatty acids or other signaling molecules (Gao et al, *J. Biol. Chem.* 284:22087-98, 2009). Although omega-3 fatty acids and lipoxygenases are of widespread occurrence, this appears to be the first description of a LOX-catalyzed oxygenation that specifically utilizes the terminal pentadiene of omega-3 fatty acids.

Keywords

Acaryochloris marina; lipoxygenase; hydroperoxide; omega-3 fatty acids; linolenic acid; stearidonic acid; GC-MS; chiral analysis

1. Introduction

Typically, individual members of the lipoxygenase (LOX) gene family of non-heme iron dioxygenases catalyze a specific oxygenation of their polyunsaturated fatty acid substrates [1–3]. The substrates all contain one or more pentadiene units composed of CH₂-interrupted *cis* double bonds. LOX catalysis is initiated by the stereospecific removal of one of the CH₂

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hydrogens, molecular oxygen is added to the opposite face of the reacting substrate at the 1 or 5 position of the pentadiene, and the resulting peroxy radical is reduced to the corresponding hydroperoxide, completing the reaction cycle. In the simplest LOX substrate linoleic acid (C18.2 ω 6), there are two available positions for oxygenation, at C9 and C13, and the possibility of *R* or *S* stereochemistry in each, giving four potential hydroperoxide products (9*R*, 9*S*, 13*R*, or 13*S*). Each of these has been found as a specific LOX product: both 9*S*-LOX and 13*S*-LOX are widespread in plants [4], 9*R*-LOX is found in cyanobacteria and lower animals [5–7], and 13*R*-LOX activity, while not naturally occurring, is known from specific mutation of 9*S*-LOX [8]. In more complex polyunsaturated fatty acids there are more potential positions for oxygenation, and many of these potential LOX activities have been found. For example, with arachidonic acid (C20.4 ω 6), ten of the twelve possibilities are known [3].

The ω 3 polyunsaturated fatty acids such as α -linolenate, stearidonate, eicosapentaenoate or docosahexaenoate have an extra pentadiene that includes the terminal ω 3 double bond. Heretofore there are no reports of primary LOX reactions being centered on the four available positions around this extra pentadiene. (A multifunctional lipoxygenase of the moss *Physcomitrella patens*, which acts as an arachidonate 12-LOX and linole(n)ate 13-LOX and which also exhibits strong hydroperoxidase and lyase activity, does produce small amounts of 12- and 16-HPOTE among the minor products from α -linolenic acid [9]. Also, although not a site of initial oxygenation, the 16 position of α -linolenate is oxygenated by soybean LOX-1 or potato LOX in further conversion of 9*S*-HPOTrE to a major 9,16-dihydroperoxide [10,11]). We report here a new LOX activity specific for *R*-oxygenation at the n-7 carbon of ω 3 substrates. The LOX enzyme in question is part of a natural fusion protein encoded in the genomic DNA of the cyanobacterium *Acaryochloris marina*, a photosynthetic organism of interest for its use of the unusual chlorophyll d [12–14]. The *A. marina* fusion protein consists of a N-terminal heme domain with sequence homology to catalase and a C-terminal LOX domain. In a separate report we have characterized the heme domain as an allene oxide synthase [15]. Here we characterize the novel activity of this *A. marina* LOX domain.

2. Materials and methods

2.1. Materials

Fatty acids and the authentic GC-MS standards were purchased from NuChek Prep Inc. (Elysian, MN). 3-[(3-Cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate and n-Octyl β -D glucopyranoside were from Sigma-Aldrich. Standards of racemic HPODEs, HODEs, HPOTrEs, HOTrEs, HPETEs and HETEs were prepared by vitamin E-controlled autoxidation [16].

2.2. Cloning, expression and purification of the C-terminal LOX domain of *Acaryochloris marina*

As described previously, a hypothetical fusion protein designated as ABW27596.1 (accession number CP000828.1) was cloned by PCR from the genomic DNA (*Acaryochloris marina* MBIC11017), a kind gift of Dr. Robert E. Blankenship of Washington University in St. Louis [15]. The C-terminal LOX domain was cloned separately by PCR using a forward primer either containing a N-terminal (His)₆ tag or without one: 5'-TCCATATGCATCACCATCACCATCACGATTGGGGCAGCGGTCACCAG-3' (*Nde*I restriction site followed by an added (His)₆ tag and then the natural amino acid sequence DWGSGHQ) or 5'-TCACTAGTCATATGGATTGGGGCAGCGGTCACCAG-3' (*Nde*I restriction site followed by the natural amino acid sequence MDWGSGHQ). The downstream primer in each case was 5'-GACTCGAGTTAGATATTGGTGCTCATCATAAG-3' (with added *Xho*I restriction site). The PCR products were subsequently cut with *Nde*I and *Xho*I restriction enzymes and inserted into the same sites of the expression vector pET17b. DNA

