Structure of a Calcium-dependent 11R-Lipoxygenase Suggests a Mechanism for Ca\(^{2+}\) Regulation

Received for publication, January 16, 2012, and in revised form, May 7, 2012 Published, JBC Papers in Press, May 9, 2012, DOI 10.1074/jbc.M112.343285

Prit Eek, Reet Järving, Ivar Järving, Nathaniel C. Gilbert, Marcia E. Newcomer, and Nigulas Samel

From the Department of Chemistry, Tallinn University of Technology, Akadeemia tee 15, 12618 Tallinn, Estonia and the Department of Biological Sciences, Louisiana State University, Baton Rouge, Louisiana 70803

Background: Lipoygenases vary in their catalytic specificity and regulation.

Results: 11R-LOX, strictly Ca\(^{2+}\)-dependent, displays novel structural features in the membrane-binding domain.

Conclusion: A model for how access to an enclosed active site is linked to Ca\(^{2+}\)-dependent membrane binding is proposed.

Significance: The 11R-LOX model provides structural insights into the allosteric regulation of lipoxygenases.

Lipoxygenases (LOXs) are a key part of several signaling pathways that lead to inflammation and cancer. Yet, the mechanisms of substrate binding and allosteric regulation by the various LOX isoforms remain speculative. Here we report the 2.47-Å resolution crystal structure of the arachidonate 11R-LOX from Gersemia fruticosa, which sheds new light on the mechanism of LOX catalysis. Our crystallographic and mutational studies suggest that the aliphatic tail of the fatty acid is bound in a hydrophobic pocket with two potential entrances. We speculate that LOXs share a common T-shaped substrate channel architecture that gives rise to the varying positional specificities. A general allosteric mechanism is proposed for transmitting the activity-inducing effect of calcium binding from the membrane-targeting PLAT (polycystin-1/lipoxygenase/toxin) domain to the active site via a conserved \(\pi\)-cation bridge.

Lipoxygenases (LOXs)\(^2\) are non-heme iron dioxygenases that catalyze the stereo- and regiospecific hydroperoxidation of polyunsaturated fatty acids (1). LOX catalysis products of arachidonic acid (AA), which is the main substrate in animals, are hydroperoxyeicosatetraenoic acids (HpETEs), these lipid mediators and their metabolites have been implicated in cancer (2), atherosclerosis (3), and allergic inflammation (4). Consequently, LOXs are targets for drug design. A complicating factor in the development of LOX inhibitors is that there are several LOX isoforms in an organism, all with equivalent catalytic machinery and chemical mechanism (5). Thus, differences in regiospecificity or regulation must be exploited to design isoform-specific inhibitors. Given the limited amount of structural information of arachidonate-metabolizing LOXs, each new structure provides crucial details related to LOX catalysis mechanism.

LOX catalysis begins with a stereoselective hydrogen abstraction by the catalytic non-heme iron from the methylene carbon (\(\text{CH}_2\)) of the selected 1,4-cis,cis-pentadiene unit on the fatty acid substrate, and is followed by regioselective dioxygen addition on the opposite face of the substrate either at \(-2\) or \(+2\) carbon (Fig. 1) (6). For such specific reactions to take place, a very distinct substrate channel that goes past the non-heme iron must position the fatty acid. This binding site must also vary among LOX isoforms to facilitate the different catalytic properties. The substrate-binding cavity has been described as “boot-shaped”; it is directly accessible from the surface of the protein and ends with a hydrophobic pocket (7). The pocket residues of several 12/15-LOXs have been mutated to demonstrate that bulkier side chains favor 15-lipoxygenation, whereas less space-filling residues, which would allow the fatty acid tail to penetrate deeper into the cavity, confer 12-LOX activity. These results are consistent with aliphatic tail-first entry (8–12). Also, a cationic arginine near the entrance of the cavity has been shown to stabilize the carboxylate head of the fatty acid (13). Computational docking studies based on x-ray crystallography data further support the boot-shaped substrate channel (14, 15). For some LOXs, carboxylate head-first binding has been suggested to explain differing specificity or double dioxygenation of AA (16, 17). In the light of the coral 8R-LOX crystal structure, however, a novel binding model was proposed with an alternative U-shaped channel that neglects the hydrophobic pocket (18). According to this hypothesis, the substrate is bound in a culvert that runs under a conserved arched helix; distinct lipoxygenases allow access to the catalytic iron from one of two possible directions. Although several lipoxygenase crystal structure models have been published, including rabbit 12/15-LOX (19, 20), coral 8R-LOX (18, 21), and recently a modified human 5-LOX (22) representing Animalia, the lack of experimental evidence on substrate binding, such as a crystalized enzyme-substrate complex, has precluded the emergence of a uniform theory.

