Crystal structure of a lipoxygenase from *Cyanothece* sp. may reveal novel features for substrate acquisition

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Abstract In eukaryotes, oxidized PUFAs, so-called oxygenated lipids, are vital signaling molecules. The first step in their biosynthesis may be catalyzed by a lipoxygenase (LOX), which forms hydroperoxides by introducing dioxygen into PUFAs. Here we characterized CspLOX1, a phylogenetically distant LOX family member from *Cyanothece* sp. PCC 8801 and determined its crystal structure. In addition to the classical two domains found in plant, animal, and coral LOXs, we identified an N-terminal helical extension, reminiscent of the long α-helical insertion in *Pseudomonas aeruginosa* LOX. In liposome flotation studies, this helical extension, rather than the β-barrel domain, was crucial for a membrane binding function. Additionally, CspLOX1 could oxygenate 1,2-dihaedonyl-sn-glycero-3-phosphocholine, suggesting that the enzyme may act directly on membranes and that fatty acids bind to the active site in a tail-first orientation. This binding mode is further supported by the fact that CspLOX1 catalyzed oxygenation at the n-10 position of both linoleic and arachidonic acid, resulting in 9Z- and 11R-hydroperoxides, respectively. Together these results reveal unifying structural features of LOXs and their function. While the core of the active site is important for lipoxygenation and thus highly conserved, peripheral domains functioning in membrane and substrate binding are more variable.—Newie, J., A. Andreou, P. Neumann, O. Einsle, I. Feussner, and R. Ficner. Crystal structure of a lipoxygenase from *Cyanothece* sp. may reveal novel features for substrate acquisition. *J. Lipid Res.* 2016. 57: 276–287.

**Supplementary key words** arachidonic acid • eicosanoids • enzymology • fatty acid/oxygenation • lipids/peroxidation • membrane • phospholipids/metabolism • X-ray crystallography

PUFA oxygenation is a process that leads to the formation of bioactive lipid compounds with a diversity of biological functions in plants and animals (1, 2). Oxidation of PUFAs may be catalyzed by two major classes of enzymes, cyclooxygenases or α-dioxygenases and lipoxygenases (LOXs) (5). Of the two, LOXs are nonheme iron-containing dioxygenases that are widely found in higher plants and animals, but have also been detected in some corals, mosses, fungi, and a number of bacteria (4, 5). Members of the LOX family catalyze the regio- and stereospecific oxygenation of PUFAs with one or more (1Z,4Z)-pentadiene moieties leading to the formation of hydroperoxy PUFAs (6). LOX hydroperoxide products are precursors of important signaling compounds such as aldehydes and jasmonates in plants and leukotrienes, resolvins, and lipoxins in mammals (3). These signaling molecules play an important role in wound and defense responses as well as in aspects of plant development (7), while in mammals they function in inflammation, asthma, and the development of atherosclerosis and cancer (1). Other than in higher organisms, very little is still known regarding the overall function of LOX products in prokaryotes and fungi (4, 5).

As the regio- and stereospecificity of the LOX reaction has an influence on the biological function of the product, many studies have focused on the molecular basis of this specificity. In the case of arachidonic acid [20:4(n-6)], which is a typical mammalian LOX substrate, several

**Abbreviations:** CD, circular dichroism; CP-HPLC, chiral phase HPLC; CspLOX1, *Cyanothece* sp. lipoxygenase 1; HEPE, hydroxyeicosapentaenoic acid; HOT-E, hydroxyoctadecatrienoic acid; ID, identification; LOX, lipoxygenase; PDB, Protein Data Bank; PLAT, polycystin-1 lipoxygenase α-toxin; RP-HPLC, reversed phase HPLC; SF-HPLC, straight phase HPLC; 11-HETE-Me, methylated 11-HETE; 18:2(n-6), linoleic acid; 18:3(n-3), α-linolenic acid; 20:4(n-6), arachidonic acid; 20:5(n-3), eicosapentaenoic acid.

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The online version of this article (available at http://www.jlr.org) contains a supplement.

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pentadiene systems are available for the LOX reaction. Depending on the depth of the substrate binding channel, either C-11 or C-14, for example, is targeted for hydrogen abstraction, due to a frameshift of the substrate (8) (Fig. 1A). Additionally, the substrate orientation was proposed to have an effect on the specificity of the hydrogen abstraction and dioxygen insertion, because another side of the substrate is exposed to the catalytic iron when the substrate binds in a reversed orientation, i.e., with the carboxy group of the fatty acid first (9). While most LOXs bind their substrate with the methyl end first, the 9S-lipoxygenation products of plant LOXs (10, 11), as well as the specificities of 5S, 12R- and 8S-LOX in mammals, were explained with the reversed orientation of the substrate (12) (Fig. 1B). Additionally, another position in the active site, also referred to as the Gly/Ala switch, may function as a switch for the regiospecificity of the reaction (13). As illustrated in Fig. 1C, the larger alanine residue in this position favors formation of the 12S product, while its replacement with the smaller glycine switches reaction specificity to 8R-lipoxygenation.

Mammalian LOXs have a molecular mass of 75–80 kDa, while LOXs from land plants are larger with a polypeptide chain of 94–104 kDa (14). Even though the sequence homology between eukaryotic isozymes is low, there is a high degree of conservation of the overall protein fold and geometry of the nonheme iron binding site (3). In general, LOXs consist of a single polypeptide chain folded in a two-domain structure with an N-terminal β-barrel domain and a C-terminal catalytic domain, which is mostly α-helical and contains the active site iron (14). The iron is octahedrally coordinated by conserved amino acid residues, which are three histidines and one asparagine [or a fourth histidine in the case of some mammalian LOXs (supplementary Fig. 1)] and the carboxylate of the C-terminal isoleucine. The N-terminal β-barrel domain, which is also referred to as the polycystin-1 LOX α-toxin (PLAT) domain, exhibits structural similarities with the C-terminal domain of human pancreatic lipase, suggesting its possible role in membrane binding (15, 16). This domain is, nonetheless, not essential for the catalytic activity because the so-called mini-LOXs, consisting only of the C-terminal domain, are enzymatically active. These mini-LOXs are naturally found in a number of cyanobacteria (17–20), but are also generated by genetic removal or by limited proteolysis (16, 21).