sequencing confirmed there were no PCR errors compared to the published sequence in GenBank (accession number CP000828.1, gene AM1-2589, protein ABW27596.1) and at CyanoBase (bacteria.kazusa.or.jp/cyanobase/).

The LOX domain was expressed at 14 °C using 2 × YT media. A single colony was inoculated into 100 ml 2 × YT media (100 µg/ml Amp) and incubated at 37°C. When the OD₆₀₀ value reached ~1.0, the culture was cooled to 14 °C and IPTG (0.5 mM final concentration) was added to induce the protein expression. Cultures were incubated at 14 °C for two days then harvested by the procedures described previously [17]. The His-tag containing LOX domain was run over a nickel-NTA column as described before [17], although as noted in Results this failed to capture the enzyme. Alternatively, frozen pellets from a 100 ml bacterial culture were resuspended using a glass Dounce homogenizer (Wheaton) in 10 ml of BugBuster® Protein Extraction Reagent (Novagen) containing Triton X-100 reduced (1%), 25 U/ml benzonase, 1 mM PMSF and one complete Mini EDTA-free protease inhibitor cocktail tablet (Roche Diagnostics). The extract was then centrifuged at 16,000 × g at 4°C for 30 min. The supernatant was fractionated by precipitation with ammonium sulfate (5%-almost no pellet, and 20%). A spectrophotometric LOX assay (UV-235 nm) using C18.3ω3 as substrate as well as a subsequent SDS-PAGE analysis indicated that essentially all LOX activity and protein was recovered in the 20% ammonium sulfate pellet. The pellet was resuspended in 50 mM potassium phosphate buffer pH 7, and dialyzed overnight at 4°C against 50 mM Tris, pH 7.5 containing 150 mM NaCl and 20% glycerol. After dialysis, a 10 min spin at 16,000g was performed to remove the insoluble protein, and aliquots of the solution were stored in -80 °C for later use. The same ammonium sulfate procedure was used to purify the His-tagged LOX protein.

2.3. Analysis of the fatty acid composition of *A. marina*

Into a 5 ml reaction vial, 38 mg of a frozen cell pellet of *A. marina* was resuspended with 500 ml Tris buffer. Methanol (1.26 ml) and chloroform (633 µl) were added proportionally to the vial and stirred for 1 hour at 4 °C to extract the lipid. For the phase separation, 625 ml of chloroform and 625 ml of water were added, mixed by vortex, and separated by centrifugation at 1000 rpm for 3 min. The lower phase was transferred into a new vial and dried under nitrogen. 50 ml of methanol was added to the vial to redissolve the lipids, and the transesterification was performed by addition of 50 ml of 0.5 M sodium methoxide. After 10 min, the solution was neutralized by 0.1 N HCl and extracted immediately with 2 ml of hexane two times. The pooled extraction was washed with 500 ml water and centrifuged at 1000 rpm for 3 min. The upper layer was transferred to a new vial, dried and stored in 1 ml methanol at -80 °C. The fatty acid composition was measured by GC-MS in the electron impact mode (see section below) using the conditions reported in comparison to a standard mixture of saturated and unsaturated fatty acid methyl esters (Nu-Chek Prep, GLC reference standard 87). The identity of the polyunsaturated fatty acids was confirmed by the GC retention time and the appropriate molecular ion, and also by HPLC retention time with UV detection (205 nm) using a Waters Symmetry C18 column (2.1×150mm), eluting with a solvent system of acetonitrile/water/ammonium acetate in the proportions 60/40/10mM (v/v/concentration) at a flow rate of 0.2 ml/min.

2.4. *A. marina* LOX activity assay

The LOX protein was quantified using a specific in-gel assay method reported previously [8], and the LOX activity assay of *A. marina* was carried out by following the absorbance increase at 235 nm due to the formation of conjugated diene chromophore in 50 mM Tris buffer, pH 7.5, 150 mM NaCl using a Lambda 35 UV/Vis spectrophotometer (PerkinElmer). Polyunsaturated fatty acids (C18.4ω3, C18.3ω3, EPA, DHA, C18.2ω6 and C20.4ω6) were

assayed at a range of concentrations (2–120 μ M) in a 1 ml cuvette. The initial rate was expressed as nanomoles of hydroperoxide formed per nanomole of the LOX enzyme per second.