The activity of various LOXs depends more or less on the presence of \(\text{Ca}^{2+}\) that promotes interactions with the lipid...
Regulation of 11R-Lipoxygenase Catalysis

FIGURE 1. All 12 possible lipoxygenation positions in AA. The catalysis begins with stereoselective hydrogen abstraction from C7, C10, or C13 (labeled proS/R), followed by antarafacial di-oxygen addition either at −2 or +2 carbon (grouped by colors). Based on data from Ref. 6.

membrane, from where the enzyme obtains its fatty acid substrate (21, 23). The human 5-LOX is effectively translocated to the nuclear envelope upon Ca\(^{2+}\) release, the C2-like PLAT domain being the selective membrane-targeting module (24). The calcium-binding sites of the PLAT domain appear to be conserved among human 5-LOX, coral 8R-LOX, gangrene α-toxin, as well as coral 11R-LOX, which are all induced by Ca\(^{2+}\), but not in rabbit 12/15-LOX, which is only mildly affected (21, 25). The molecular mechanism of Ca\(^{2+}\)- and membrane-induced allosteric regulation is not clear. Although many mammalian lipoxygenases retain their reaction specificity after PLAT domain truncation, this is accompanied by reduced turnover rates (26). Moreover, tight association of PLAT and catalytic domains has been shown to be important for protein stability and catalytic activity (27). A possible structural element that may be under allosteric control is the α2 region that forms a “lid” over the putative substrate channel entrance, which can adopt different conformations, thereby either opening or closing the orifice (20, 28, 29). Another plausible allosteric mechanism could involve oligomerization, which has been noticed in case of human platelet 12-LOX and rabbit 12/15-LOX (30, 31), but no definite assembly has been described to date.

The arachidonate 11R-LOX from the white sea coral Gersemia fruticosa is the first described lipoxygenase with 11R-specificity (25). Based on primary structure comparison it is most closely related to the 8R-LOX from the allene oxide synthase-lipoxygenase fusion protein of the Caribbean sea whip coral Plexaura homomalla (42% identity) (32) and the analogous enzyme from G. fruticosa (43%) (33). The closest mammalian counterparts are 5-LOXs (about 33%). Experiments with coral 11R-LOX from the Caribbean sea whip coral 11R-LOX (34), has formed into a boot-shaped cavity was conducted to address questions regarding substrate binding. A general hypothesis of possible substrate orientations in the active site is described.

EXPERIMENTAL PROCEDURES

Expression and Purification—Recombinant G. fruticosa 11R-LOX with an N-terminal His\(_4\) tag in pET-11a vector was transformed into Escherichia coli BL21(DE3) cells (Novagen). Colonies were grown overnight in 25 ml of LB containing 100 μg/ml of ampicillin at 37 °C. A 500-ml volume of autoinducing medium ZYM-5052 (35) (with 100 μg/ml ampicillin) was inoculated with 5 ml of overnight culture. The culture was incubated at 37 °C for 3–4 h, followed by growth to saturation at 20 °C. Cells were harvested by centrifugation and frozen at −80 °C when the absorbance at 600 nm had remained stable for 4 h (usually 27–30 h after the inoculation).