The crystal structure of the Pseudomonas aeruginosa LOX presents the first structure of a prokaryotic LOX and reveals an unusual helical insertion, as well as a phospholipid molecule bound in the active site (22). The head group of this lipid interacts with peripheral parts of the enzyme, suggesting that substrates might be acquired directly from the membrane.

In order to analyze the evolution of LOXs, their structures, functions, and specificities, it is important to include enzymes that are phylogenetically distant. Such outgroups are important for the correct construction of the phylogenetic tree. We identified two putative LOX genes in the genome of the unicellular diazotrophic cyanobacterium, Cyanothece sp. PCC 8801, and named them CspLOX1 and CspLOX2. While CspLOX2 belongs to the group of prokaryotic mini-LOXs (17), CspLOX1 represents a rare group of LOX enzymes found in few bacteria with only very low sequence homology to all well-characterized LOXs. Here we show that despite this low sequence homology, CspLOX1 still catalyzes a typical LOX reaction. While the enzyme shares many biochemical features with plant and animal LOXs, its crystal structure reveals major differences within the N-terminal domain. Of special interest is an α-helical extension that confers binding to liposomes and could thus fulfill a function in substrate acquisition directly from membranes.

**MATERIALS AND METHODS**

**Materials**

Chemicals were obtained from Sigma and Carl Roth and Company. Agarose was from Biozym Scientific GmbH. Pentaerythritol
propoxyxylate was from Jena Biosciences. All fatty acids were from Sigma or Cayman Chemical. The 1,2-diarachidonoyl-sn-glycero-3-phosphocholine [PC-20:4(n-6)] was purchased from Sigma or Cayman Chemical. The 1,2-diarachidonoyl-propoxylate was from Jena Bioscience. All fatty acids were from MBI Fermentas.

Cyanobacterial material and growth conditions
Cyanothecae sp. (PCC 8801) was obtained from the Pasteur Culture Collection (PCC) of cyanobacteria (Paris; https://www.pasteur.fr/recherche/banques/PCC/Media.htm#BG11). Cultures were grown as described in (17).

Cloning and expression and purification of recombinant CspLOX1 in E. coli
Genomic DNA was isolated from approximately 50 mg of frozen cell material of Cyanothecae sp., as previously described (17). The csplox1 (GenBank accession number WP_012595715.1) was amplified from genomic DNA with gene-specific primers containing BamHI and XhoI restriction sites (forward primer, 5′-CGATCCATGTCTACAAACAAAGAGAAATTTC-3′; reverse primer, 5′-CAAGCTTTCAATATATATTAAAGTGCG-3′) using Phusion high-fidelity DNA polymerase (New England Biolabs). The PCR product was then cloned into the pET vector (Fermentas) according to the manufacturer’s instructions. The identity of the cloned gene was confirmed by DNA sequencing. The PCR product was further cloned into the pET28a vector (Merck). For heterologous expression of the His-tagged recombinant protein, Escherichia coli BL21 Star cells (Invitrogen) were grown in 2YT containing 25 µg/ml kanamycin at 37°C until OD₆₀₀ of 1 and induced with the addition of 1 mM isopropyl β-d-thiogalactopyranoside. The cultures were additionally supplemented with 250 µM ammonium iron(III) citrate and further cultivated by shaking at 28°C for 18 h. Alternatively, proteins were expressed in the ZYP-5052 defined medium for autoinduction using the same conditions (23). For purification of CspLOX1, frozen cell pellets from 200 ml of cultures were resuspended in 30 ml 50 mM Tris (pH 7.5) and 30 mM imidazole. Cells were incubated on ice for 30 min after the addition of 0.1 mg/ml lysozyme, 0.2 mM phenylmethylsulfonyl fluoride, 20 µg/ml DNase, and 1 mM MgCl₂, disrupted by three pulses of 30 s sonication on ice, and the cell debris was centrifuged at 27,000 g at 4°C for 20 min. The supernatant was applied to a 1 ml HisTrap FF column (GE Healthcare) previously equilibrated with 50 mM Tris (pH 7.5) and 30 mM imidazole. The column was washed with the equilibration buffer and the protein was finally eluted with 50 mM Tris (pH 7.5) and 300 mM imidazole. The eluted protein was then concentrated and further purified using a Superdex 200 HR (GE Healthcare). CspLOX1 was concentrated to 17 mg/ml and rebuffed in 10 mM HEPEs (pH 7.5) over a Spin-X UF concentrator (cutoff of 30,000, Corning). Protein purity was evaluated by SDS-PAGE and protein concentration was estimated by absorption (ε₂₈₀ = 105,575 M⁻¹ cm⁻¹). For truncated versions of CspLOX1, the desired fragments were amplified with Phusion high-fidelity DNA polymerase (New England Biolabs) using the following primer pairs: truncated version (444617), forward primer 5′-CCGGATCCGCTATACCCAAAACCCATCG-3′ and reverse primer 5′-CAAGCTTCTAAATATATTAAAGTGCG-3′; truncated version (180467), forward primer 5′-CCGGATCCGAAGAACCCCAGATGATTAC-3′ and the same reverse primer. The fragments were cloned into the pET vector (Fermentas) and into pET28a using the restriction sites BamHI and HindIII. Single or multiple amino acids were exchanged on CspLOX1 in the expression plasmid, pET28a, according to the QuikChange site-directed mutagenesis kit (Stratagene).