2.5. Incubation, extraction and HPLC product analysis

Incubations were performed at room temperature in 1 ml of 50 mM Tris, pH 7.5, 150 mM NaCl in the presence of 2 μ l *A. marina* LOX domain supernatant using the fatty acids C18.4 ω 3, C18.3 ω 3, C18.2 ω 6, C20.4 ω 6, EPA, or DHA. Reaction was monitored by UV spectrophotometry at 235 nm. The LOX product was extracted by C18 cartridge (Waters), and analyzed by RP-HPLC using a C18 Waters Symmetry 5 μ m column (0.46 $\times$ 25 cm) with solvent methanol/water/acetic acid (80/20/0.01, by volume) at a flow rate of 1 ml/min and SP-HPLC using a silica column (0.46 $\times$ 25 cm) with solvent hexane/isopropanol/acetic acid (100/2/0.1, v/v/v) at a flow rate of 2 ml/min. The reduced LOX product was achieved either by the inclusion of SnCl₂ in the incubation solution or using TPP after the product extraction.

2.6. Chiral analysis of LOX products

The LOX products of fatty acid hydroperoxides were reduced to the hydroxy fatty acid and methylated with diazomethane. Steric analysis was carried out on a Daicel Chiralpak AD column (25 $\times$ 0.46 cm) eluted with hexane/methanol (100/2) or hexane/ethanol (100/2) at a flow rate of 1 ml/min, with UV detection at 235 nm [18].

2.7. Derivatization and CD measurement

The enantiomers of the racemic 12-HOTrE methyl ester eluted from the Chiralpak AD column were labeled as peak 1 for the first eluting enantiomer and peak 2 for the second eluting enantiomer. In the determination of stereoconfiguration of 12-hydroxy group, a naphthoyl chromophore was attached to the hydroxyl end in the assistance of Cotton Effect. Procedures for the derivatization followed previous reports with some modifications [19,20]. In a 2 ml reaction vial, 33 μ g of each enantiomer was dried under nitrogen, redissolved in 25 μ l acetonitrile, and 1 μ l of DBU, a few grains of 2-naphthoyl chloride and 4-dimethylaminopyridine were added. The reaction was kept overnight at room temperature, and then dried under nitrogen. The residue in each vial was extracted into 1 ml of dichloromethane and washed three times with water. The naphthoate derivatives were purified using RP-HPLC with solvent system of methanol:water (95:5, v/v) at 1 ml/min. For CD measurement, an appropriate amount of each derivative was dissolved in acetonitrile and adjusted to 1 AU for a good CD signal. The CD spectra were acquired on a JASCO J-700 spectropolarimeter.

2.8. Derivatization and GC-MS analysis

Methyl esters were prepared using ethereal diazomethane/methanol. Catalytic hydrogenations were performed in 100 μ L of ethanol using ~1 mg of palladium and bubbled with hydrogen for 2 min at room temperature. The hydrogenated products were extracted into ethyl acetate in the addition of water. Methoxime derivatives were prepared by treatment with 5 μ l of methoxylamine hydrochloride in pyridine (10 mg/ml) overnight at room temperature. Trimethylsilyl ether was prepared using *bis*(trimethylsilyl)-trifluoroacetamide (10 μ L) at room temperature for 2 hours or overnight, and the residue was dried, redissolved in hexane for GC-MS analysis. The GC-MS spectra were acquired in the positive ion electron impact mode (70 eV) using a 30 meter Agilent J&W HP-5 column (0.25 mm inner diameter, 0.25 μ m film thickness) connected to a trace DSQ mass spectrometer. Samples were injected at 150 $^{\circ}$ C, and after 1 min the temperature was programmed to 300 $^{\circ}$ C at 20 $^{\circ}$ C/min increment.