Cell pellets were resuspended in Bugbuster (Novagen) with added DNase I, pepstatin, and PMSF. The suspension was stirred and incubated on ice for 30 min, lysed in a French pressure cell, and centrifuged at 39,000 × g for 40 min at 4 °C. The supernatant was applied onto a HisTrap Ni-Sepharose column (GE Healthcare) with 20 mM Tris-HCl, pH 8.0, and 150 mM NaCl in a Sephadex G-25 Fine column (Amersham Biosciences). The protein was eluted with an imidazole gradient from 20 to 200 mM. Protein fractions were dialyzed overnight against 20 mM Tris-HCl, pH 8.0, or desalted in a Sephadex G-25 Fine column (Amersham Biosciences). The sample was then applied onto a Mono Q anion exchanger (GE Healthcare), washed with binding buffer (50 mM Tris-HCl, 500 mM NaCl, 20 mM imidazole, pH 8.0) on an ÄKTA FPLC system (GE Healthcare). The protein was eluted with an imidazole gradient from 20 to 200 mM. Protein fractions were dialyzed overnight against 20 mM Tris-HCl, pH 8.0, or desalted in a Sephadex G-25 Fine column (Amersham Biosciences). The sample was then applied onto a Mono Q anion exchanger (GE Healthcare), washed with binding buffer (50 mM Tris-HCl, 500 mM NaCl, 20 mM imidazole, pH 8.0) on an ÄKTA FPLC system (GE Healthcare). The protein was eluted with an imidazole gradient from 20 to 200 mM. Protein fractions were dialyzed overnight against 20 mM Tris-HCl, pH 8.0, or desalted in a Sephadex G-25 Fine column (Amersham Biosciences). The sample was then applied onto a Mono Q anion exchanger (GE Healthcare), washed with binding buffer (50 mM Tris-HCl, 500 mM NaCl, 20 mM imidazole, pH 8.0) on an ÄKTA FPLC system (GE Healthcare). The protein was eluted with an imidazole gradient from 20 to 200 mM. Protein fractions were dialyzed overnight against 20 mM Tris-HCl, pH 8.0, or desalted in a Sephadex G-25 Fine column (Amersham Biosciences).
above and serial dilutions of seed stock as described in the Seed Bead user guide. Larger single crystals grew in 2–3 days. For cryoprotection, crystals were transferred into 0.1 M bis-Tris, pH 7.2, 12% (w/v) PEG 3350, 20–25% (w/v) sucrose in two consecutive steps and then frozen in liquid nitrogen or a 100 K cryostream.

Data Collection and Structure Determination—Preliminary screens for crystal diffraction were conducted at the Gulf Coast Protein Crystallography Consortium beamline at the Center for Advanced Microstructures and Devices (CAMD, Louisiana State University). A full dataset was collected at the NE-CAT beamline 24-ID-E at the Advanced Photon Source (Argonne, IL) using 0.98-Å radiation at 100 K. Data were processed to a resolution of 2.47 Å (Table 1) using xia2 (36). The structure was determined by molecular replacement with MrBUMP (37, 38) using 3.2 Å P. homomalla 8R-LOX model (PDB code 2fnq). The initial refinement cycles were performed with REFMAC5 (39). Both MrBUMP and REFMAC5 are part of the CCP4 suite (40). Manual model building was done with COOT (41) and further refinement in PHENIX (42) using the program phenix.refine with non-crystallographic symmetry and Ramachandran restraints, individual isotropic atomic displacement factors, and automatic water picking. For the final refinement, hydrogen atoms were added to the model, Ramachandran restraints were released and both stereochemistry and atomic displacement weights were optimized. Illustrations were prepared with UCSF Chimera (43), surfaces were obtained with MS-MS (44). The dimerization interface was analyzed using PISA (Protein Interfaces, Surfaces and Assemblies) (European Bioinformatics Institute) (45). Sequences were aligned with ClustalW2 (46) and rendered with ESPript (47).