Cloning and expression and purification of recombinant AmLOX1 in E. coli
Acaryochloris marina (MBIC11017) was obtained from the NITE Biological Resource Center, Department of Biotechnology, Japan. Genomic DNA was isolated from approximately 50 mg of frozen cell material, as previously described (17). For the amplification of the genomic DNA of the fusion protein of LOX and catalase-related hemoprotein (24), forward and reverse primers containing BamHI and HindIII restriction sites were designed (forward primer, 5′-CGATCCATGGATAGTCGTGATCCGCGCACTG-3′ and reverse primer, 5′-CAAGCTTTTAGATATTGGTGCTCATCATA-3′). Cloning and expression were further carried out as described above.

LOX activity assay and HPLC product analysis
For activity assays, 15 µg of CspLOX1 were added to 900 µl 50 mM Tris buffer (pH 7.5) containing 250 µg of the respective substrate. The reaction was allowed to proceed at 23°C for 30 min. The hydroperoxides formed were reduced to their corresponding hydroxides with the addition of 900 µl of 50 mM SnCl₂ dissolved in methanol. After acidification to pH 3.0 with glacial acetic acid, fatty acids were extracted as described in (25), resuspended in 80 µl methanol/water/acidic acid (85:15:0.1, v/v/v), and subjected to HPLC analysis. Hydroxy fatty acids were separated from fatty acids with reversed phase (RP)-HPLC using an EC250/4 Nucleosil 120-5 C₅₆ column (250 × 2.1 mm, 5 µm particle size; Macherey-Nagel). Elution was carried out with a solvent system of A [methanol/water/acidic acid (85:15:0.1, v/v/v)] and solvent B [methanol/acidic acid (100:0.1, v/v)] using the following gradient elution profile: flow rate of 0.2 ml/min, 0–10 min, 60% A; 10–12.5 min from 60% A to 100% B, flow rate increase to 0.4 ml/min; 12.5–22 min, 100% B; 22–25 min from 100% B to 60% A; and 25–27 min, 60% A. Straight phase (SP)-HPLC of hydroxy fatty acid isomers was carried out on a Zorbax Rs-SIL column (150 × 2.1 mm, 5 µm particle size; Agilent) eluted with a solvent system of n-hexane/2-propanol/acidic acid (100:1:0.1, v/v/v) at a flow rate of 0.2 ml/min. Chiral phase (CP)-HPLC of the hydroxy fatty acids was carried out on a Chiralcel OD-H column (150 × 2.1 mm, 5 µm particle size; Daicel, VWR) with a solvent system of n-hexane/2-propanol/trifluoroacetic acid (100:5:0.1, v/v/v) for C18 fatty acid hydroxides and (100:20:1, v/v/v) for C20 fatty acid hydroxides at a flow rate of 0.1 ml/min. Absorbance at 234 nm was recorded for the detection of conjugated hydroxy fatty acids, respectively, during all chromatographic steps. Activity of CspLOX1 against PC esters was measured as previously described (17). Methyl esters were separated by RP-HPLC using a column as described above and with a solvent system of solvent A [methanol/water/acidic acid (75:25:0.1, v/v/v)] and solvent B [methanol/acidic acid (100:0.1, v/v)] using the following gradient: flow rate of 0.18 ml/min, 0–5 min, 100% A; 5–10 min from 100% A to 100% B and flow rate increase to 0.36 ml/min; 10–20 min 100% B; 20–25 min from 100% B to 100% A; 25–30 min 100% A. SP-HPLC analysis was carried out using a column as described above with a solvent system of n-hexane/2-propanol/trifluoroacetic acid (100:1:0.02, v/v/v) at a flow rate of 0.1 ml/min and CP-HPLC analysis was carried out as in the case of free hydroxylated fatty acid.

GC/MS analysis
The identity of the hydroxy fatty acids was verified by GC/MS. For this purpose, after reduction with SnCl₂, samples were methylated and analyzed by GC/MS as previously described (17).

Determination of kinetic parameters
For analysis of the initial reaction rate data, the formation of the conjugated double bond of the hydroperoxide products was

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monitored at 234 nm ($\epsilon = 2.5 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$) with a CARY 100 Bio spectrophotometer (Varian) at different 18:2 concentrations. The kinetic experiments were conducted at 30°C in 200 mM sodium borate buffer (pH 9.0). Reactions were initiated by the addition of 10 μg of purified enzyme to 1 ml of the buffer containing a defined amount of substrate. The kinetic parameters were calculated by nonlinear regression fitting of the experimental points to the Michaelis-Menten equation.

**Protein crystallization and X-ray data collection**

Initial screening was performed using the sitting drop vapor diffusion method with commercial screens (Hampton Research) at 4°C and 20°C by mixing 1 μl of CspLOX1 [17 mg ml$^{-1}$ in 10 mM HEPES (pH 7.5)] with 1 μl reservoir solution. Diffraction quality crystals were obtained in 40% pentane thiol propoxylate (3/4 PO/OH), 0.14 M KCl, and 50 mM HEPES (pH 7.5) at 4°C. Crystals usually appeared after 2–5 days and grew to the size of 0.1 × 0.15 × 0.25 mm. As the crystallization droplet contained high amounts of pentane thiol propoxylate, the crystals did not need any further cryoprotection and were flash-cooled by plunging them directly from their native drops into liquid nitrogen prior to the data collection at 100 K. The gold derivative crystals were obtained by soaking in mother liquor containing 8 mM potassium tetrachloroaurate(III) hydrate. The oscillation photographs of the native and gold derivative crystals were collected at the beamline X12 (DESY-EMBL outstation in Hamburg) equipped with a MAR Mosaic CCD 225 detector. The diffraction images were integrated and scaled using the XDS package (26). The CspLOX1 protein crystallized in the hexagonal space group P6$_1$2$_2$ ($a = 121.10$ Å, $b = 121.10$ Å, $c = 235.60$ Å, $\alpha = 90^\circ$, $\beta = 90^\circ$, $\gamma = 120^\circ$) with one molecule in the asymmetric unit.