3. Results

3.1. Cloning, expression and purification of the LOX protein

BLAST searches of the *A. marina* genome revealed the presence of DNA encoding a putative fusion protein of a lipoxygenase and a catalase-related hemoprotein (a hypothetical protein designated as ABW27596.1). The cDNA of the full-length fusion protein and the C-terminal LOX domain (with and without an added N-terminal His-tag) were cloned and expressed in *E. coli*. The activity of the N-terminal catalase-related domain and its characterization as an allene oxide synthase was reported separately [15]. Catalytic activities of the LOX domain were monitored in the full-length protein by trapping the intermediate hydroperoxide as the hydroxy derivative by including SnCl_2 (2 mM) in the incubation. The primary hydroperoxide products were isolated by incubations using the LOX domain alone. When the LOX domain was expressed on its own with culture of the *E. coli* at 28 °C, although there was LOX activity in the bacterial cell lysate, most of the protein was insoluble and centrifuged down in the cell pellet. Considerably more protein remained soluble when expression was conducted at 14 °C and the supernatant retained very strong LOX activity, particularly using the His-tagged construct from which 1 μl of supernatant could fully metabolize 20 μg of polyunsaturated fatty acid. For reasons that were not determined, the LOX protein containing the N-terminal His-tag was not efficiently captured by a Ni-NTA affinity column, and therefore an alternative procedure was developed. A remarkable purification was achieved using precipitation with 20% ammonium sulfate, a procedure that gave almost pure protein from either the His-tagged or natural LOX protein constructs (Fig. 1). No significant activity remained in the supernatant from the 20% ammonium sulfate fractionation, confirmed by the absence of any visible band of LOX protein in SDS-PAGE analysis (not shown). For the non-His-tagged protein, spectrophotometric LOX assay (increase in absorbance at 235 nm) indicated a 33-fold enrichment on the LOX specific activity in the 20% ammonium sulfate fraction.

3.2. Fatty acid composition of *A. marina*

The natural content of polyunsaturated fatty acids in *A. marina* was determined after lipid extraction of a cell pellet, transesterification to the methyl esters and analysis by GC-MS, HPLC-UV and LC-MS. Compared with the fatty acid standards, the GC-MS chromatogram showed that the most prominent single fatty acid was C16:0, although there were no C16 unsaturated fatty acids. A mixture of C18 unsaturated fatty acids were present, predominantly stearidonic acid (C18:4 ω 3), along with lower levels of C18:3 ω 3, C18:3 ω 6, two C18:2 species including linoleic acid, and two C18:1 fatty acids including the oleate (Table 1). C18:0 saturated fatty acid was present in the least amount. C20 and higher fatty acids were absent. HPLC-UV analysis compared to authentic standards confirmed the identity of the polyunsaturated fatty acids.

3.3. Substrate specificity of the LOX domain

The kinetic parameters for C18:4 ω 3, C18:3 ω 3, EPA, DHA, C18:2 ω 6 and C20:4 ω 6 were determined using the purified natural LOX protein in the concentration range of 2 to 120 μM by measurement of the absorbance increase at 235 nm due to formation of the conjugated diene chromophore of the hydroperoxide product (Table 2). The results demonstrate that the ω -3 fatty acids, generally, showed a higher value of k_{cat} , C18:4 ω 3 and C18:3 ω 3 exhibited a lower K_{M} value (5 μM) and therefore exhibit a higher catalytic efficiency. Compared to the ω -3 fatty acids, the ω -6 fatty acids showed lower values of k_{cat} and higher K_{M} , C18:2 ω 6 having the lowest catalytic efficiency. The most abundant polyunsaturated fatty acid in *A. marina*, stearidonic acid, was found to be the optimal substrate for the LOX domain, with a turnover number (k_{cat}) of 36 s^{-1} .