Site-directed Mutagenesis—The V430A, L431A, V609A, and V609W mutations were introduced using whole plasmid PCR primed with complementary primers that additionally contained silent mutations for restriction analysis. The M606A mutant was obtained by separately cloning the upstream and downstream fragments of the recombinant cDNA using mutagenic primers with M606A mutation: 5’-AA GGA TCC ATG CAT CAC TTC GAC CGC C-3’ (M606A upstream) and 5’-GAC ACC ATG CTG GTC AAC GGA G-3’ (M606A downstream) for the upstream fragment, 5’-CAA GGT GTT ACA GCT TCT GTT GTG AAT GCG C-3’ (M606A upstream) and 5’-GAT GGA TCC TTA GAT GGC AAT ACT GTT CGG-3’ (11R-LOX cDNA downstream) for the downstream fragment.

Enzyme Assay—Initial enzyme specificity experiments were conducted on crude bacterial lysates. BL21-CodonPlus(DE3)-RP cells (Stratagene) were transferred and grown in LB medium with 100 μg/ml of ampicillin at 37 °C. Following 500 μM isopropyl β-4-thiogalactoside induction at $A_{490\text{nm}} = 0.6$, cells were grown for 18–18 h at 10 °C. Cells were harvested by centrifugation at 4 °C, washed with 50 mM Tris-HCl, pH 8.0, aliquoted, and frozen at −80 °C.

For product analysis, aliquots of 2.5 ml of culture were resuspended in 500 μl of 50 mM Tris-HCl, pH 8.0, with 1 mM PMSF and sonicated 3 × 5 s using a Torbeo 36810-Series cell disruptor (Cole Parmer) at a setting of 5. The suspension was centrifuged at 13,000 × g for 20 min at 4 °C and the supernatant was harvested. CaCl₂ was added to the enzyme solution in final concentration of 10 mM. Incubations of 1–20 ml were carried out in 50 mM Tris-HCl, 250 mM NaCl, pH 9.0 buffer with 50 μM arachidonic acid at room temperature for 5 min with constant stirring. [1-14C]-Labeled arachidonic acid (GE Healthcare) was used in 1-ml incubations. HpETEs were reduced to corresponding hydroxy acids (HETEs) with 10 mM SnCl₂, the mixture was acidified with KH₂PO₄/HCl (1:1) to pH 6 and the products were extracted using ethyl acetate. Incubation products (10–20 ml volumes) were purified prior to HPLC analysis using thin layer chromatography: the extract was applied on a silica gel plate, eluted with a hexane/ethyl acetate/acetic acid (3:4:2.1) solution. The arachidonic acid (Cayman) was varied from 2 to 200 μM. The reaction was initiated by adding 6 nM wild-type enzyme, or up to 32 nM for the less active mutants. The reaction velocity was determined from the slope of the linear portion of the curve. $K_m$ and $k_{cat}$ values were obtained by nonlinear regression analysis with the Michaelis-Menten equation. As 11R-LOX exhibits very strong substrate inhibition and, thus far, no suitable kinetic model has been derived, only the ascending part of the curve (2–30 μM AA) was used for fitting.

HPLC-MS Analysis—Catalysis products were analyzed by reverse phase HPLC using an Agilent Eclipse 3.5 μm 150 × 2.1-mm ODS column thermostatted at 35 °C. The sample was eluted isocratically with methanol/water/acetic acid (75:25:0.01, v/v/v) at 0.25 ml/min on an Agilent 2200 Series HPLC system. Products were detected using a diode array detector monitoring wavelengths 210–280 nm, followed by an Agilent LC/MSD Trap XCT mass spectrometer. The MS/MS spectra of arachidonic acid derivatives were obtained in negative mode using an APCI interface.