**Structure determination and refinement**

The Molecular Replacement search with the PHASER (27) program using the structure of LOX1 [Protein Data Bank (PDB) identification (ID): 1YGE] as the search model yielded a solution in the P6$_1$2$_2$ space group. The resulting electron density map, however, was of poor quality and did not allow manual rebuilding of the model. After unsuccessful trials of the quick-soak approach using bromide or iodide anions (28), soaks with different heavy metals, as well as Magic Triangle compounds (29, 30), were undertaken to obtain derivatized CspLOX1 crystals. Diffraction data from several potentially derivatized crystals were measured, but most of them appeared not to be suitable for single isomorphous replacement or single-wavelength anomalous diffraction approaches. Among them a peak data set of a gold derivatized crystal was collected (highest resolution limit of 2.7 Å). However, a weak anomalous signal (mean anomalous correlation of 26% at 4.40 Å resolution and its estimated standard deviation of 1.123, as reported by XDS) did not allow heavy metal sites to be found using the single-wavelength anomalous diffraction approach. The phase problem was finally solved by the means of single isomorphous replacement with anomalous scattering. The reflection file for the heavy atom substructure search was prepared with a free demo version of XPREP (courtesy of G. M. Sheldrick and B. Nonius). Several hundred SHELXD runs (31) with different parameters (combining the highest resolution limits from 3.2 to 4.2 Å in steps of 0.1 Å with a different number of sites and different maximum E values) were attempted. For the best 20 heavy atom substructures, several runs of density modification and phase extension with different solvent content were tried with SHELXE (32), none of which resulted in an interpretable electron density map. Finally SHARP/autoSHARP (33) was tried using the best heavy atom sites from previous SHELXD runs. This resulted in obtaining an interpretable electron density map, which was used by ARP/wARP (34) to build an initial model, which was then rebuilt in O (35). The structure was initially refined using CNS (36) against the MLHL target (maximum likelihood, Hendrickson-Lattman coefficients) and verified against simulated annealing omit maps, as well as SIGMA$_A$-weighted electron density maps. The model was further rebuilt in Coot (37) and refined in PHE-NIX (38). The final model, consisting of residues 1 to 668, one iron ion, one sodium ion, and 51 water molecules was refined at the resolution of 2.7 Å to $R$ and $R_{	ext{free}}$, values of 20.6 and 25.24%, respectively. The refined model had good geometry as judged by MOLPROBITY (39): 95% of all residues were in preferred regions and 0.75% (residues) in the disallowed. Molecular graphics were carried out using PyMOL (DeLano Scientific LLC, www.pymol.org). Interactions between different domains were identified using the PISA program (40). The CspLOX1 structure was deposited in the PDB with the code 5EK8.

**Circular dichroism spectroscopy**

The secondary structures of CspLOX1 variants were analyzed and compared with the wild-type protein using far-UV circular dichroism (CD). Samples were dialyzed with 10 mM potassium phosphate buffer (pH 7.5) using a 30 kDa molecular mass cutoff Vivaspin concentrator (Sartorius). CD spectra were recorded with an Applied Photophysics Chirascan spectropolarimeter over a wavelength range of 190–260 nm using a 1 nm data pitch. The corresponding buffer spectrum was subtracted from each sample spectrum.

**Membrane binding assay**

Liposomes were prepared with 1,2-dipalmitoyl-sn-glycero-3-phosphocholine. The lipid was dissolved to a concentration of 1 mg/ml in chloroform and evaporated at 55°C under a nitrogen stream. Remnants of chloroform were removed by lyophilization and the lipid layer was dissolved in 50 mM Tris (pH 8) to a concentration of 10 mM 1,2-dipalmitoyl-sn-glycero-3-phosphocholine. The solution was alternately heated to 60°C and mixed before three cycles of heating to 60°C and freezing to −80°C. The liposomes were finally prepared with an extruder (Avanti Polar Lipids). The ability of LOX to bind to liposomes was then assayed in a flotation experiment, as described by Bigay and Antony (41). After centrifugation in a sucrose gradient, the protein from different fractions was precipitated with trichloroacetic acid and analyzed by SDS-PAGE.

**RESULTS**

CspLOX1 exhibits only a low sequence similarity with other LOXs

The only PUFA that was found in *Cyanothece* sp. PCC 8801, and could therefore serve as a potential substrate, was linoleic acid [18:2(n-6)] (17). Analysis of endogenous oxylipins from the cyanobacterium, *Cyanothece* sp. PCC 8801, as well as incubation of crude cell extracts with 18:2(n-6), showed the formation of predominantly 9-LOX products, suggesting the presence of an enzyme with 9-LOX activity. When we tried to identify the responsible gene(s) for the observed activity, only one LOX gene encoding CspLOX2 was identified by BLAST search analysis. However, this enzyme was characterized as LOX that forms mainly the bis-allylic 11-hydroperoxide and almost equal amounts of 9- and 13-hydroperoxides (17). Therefore, it seemed likely that another LOX exists that contributes to the predominant 9-lipoxygenation. Further search

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could finally identify another hypothetical LOX, namely CspLOX1 (GenBank accession number WP_012595715.1), that exhibits only a very low overall sequence identity of less than 12% with other characterized LOXs from soybean, rabbit, or the coral *Plexaura homomalla* (supplementary Fig. 1). In a phylogenetic tree, CspLOX1 is therefore placed at a very distant position from other characterized LOXs and may form a rare group of LOX enzymes found in few cyanobacteria together with the LOX domain of the *Nostoc punctiforme* 9R-LOX (Fig. 2). The enzyme is more similar to a hypothetical protein from the cyanobacterium *Coleofasciculus chthonoplastes* (GenBank accession number WP_006102195.1) with 61% sequence identity and to a predicted LOX from the cyanobacterium *Microcystis aeruginosa* (GenBank accession number WP_046663104.1) with 40% sequence identity. CspLOX1, which has a calculated molecular mass of 76.5 kDa, is rather small compared with other eukaryotic LOXs, ranging from approximately 75–80 kDa for animal LOXs to 94–104 kDa for plant LOXs. While the C-terminal part was identified as partial LOX domain with the conserved metal ligands His359, His364, His570, Asn574, and Ile668, no conserved domain was identified for the first 150 N-terminal amino acids.