3.4. Characterization of the oxygenation products from ω 3 fatty acids

As judged by HPLC and UV analyses, the full-length fusion protein, the His-tagged or non-tagged LOX protein had indistinguishable catalytic specificities. Using the full-length hemoprotein-LOX fusion protein, the primary LOX products were trapped as the hydroxy fatty acid by including SnCl_2 in the enzyme incubation, while the primary hydroperoxide products of the expressed LOX domain were extracted and chromatographed intact, or reduced by triphenylphosphine treatment of the product extract prior to HPLC analysis. The single main LOX product from C18.3 ω 3 did not co-chromatograph on HPLC with 9-hydroxy or 13-hydroxy standards prepared using the typical plant 9- and 13-LOX enzymes (Fig. 2A). When C18.4 ω 3 was assayed with the LOX domain, it too gave a single main product (Fig. 2B). By SP-HPLC comparison to a mixture of four authentic HOTrEs prepared by autoxidation (the four standards eluted in the order 13-, 12-, 16-, and 9-hydroxy) [21], the natural product from C18.3 ω 3 co-chromatographed with the second-eluting standard and displayed an identical UV spectrum. Accordingly, the second peak from the autoxidation mixture and the *A. marina* product were collected and analyzed by GC-MS before and after hydrogenation. The mass spectrum of the hydrogenated derivative (methyl ester TMS ether) showed ions of m/z 187 (100%, α -cleavage between C11 and C12), 301 (40%, α -cleavage between C12 and C13), 73 (67%, TMS), 386 (0.1%, M^+), 371 (0.2%, M-15), and 355 (1%, M-31), which matches the published spectrum of 12-hydroxystearate [22]. The methyl ester TMS derivative of the enzymatic product and the second peak from autoxidation also showed identical spectra on GC-MS, confirming the identity of the enzymatic product as 12-hydroxylinolenate. Based on the known structure of the products from vitamin E-controlled autoxidation and the expected double bond configurations from a typical lipoxygenase reaction, the precise structure can be designated as 12-hydroxy-octadeca-9Z,13E,15Z-trienoic acid.

With stearidonic acid as substrate, the directly analogous product was formed. The mass spectrum of the TMS ester TMS ether derivative showed ions of m/z 183 (100%, α -cleavage between C11 and C12), 355 (37%, α -cleavage between C12 and C13) and 73 (68%, TMS). The mass spectrum of the hydrogenated derivative (TMS ester TMS ether) showed ions of m/z 187 (70%, α -cleavage between C11 and C12), 359 (100%, α -cleavage between C12 and C13) and 73 (32%, TMS). Together with the UV spectral data which indicated a *cis-trans* conjugated diene (λ_{max} 236 nm), the lipoxygenase product of stearidonic acid was identified as 12-hydroxy-octadeca-6Z,9Z,13E,15Z-tetraenoic acid (12-hydroxystearidonate). EPA and DHA were similarly metabolized by the *A. marina* LOX domain and the oxygenation products identified as 14-hydroxy-eicosa-5Z,8Z,11Z,15E,17Z-pentaenoic acid and 16-hydroxy-docosa-4Z,7Z,10Z,13Z,17E,19Z-hexaenoic acid by GC-MS of the hydrogenated TMS ester TMS ether derivatives (data not shown).

3.5. Chirality of the novel LOX products

On the Chiralpak AD column with the solvent of hexane/alcohol, the enantiomers of all HETEs and HODEs are well resolved and are known to elute in the order of *R* then *S* [18,23]. However, in the case of 12-HOTrE no authentic standards were available to confirm this, and therefore the two enantiomers were resolved on the chiral column and their absolute stereochemistry established by a circular dichroism method [19]. Each enantiomer (as the methyl ester) was derivatized on the free hydroxyl to the naphthoate ester and repurified. The UV absorbance of each enantiomer derivative was adjusted to ~1 AU at 235 nm in acetonitrile, and the CD spectra were recorded. The early eluting 12-HOTrE enantiomer (peak 1) exhibited a negative first and positive second Cotton effect while a positive first and negative second Cotton effect was shown for the peak 2 enantiomer (Fig. 3A). This allowed assignment of the absolute stereoconfigurations of peak 1 as 12*R* and peak 2 as 12*S* [19], the same order of *R* before *S* as with other HODEs and HETEs. Fig. 3B shows the chiral column HPLC profile of the 12-

hydroxy product of C18.3 ω 3 compared to the newly assigned *R* and *S* standards, thus establishing the stereoconfiguration as 12*R*.

3.6. Characterization of the oxygenation products from ω 6 fatty acids

Linoleic acid and arachidonic acid were each oxygenated to a single main LOX product. These were identified by co-chromatography on RP-HPLC, SP-HPLC and chiral phase HPLC with authentic standards and by a perfect match of their UV spectra with standards. By comparison with authentic standards, including on chiral column HPLC, the hydroxy product from linoleic acid was identified as 9*R*-HODE; (the ratio of 9:13 products was approximately 87:13). Similarly, the major product of arachidonic acid was identified as 11*R*-HPETE (Fig. 4A, B, C and D).