[1-14C]-Labeled products were additionally analyzed using a Radiomatic 500TR Flow Scintillation Analyzer (Packard Bioscience) preceded by an Agilent Eclipse 5 μm 150 × 4.6-mm ODS column thermostatted at 35 °C and a diode array detector. The same eluent was used at a flow rate of 1 ml/min.
Regulation of 11R-Lipoxygenase Catalysis

Like the majority of lipoxygenases, 11R-LOX consists of two distinct domains: the N-terminal C2-like PLAT domain (residues 1–115) and a larger mainly α-helical catalytic domain (residues 129–679), which are connected by a small linker region (residues 116–128). The catalytic domain contains the non-heme iron, which is coordinated by three highly conserved histidines, His^{372}, His^{378}, and His^{556}, and the carboxylate group of the C-terminal Ile^{679}. In general, the structure is very similar to that of the 8R-LOX domain of *P. homomalla* allene oxide synthase-LOX fusion protein, a root mean square deviation of 644 C$_\alpha$ pairs being 1.37 Å. The greatest differences are found in the putative entrance to the active site, which more closely resembles the recently published human Stable-5-LOX (22). Similarly to 5-LOX, the α2-helix of 11R-LOX that covers the putative entrance is only about 2 turns long and is flanked by loops and small 3$_{10}$-helices. It should also be mentioned that the overall similarity between the 11R- and 5-LOX is very high (root mean square deviation of 641 C$_\alpha$ pairs is 1.49 Å).

Another heterogeneous region includes the putative Ca$^{2+}$-binding sites in the PLAT domain. The PLAT domain is a β-sandwich consisting of two antiparallel 4-strand β-sheets. At the sheets’ ends proximal to the catalytic domain (opposite to the N terminus) there are four loops that connect the two sheets, three of the loops being rather extensive, this is the region that contains the Ca$^{2+}$-binding sites. The electron density map is less clear in the region of the PLAT domain, which is characterized by higher mean B-factors, 37.3 Å$^2$ for the catalytic domain versus 70.5 Å$^2$ in the PLAT domain. The least well defined densities are found in the putative calcium-binding loops, especially residues His$^{45}$–Glu$^{57}$ and Gly$^{57}$–Lys$^{77}$; whereas main chain density is not ambiguous, several side chains were modeled primarily according to optimal geometry (side chains present at ≥0.5 σ). For refinement, all occupancies were set to 1. The apparent mobility of these residues is described by their elevated B-factors.

**Dimerization**—Size exclusion chromatography indicated that 11R-LOX appears as a dimer in a calcium-free buffer. The purified enzyme eluted with aldolase (158 kDa), which is double the molecular mass of the recombinant protein (79 kDa) (*supplementary Fig. S1*). Based on ultrafiltration assays, it was previously concluded that 11R-LOX is in a monomeric state in solution: in calcium-free conditions the enzyme passed a filter with a 100-kDa cutoff without major losses (25). The findings presented here clearly dispute those claims. In the presence of 10 mM CaCl$_2$, however, size exclusion chromatography analysis indicated the formation of large aggregates, as the protein eluted with the void volume (data not shown). Similar results were obtained using ultrafiltration (25). Potential dimerization interfaces were searched among the crystal contacts using PISA (45), but according to the criteria established by the algorithm, no significant assemblies were found.

**Substrate Channel and Its Entrances**—There is an array of consecutive, mostly hydrophobic cavities concealed in the catalytic domain alongside the coordinated non-heme iron (Fig. 2). These cavities are covered by a conserved arched helix (α10–α11 in *G. fruticosa*), and the potential entrances on either side are blocked by short helices and loops. The arched helix harbors the R/S-stereospecificity determinant Gly$^{416}$ (34).