Fig. 2. Phylogenetic tree of LOXs from different kingdoms. The organisms are indicated as well as their major specificities. The enzyme of this study, *Cyanothece* sp. LOX1, is only distantly related to other LOXs and to its isozyme, *Cyanothece* sp. LOX2. The tree was constructed with the program Geneious using the BLOSUM matrix. The proteins mentioned in the tree refer to the corresponding accession numbers in the GenBank. *A. thaliana* LOX1 (9S) (NP_175900.1), *A. thaliana* LOX2 (13S) (NP_566875.1), *A. thaliana* LOX3 (13S) (NP_564021.1), *A. thaliana* LOX4 (13S) (NP_177396.1), *A. thaliana* LOX5 (9S) (NP_188879.2), *A. thaliana* LOX6 (13S) (NP_176923.1), *B. thailandiensis* (13S) (ABC36974), *Cyanothece* sp. LOX1 (9R) (WP_012595715.1), *Cyanothece* sp. LOX2 (9R/11R) (WP_012596348.1), *F. oxysporum* LOX (13S) (EXK38530), *F. oxysporum* Mn-LOX (FOXB_09004), *G. fructosovora* LOX (11R) (AAP98506.1), *G. graminis* Mn-LOX (13R) (AAK81882.1), *G. max* LOX1 (13S) (NP_001236153.1), *G. max* LOX2 (9/13S) (NP_001237685.1), *G. max* LOX3 (9/13S) (CAA30016), *H. sapiens* LOX2 (15S) (AAB61706.1), *H. sapiens* LOX (5S) (NP_000688.2), *H. sapiens* LOX (15S) (NP_001131.3), *M. salvinii* Mn-LOX (9S) (CAD61974.1), *M. salvinii* Mn-LOX (9S) (CAD61974.1), *N. punctiforme* LOX (9R) (WP_010994078.1), *N. punctiforme* LOX1 (13S) (NP_001139620.1), *O. cuniculus* (15S) (NP_001139620.1), *O. cuniculus* (15S) (NP_001273678.1), *O. cuniculus* (9/13S) (NP_001273678.1), *O. sativa* LOX2 (13S) (NP_001273678.1), *O. sativa* LOX3 (13S) (NP_001273678.1), *P. aeruginosa* LOX (15S) (WP_021424774.1), *P. aeruginosa* LOX (15S) (WP_021424774.1), *P. homomalla* LOX (8S) (AXC47743), *P. patens* LOX1 (13S) (XP_001784705.1), *P. patens* LOX2 (13S) (ABF66648), *P. patens* LOX3 (13S) (XP_001785004.1), *P. patens* LOX4 (13S) (ABF66650.1), *P. patens* LOX5 (13S) (ABF66651.1), *P. patens* LOX6 (13S) (ABF66652.1), *P. patens* LOX7 (13S) (ABF66653.1), *S. tuberosum* LOX (9S) (NP_001275357.1), *S. tuberosum* LOX (13S) (NP_001275357.1), *S. tuberosum* LOX2 (13S) (NP_001275115.1).
CspLOX1 determines the position of dioxygenation of free PUFAs from the methyl end

Knowing that the sequence similarity to other characterized LOXs is very low, it was interesting to determine the catalytic activity of CspLOX1. We therefore expressed the N-terminally His-tagged protein in *E. coli* cells, purified it to homogeneity, and tested the enzyme for LOX activity with various free PUFAs. The enzyme showed activity not only with the endogenous 18:2(n-6) (17), but also with longer and more unsaturated substrates, such as α-linolenic acid [18:3(n-3)], 20:4(n-6), and eicosapentaenoic acid [20:5(n-3)]. To determine the specificity of the enzyme, generated reaction products were reduced to the derivative hydroxides and analyzed by HPLC. The (n-6) fatty acids, 18:2(n-6) and 20:4(n-6), yielded only single products, namely 9R-HODE and 11R-HETE, respectively (Fig. 3A, C). In both substrates, dioxygen was thus inserted at the n-10 position of the fatty acid (Fig. 3E). In contrast, when (n-3) fatty acids, 18:3(n-3) and 20:5(n-3), were tested as substrates, a mixture of two products was formed (Fig. 3B, D).

The hydroxy derivatives were identified as 9R-hydroxyoctadecatrienoic acid (9R-HOTE) and 12R-HOTE from 18:3(n-3) and 11-hydroxyeicosapentaenoic acid (11-HEPE) and 14-HEPE from 20:5(n-3), respectively (Fig. 3B, D). Thus, one of the products resulted again from dioxygenation at the n-10 position of the fatty acid, while the second one corresponded to dioxygen insertion at n-7 (Fig. 3E). The products were identified by comparison with original standards or by LC/MS as well as GC/MS analysis of the TMS ether derivative, in the case of 12-HOTE (data not shown). The stereochemistry of the rare oxylipin, 12-HOTE, was assigned by comparison to 12R-HOTE produced by the LOX domain of a fusion protein from *A. marina* (24) (supplementary Fig. 3). Due to the lack of authentic standards, the chirality of HEPEs could not be assigned. Together these results hint that all substrates penetrate the active site with their methyl end first because dioxygen insertion always occurs at the same position measured from the methyl end and from the same side of the substrate molecule (Fig. 3E).