3.7. Summary of the positional specificity of the *A. marina* LOX

The oxygenation products of the LOX domain were formed on the last available pentadiene in either omega-3 or omega-6 fatty acids. Fig. 5 schematically shows *A. marina* LOX product structures from omega-3 fatty acids (C18.3 ω 3, C18.4 ω 3, C20.5 ω 3, and C22.6 ω 3) and omega-6 fatty acids (C18.2 ω 6 and C20.4 ω 6). Omega-3 fatty acids were oxygenated specifically at the n-7 position, which is novel, while omega-6 fatty acids were oxygenated at n-10. These hydroperoxides are mainly further converted to allene epoxides by the catalase-related heme domain of the *A. marina* fusion protein [15].

4. Discussion

The *Acaryochloris marina* fusion protein has some features in common with the first of the catalase-related hemoprotein-lipoxygenase fusion proteins to be described, the type in corals including *Plexaura homomalla* and *Gersemia fruticosa* [24,25], (and probably including the reported sequence from *Clavularia viridis* with GenBank accession number AB188528). They all catalyze consecutive LOX and AOS reactions [15,24,25]. However, in contrast to the coral proteins that have 8*R*-LOX activity with C20 fatty acids and weak activity with C18 substrates [26], the *A. marina* LOX domain has a novel catalytic activity and metabolizes C18 polyunsaturates very well. The LOX domain of the *A. marina* fusion protein is also unusually small, only 52 kD (similar to that in *Anabaena*, only 49 kD [5,6]). This compares with 75–80 kD for typical animal LOX including the coral LOX domains and ~94–104 kD for plant LOX [1]. The “missing” N-terminal beta-barrel or C2-like subdomain accounts for part of the difference but there are also many shorter stretches throughout the rest of the primary structure. Despite this, *A. marina* LOX domain has typical ligands to the non-heme iron (three His, an Asn, and the C-terminal Ile). At the position of the Gly-or-Ala determinant of *R* or *S* product stereospecificity, *A. marina* has Gly (G586 in the full-length fusion protein), consistent with its *R*-specificity [27,28]. (The only known exception to the rule that Gly signifies *R* stereospecificity and Ala signifies *S* is in the *Anabaena* LOX domain in which the equivalent *R* determinant is Ala and substitution with Val switches specificity in favor of *S* [5,6]).

We found that the fatty acid composition of *A. marina*, in common with other cyanobacteria, includes typical eukaryotic all-*cis* polyunsaturated fatty acids (cf. refs [29–31]). Although some cyanobacteria contain C18 and C20 polyunsaturates, only C18 were detected in *A. marina*, with stearidonic acid (C18.4 ω 3) predominating. Consistent with this, stearidonic acid is an excellent substrate for the LOX domain, and its hydroperoxide product is readily transformed to a novel allene oxide by the catalase-related domain [15]. The specific oxygenation catalyzed by the *A. marina* LOX domain places the oxygen on the n-7 carbon of ω 3 polyunsaturated fatty acids, a novel LOX activity, producing 12*R*-hydroperoxides from C18.3 ω 3 and C18.4 ω 3 substrates, 14*R* from C20.5 ω 3, and 16*R* from C22.6 ω 3. Omega-6 fatty acids cannot be oxygenated at the n-7 carbon and instead the reaction is “frame-shifted” along the fatty acid

chain, resulting in 9*R*-hydroperoxide from C18.2ω6 and 11*R* from C20.4ω6. Although there are no C20 or C22 fatty acids in *A. marina*, the LOX domain accepts these as suitable substrates. Nonetheless, based on the natural predominance of stearidonic acid, the presumption is that the biosynthetic pathway involving this fusion protein in *A. marina* produces the allene oxide derivative of 12*R*-hydroperoxy-C18.4ω3 either as a signaling molecule in its own right, or as a precursor of end products equivalent to the cyclopentenone/cyclopentane derivatives of the jasmonate pathway in plants [4].

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Abbreviations

H(P)ODE	hydro(pero)xyoctadecadienoic acid
H(P)OTrE	hydro(pero)xyoctadecatrienoic acid
H(P)ETE	hydro(pero)xyeicosatetraenoic acid
PMSF	phenylmethylsulfonyl fluoride
LOX	lipoxygenase
TPP	triphenylphosphine
EPA	eicosapentaenoic acid
DHA	docosahexaenoic acid
DBU	1,8-Diazabicyclo[5,4,0]undec-7-ene
CD	circular dichroism
HPLC	high pressure liquid chromatography
GC-MS	gas chromatography mass spectrometry
LC-MS	liquid chromatography mass spectrometry

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Fig. 1. SDS-PAGE showing the supernatant and the 20% ammonium sulfate fractionation of *A. marina* LOX protein

Lane 1: molecular weight standards. Lanes 2 and 3: the non-His-tagged LOX enzyme, before and after 20% ammonium sulfate fractionation. Lanes 4 and 5: His-tagged LOX enzyme, before and after 20% ammonium sulfate fractionation.