The kinetic parameters of CspLOX1 were then determined with different concentrations of 18:2(n-6), which is the only endogenous substrate (17). We determined a $k_{cat}$ of $19.0 \pm 1.3 \text{ s}^{-1}$ and a $K_m$ of $14.7 \pm 4.4 \text{ M}$ for 18:2(n-6) by fitting the data to the Michaelis-Menten equation.

![Fig. 3. SP-HPLC analyses of hydroxide derivatives of the products formed from the reaction of CspLOX1 with 18:2(n-6) (A), 18:3(n-3) (B), 20:4(n-6) (C), and 20:5(n-3) (D). Two hundred fifty micrograms of the respective fatty acid substrate were incubated with 15 μg of CspLOX1 for 30 min, the reaction products were reduced by SnCl₂ and initially separated by RP-HPLC. Hydroxides were then analyzed using a Zorbax RxSIL column (150 × 2.1 mm, 5 μm particle size, Agilent) with a solvent system of n-hexane/2-propanol/trifluoroacetic acid (100:1:0.1, v/v/v) at a flow rate of 0.2 ml/min. E: Model of the CspLOX1 specificities with different substrates.](image-url)
Conversion of phosphatidylcholine suggests tail-first binding mode

A significant difference observed between various LOX isozymes concerns their ability to oxygenate fatty acids, which are esterified to a bulky head group. The activity, or lack thereof, has been connected with different orientations of fatty acid binding to the enzyme active site (11, 42, 43). So far, only enzymes that bind their substrate with the methyl end first have been described to oxygenate complex lipids. To obtain additional information regarding the binding mode of fatty acids to the CspLOX1 active site, we monitored the activity of the enzyme against 1,2-diara-chidonyl-sn-glycero-3-phosphocholine [PC-20:4(n-6)], as previously described (42). For product analysis, fatty acids were reduced, transmethylated, and separated by SP-HPLC and CP-HPLC. Two major products could be identified, namely, methylated 11-HETE (11-HETE-Me) and 15-HETE-Me. Of these, 11-HETE-Me consisted only of the $R$ stereoisomer and was produced exclusively in samples containing CspLOX1, while 15-HETE-Me consisted of a racemic mixture of the two enantiomers and was produced in comparable amounts also in the absence of enzyme, suggesting that it is a byproduct attributed to autoxidation (Fig. 4). Therefore, 11$R$-hydroperoxycicosatetraenoic acid is the major product for both free and esterified 20:4(n-6), strongly suggesting that substrates penetrate the active site of CspLOX1 with their methyl end first.

The X-ray structure reveals a modified β-barrel domain and an N-terminal α-helical extension

CspLOX1 is phylogenetically distant from other LOXs, but still catalyzes a typical LOX reaction. It was therefore interesting to analyze structural similarities of CspLOX2 with other LOXs to understand which features are necessary for LOX catalysis. CspLOX1 crystals diffracting to 2.7 Å could be obtained, but due to the low similarity of CspLOX1 to other LOXs, a structure solution by Molecular
α1 of the human 15-LOX1, the soybean LOX1, or many other LOXs, which in CspLOX1 corresponds structurally to an elongated loop region (Fig. 6). The catalytic iron is buried approximately 13 Å from the protein surface and is coordinated octahedrally by the conserved amino acid residues already identified by sequence alignment (supplementary Fig. 2). Using the CAVER2.0 plugin for PYMOL (44), a putative substrate tunnel leading from the surface to the catalytic iron could be identified (Fig. 5, blue). Two arginines, Arg399 and Arg400, are positioned at the entry point of this channel, while the bottom is defined by Tyr360, Val406, and Phe413 (Fig. 5B). The “ceiling” of the putative active site is formed by the so-called arched helix α12 (Fig. 5B, yellow) and its walls are lined by a number of hydrophobic amino acid residues, including several leucines and isoleucines.

The additional 40 amino acid extension, connected to the N-terminus of the β-barrel by a linker region, is comprised of two amphipathic α-helices (supplementary Fig. 4A). Two salt bridges and six hydrogen bonds are formed between the α-helical extension and the catalytic domain and an area of 875 Å², which corresponds to 23% of the total area of the two N-terminal α-helices, is buried at the interface. Additionally, this helical extension is on the opposite side interacting with the same helices of a neighboring molecule in the crystal lattice (supplementary Fig. 4B). At this interface, four hydrogen bonds are formed and a surface area of 790 Å² is buried (21% of the total area of helical extension).

Although the β-barrel domain is a typical feature of LOX enzymes, it exhibits some unusual characteristics in the CspLOX1 structure. Comparison of this domain with those from LOXs of different organisms reveals that the topology is fairly similar despite a low sequence similarity (Fig. 6). In CspLOX1, however, two antiparallel β-strands are strongly extended and form a cleft with the β-strands on the opposite site of the barrel (Fig. 6C). Furthermore, this cleft of the β-barrel domain interacts with the catalytic domain where the α-helix is lacking in CspLOX1, resulting in an altered arrangement of the β-barrel domain relative to the catalytic domain (supplementary Fig. 4A). While the β-barrel is oriented rather parallel to the cylindrical-shaped catalytic domain in all other LOX structures, it is rotated by more than 90° in CspLOX1 (Fig. 6).

**Mutational studies suggest conserved function of the Gly/Ala switch**

An interesting and highly conserved residue responsible for LOX specificity is Gly401 at the position that functions as the so-called Gly/Ala switch (13). Exchanging this residue to the alternative alanine (Gly401Ala) causes the specificity of CspLOX1 to be changed from 9R- to almost exclusively 13S-lipoxygenation (Fig. 7). This is in agreement with both of our previous observations and the accepted model that the substrate binds with the methyl-end or tail-first in the hydrophobic channel.