Fig. 2. SP-HPLC analysis of *A. marina* lipoxxygenase products from (A) C18.3ω3 and (B) C18.4ω3 α-Linolenic acid was reacted with the full-length hemoprotein-lipoxygenase fusion protein in the presence of 2 mM SnCl₂, allowing the primary lipoxygenase product to be trapped as the hydroxy derivative. Stearidonic acid was incubated with the LOX domain only including 2 mM SnCl₂ to reduce the hydroperoxide to the hydroxy derivative. The analysis was performed using a Beckman Ultrasphere silica column (0.46 × 25 cm), eluted with a solvent system of hexane/isopropanol/acetic acid in the proportions of 100/2/0.1 (v/v/v) at a flow rate of 2 ml/min and detected at 235 nm. Arrows in panel A show the retention times of 13-HOTrE and 9-HOTrE standards; the other small peaks are not hydroxy-fatty acids.

Fig. 3. Chiral analysis and absolute stereochemistry of 12-HOTrE

(A) CD spectra of peaks 1 and 2 from the Chiralpak AD column. The enantiomers of 12-HOTrE were collected as the methyl ester from the Chiralpak AD column, derivatized to the 12-O-naphthoyl ester, repurified and analyzed in acetonitrile using a JASCO J-700 spectropolarimeter. (B) Chiral HPLC analysis of 12-HOTrE (methyl ester) from the *A. marina* LOX (middle) and racemic 12-HOTrE methyl ester (bottom). The Chiralpak AD column (25 × 0.46 cm) was eluted with a solvent of hexane/methanol (100/2, v/v) at a flow rate 1 ml/min.

Fig. 4. Chiral HPLC analysis of *A. marina* LOX products from $\omega 6$ fatty acids

Product methyl esters were analyzed using a Chiralpak AD column (25×0.46 cm) eluted with a solvent of hexane/ethanol (100/2, v/v) at a flow rate 1 ml/min. (A) The LOX product of C18:2 $\omega 6$, (B) 9RS-HODE methyl ester standard, (C) The LOX product of C20:4 $\omega 6$ and (D) 11RS-HETE methyl ester standard.

Fig. 5. Oxygenation specificity of the *A. marina* LOX domain

Omega-3 fatty acids are oxygenated at the n-7 position in the *R*-configuration, i.e. at C12 for C18.3 ω 3 and C18.4 ω 3, at C14 for C20.5 ω 3, and at C16 for C22.6 ω 3. Omega-6 fatty acids are oxygenated at n-10, i.e. 9*R* in C18.2 ω 6 and 11*R* in C20.4 ω 6.

Table 1Fatty acid composition of *A. marina*.

Fatty acids		Percentage (%)
Saturated fatty acids	C16:0	52
	C18:0	1
Unsaturated fatty acids	C18:4 ω 3	19
	C18:3 ω 3	5
	C18:3 ω 6	4
	C18:2 ω 6	3
	C18:2	3
	C18:1 ω 9	7
	C18:1	6

Table 2Kinetic parameters for the LOX protein of *A. marina*. (mean $\pm$ S.E., n = 3)

Substrates	K_M (μ M)	k_{cat} (s^{-1})	k_{cat}/K_M ($M^{-1}s^{-1}$)
C18.4 ω 3	5.2 $\pm$ 0.8	35.8 $\pm$ 1.2	6.9 $\times 10^6$
C18.3 ω 3	5.1 $\pm$ 1.2	23.4 $\pm$ 1.4	4.6 $\times 10^6$
EPA	6.5 $\pm$ 1.8	28.0 $\pm$ 2.4	4.3 $\times 10^6$
DHA	12.5 $\pm$ 5.5	24.2 $\pm$ 4.8	1.9 $\times 10^6$
C18.2 ω 6	19.0 $\pm$ 5.0	13.4 $\pm$ 1.2	7.1 $\times 10^5$
C20.4 ω 6	9.2 $\pm$ 1.8	16.0 $\pm$ 1.0	1.7 $\times 10^6$