Because a tail-first orientation of the substrate would result in a positioning of the fatty acid carboxyl group close to the entrance of the channel, we investigated whether the positively charged amino acids, Arg399 and Arg400, could be involved in positioning of the fatty acid substrates for hydrogen abstraction by interaction with the negatively charged carboxyl group of the fatty acid. Both single and double mutants of the two arginines were expressed in comparable amounts as the wild-type enzyme in E. coli, but the isolated proteins exhibited no measurable dioxygenase activity with any of the substrates tested in photometrical assays. In order to determine whether the lack of activity was due to misfolding of the protein variants, CD spectra of wild-type and mutated enzymes were recorded in the far-UV and near-UV regions. The CD spectra of variant and wild-type structures point, however, to very similar
by the truncation of the first 179 amino acids, is also conserved in other LOXs. Here, this loop is found C-terminal of the helix α9251 in the catalytic domain that is missing in CspLOX1. The cleavage of this loop region may explain the observed loss of activity (supplementary Fig. 5B).

**DISCUSSION**

A number of LOX structures from different organisms have been solved during the last 20 years, and available 3D models exhibit a high degree of similarity to each other. With an outsider like the cyanobacterial enzyme CspLOX1 (as identified by sequence alignments), we aimed to investigate what had remained conserved from a common LOX ancestor and which features had been more variable during enzyme evolution and were thus dispensable for LOX catalysis. In this structural and biochemical study, we showed that the distantly related CspLOX1 from *Cyanothece* sp. PCC8801 possesses, indeed, LOX activity and shares many biochemical features with eukaryotic LOXs (Figs. 3, 4). But while the catalytic core of the enzyme is secondary structures, suggesting that the lack of activity of the protein variants is not due to protein misfolding.

The α-helical extension at the N-terminus facilitates binding to liposomes

It has been reported that the β-barrel domain of LOXs can function in membrane binding to acquire substrates directly from the membrane (45, 46). As described above, CspLOX1 also has, in addition, an amphipathic α-helical extension at the N-terminus. To investigate the membrane binding ability of the individual parts of CspLOX1, we designed truncated versions of CspLOX1 in which we cut off the N-terminal α-helices, or both the N-terminal α-helices and the β-barrel, to obtain only the catalytic domain of the enzyme (Fig. 8). We tested to determine whether these truncated versions and full-length CspLOX1 could bind to artificial membranes in a flotation experiment with liposomes consisting of phosphatidylcholine (41). In this experiment, liposomes were incubated with the protein variants to allow binding to the artificial membranes. During centrifugation in a sucrose gradient, the liposomes floated up to the top fraction with all protein that had bound to them. Under the applied conditions, only the full-length protein was able to efficiently bind to the liposomes (Fig. 8A), while a cleavage of the N-terminal α-helices already suppressed a binding (Fig. 8B) and, as expected, the catalytic domain alone was not sufficient for an effective binding to these liposomes (Fig. 8C). These results suggest that the N-terminal helical extension, which is built up by two amphipathic helices connected via a short two amino acid long linker region, may function as a membrane anchor. To assess whether the truncated versions were still structurally intact, we determined the catalytic activity of the truncated versions. While the variant with the β-barrel domain was almost twice as active as the wild-type protein, the catalytic domain alone was inactive (supplementary Fig. 5). Closer inspection of the linker region between the catalytic and β-barrel domain, however, showed that it forms a loop covering parts of the active site (residues 166 to 174). This loop, which is largely removed by the truncation of the first 179 amino acids, is also conserved in other LOXs. Here, this loop is found C-terminal of the helix α1 in the catalytic domain that is missing in CspLOX1. The cleavage of this loop region may explain the observed loss of activity (supplementary Fig. 5B).
highly conserved, the N-terminus exhibits a number of alterations from the classical LOX structure (Fig. 6A, B vs. Fig. 6C). The most interesting of these variations is an additional α-helical extension at the N-terminus that mediates binding to liposomes (Fig. 8).

To date, among the LOX structures of 12 different enzymes that have been deposited in the PDB (PDB IDs: 1YGE, 1LNH, 2IUJ, 2UG2, 2FNQ, 3VF1, 1LOX, 3RDE, 3D3L, 4NRE, and 3O8Y), 11 enzymes exhibit the characteristic two-domain LOX topology with a C-terminal catalytic domain and the β-barrel domain at the N-terminus, unless only the catalytic domain alone was crystallized (3RDE, 3D3L). So far, the only exception to this classical two-domain architecture has been observed for the 15-LOX from *P. aeruginosa*, which has an insertion of two long α-helices instead of a β-barrel domain (supplementary Fig. 6) (22). Here, we describe a LOX structure that combines both variants in its N-terminal domain; it contains a β-barrel domain and an additional α-helical extension (Fig. 5A). Our results, obtained with truncated CspLOX1 variants in a liposome-binding assay, further suggest that this α-helical extension mediates membrane binding (Fig. 8). It therefore seems likely that different structural domains can fulfill this function in LOXs. While other studies reported that the β-barrel domain mediates the binding to membranes (45, 47), this domain could not contribute to a remarkable binding to liposomes in CspLOX1 under the tested conditions. It should be noted that the three tryptophan residues, which have been discussed to be involved in selective binding to PC in human 5-LOX (47), are not conserved in the β-barrel domain of CspLOX1. In addition to these tryptophans, certain amino acids within the β-barrel domain were proposed as Ca<sup>2+</sup>-ligands in soybean LOX-1, human 5-LOX, and the coral 8R-LOX (46–48). From our experimental conditions, we cannot rule out that the β-barrel could also contribute to the membrane binding ability in CspLOX1 under high Ca<sup>2+</sup> concentrations.

Interestingly, the *Pseudomonas* 15-LOX, which consists only of the catalytic domain and two additional 70-residue-long α-helices (residues 127 to 197), is also able to bind to membranes (22). These amphipathic α-helices occupy the same position as the N-terminal helices in CspLOX1 (supplementary Fig. 6). Even though it has not been experimentally confirmed that these α-helices may function as a membrane anchor, a similar function as in CspLOX1 seems very likely, as a phospholipid ligand, which is the building block of biomembranes, interacts with the long α-helices in the crystal structure (22). In addition to this new membrane-targeting domain identified in CspLOX1, we found additional unique variations within the N-terminal domain. Two β-strands of the PLAT domain are remarkably elongated, as compared with other LOXs, and the linker region between the two domains does not possess an α-helical conformation (Fig. 6). Furthermore, the orientation of the β-barrel domain relative to the catalytic domain is altered. This might be the result of a certain rotational flexibility of the PLAT domain, as shown by small angle X-ray scattering experiments for 15-LOX1 (49). Out of many available orientations, the one which is energetically favored is, in general, represented in the crystal structure and stabilized by crystal contacts. The position of the two N-terminal amphipathic α-helices is further driven by contact with the catalytic domain. As a consequence, the hydrophobic side of the N-terminal α-helices is exposed at the surface and needs to be buried by another hydrophobic part. The N-terminal helices of another CspLOX1 molecule serve this function, as seen in the crystal lattice (supplementary Fig. 4B). Structural analysis revealed that the linker between the PLAT and catalytic domain is significantly shorter than that of other LOXs. In contrast to other LOXs, the linker region of CspLOX1 does not possess any defined secondary structure (α-helix), but adopts an extended loop conformation and interacts with the catalytic domain and, thus, structurally replaces the helical linker (supplementary Fig. 4A).

In contrast to the N-terminal parts of the enzyme, the C-terminal catalytic domain strongly resembles that of other LOXs. All long helices, except the first one, are located at similar positions and the iron coordinating residues are also highly conserved. Importantly, we could identify a channel that connects the surface of the enzyme with the catalytic iron and most likely represents the substrate binding channel (Fig. 5), as it coincides with the 20:4 binding side of the coral 8R-LOX (50). Even though the bottom part of the channel is not suitable to accommodate substrates with 18 or 20 carbons and most likely requires a rearrangement of amino acid side chains during catalysis, the upper part of the channel is found at a similar position as in other animal LOXs. Since the initial experiment by Brash, Ingram, and Harris (42), experiments with complex lipids are used to analyze the orientation of the substrate within the active site of LOXs. Indeed CspLOX1 binds its substrates with the hydrophobic methyl end at the bottom of the active site channel (Fig. 4), which is in agreement with models proposing a tail-first substrate orientation in linoleate 9R- or 13S-LOXs and arachidonate 8R- or 11S-LOXs (Fig. 1). These results were supported by conversion of substrates with different length and degree of unsaturation, in which the product composition was dependent on the fatty acid structure of the methyl end, rather than the carboxyl end (Fig. 3E). Additionally, two arginine residues positioned at the entrance of the putative binding pocket might be involved in positioning of the substrate by ionic interactions with the negatively charged carboxyl group (Fig. 5B). Though we could show that a replacement of these arginines by alanines resulted in a loss of enzymatic activity, further experiments will be required to unambiguously show whether these arginines are indeed involved in binding of the fatty acid substrate.

Overall, the enzyme can be classified as linoleate 9R-LOX (Fig. 3A). Although CspLOX1 was active with different substrates, the only endogenous substrate is 18:2(n-6) (17). This specificity was also found in other cyanobacterial and fungal LOXs (51, 52), but is otherwise rather unusual, with most plant and animal LOXs having a specificity for the S-isomer (Fig. 2). Interestingly, a highly conserved Gly/Ala switch that can change the specificity from 9R to 13S or vice versa was also structurally and functionally
conserved in CspLOX1 (Fig. 7). This residue is thought to play a decisive role in regulating the position of dioxygenation by shielding one of the two possible sites of dioxygen addition (13). As a single amino acid exchange can switch the dioxygen insertion to the other end of the pentadiene moiety, both specificities are interchangeable by only a single mutation step during evolution. Together these results suggest that most features of the active site are highly conserved and must have been acquired very early in the evolution of LOXs.

From a phylogenetic point of view, it is tempting to speculate about the loss and acquisition of domains and functions from the last common ancestor of all LOXs. We can assume from the phylogenetic tree (Fig. 2) and the available crystal structures that LOXs initially consisted of an α-helical catalytic domain and the N-terminal β-barrel domain. Because mammalian LOXs, plant LOXs, and CspLOX1 contain this domain, it is likely that the β-barrel was lost during the evolution of some bacterial mini-LOXs. The helical extension, which was up to now only reported for P. aeruginosa and now also for CspLOX1, might have been acquired independently, as their structural equivalents cannot be identified by sequence alignment in other LOXs. Some other bacterial LOXs even acquired a new enzymatic domain with a further activity (51, 53). Additionally, the iron was probably originally coordinated by three histidines, one asparagine, and the carboxyl group of the C-terminal isoleucine, as this pattern is found from CspLOX1 to plant LOXs. The coordination with four histidines and the C-terminal carboxyl group, as found in some mammalian LOXs, was probably acquired later in evolution.

The function of LOXs is primarily defined by the specificity of the enzyme. One of the critical factors that influences the product specificity is the orientation of the substrate (Fig. 1). Our results strongly support the model that the initial substrate orientation in the active site is tail-first, as suggested for most enzymes, including CspLOX1. This tail-first substrate orientation is, in general, associated with 13S or 9R-lipoxygenation of 18:2(n-6) and often with the ability to oxygenate complex lipids, as is the case for CspLOX1 to plant LOXs. The coordination with four histidines and the C-terminal carboxyl group, as found in some mammalian LOXs, was probably acquired later in evolution.

The authors thank Sabine Freitag for excellent technical support.

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