### TABLE 1
Data collection and refinement statistics

| Data collection |  |
|-----------------|-----------------|
| **Space group** | C2              |
| **Cell dimensions** | 114.80 148.71 117.33 | 90.00 119.16 90.00 |
| a (Å), b (Å), c (Å) |  |
| Resolution (Å) | 36.48–2.47 (2.54–2.47) |
| **R$_{o/w}$** | 0.072 (0.474) |
| I/σI | 10.1 (1.7) |
| Completeness (%) | 97.1 (84.6) |
| **Redundancy** | 3.0 (2.3) |

| Refinement |  |
|------------|-----------------|
| Resolution (Å) | 36.48–2.47 |
| No. of reflections | 57,423 |
| R$_{o/w}$/R$_{free}$ | 0.2019/0.2281 |
| No. of atoms |  |
| Protein | 10,880 |
| Iron | 250 |
| Sucrose | 46 |
| Water | 482 |
| B-factor (Å$^2$) | 4.6 |
| Bond lengths (Å) | 3.90 |
| Bond angles (°) | 10.80 |

$^*$ Values in the parentheses represent the highest resolution shell.

$^a$ R$_{free}$ = Σ$_{hkl}$|I$_{hkl}$| - |I$_{0hkl}$| / Σ$_{hkl}$|I$_{hkl}$|, where |I$_{0hkl}$| is the intensity of the hth observation and |I$_{hkl}$| is the mean intensity of the reflection h.

$^b$ R = Σ|Fo| - |Fc| / Σ|Fo|, where |Fo| and |Fc| are the observed and calculated structure factor amplitudes, respectively. R$_{free}$ was calculated using 3.33% of the total reflections.

To analyze the chirality of HETEs, the products were purified in a normal phase HPLC system using a Phenomenex Luna 5 μm 250 × 4.6-mm Silica column in isopropyl alcohol (1% H$_2$O)/hexane (7:93, v/v) at 1 ml/min. HETEs were methylated using diazomethane and analyzed using a Chiralcel OD-H 250 × 4.6-mm column in 0.7% isopropyl alcohol (dry), 0.7% methanol, hexane (v/v/v) at 1 ml/min.

**Liposome Preparation**—Small unilamellar vesicles were prepared from 1-α-phosphatidylcholine, 1-α-phosphatidylethanolamine, and 1-α-phosphatidylserine (Avanti Polar Lipids). A mixture of phosphatidylcholine/phosphatidylethanolamine/phosphatidylserine (40:30:30 mol %) in chloroform was dried in a round-bottom flask using a nitrogen stream to form a thin film and was incubated in vacuum for 1 h at room temperature. The suspension was shaken vigorously, sonicated 15 × 5 s on a Torbéo 36810-Series cell disruptor (Cole Parmer) with 1-min intervals at the setting of 5 in a water bath at room temperature. The sonicate was centrifuged at 13,000 × g for 20 min at 4 °C. The supernatant was saved and stored at 4 °C.

**RESULTS**

**Overall Structure**—The crystal structure of 11R-LOX in a calcium-free environment was refined at 2.47 Å with R$_{o/w}$ and R$_{free}$ values of 0.20 and 0.23, respectively (Table 1). The protein crystallized in space group C2 with two molecules in the asymmetric unit. Phasing was done with molecular replacement using *P. homomalla* 8R-LOX (PDB code 2fnq) as a template. A total of 96.1% of the residues are in the favored region of the Ramachandran plot and there are no outliers according to MolProbity validation (48).
There is a small confined chamber (about 34 Å³) next to the catalytic iron that is surrounded by several conserved aliphatic residues, which have been thoroughly discussed in P. homomalla 8R-LOX by Neau et al. (18). Of those residues, Leu374, Leu420, and Leu613 form an orifice that leads to the largest cavity (188 Å³), which is located under the arched helix toward the “rear” end of the enzyme (away from the PLAT domain). The bottom of this cavity is composed of residues Thr365, Val430, and Val609, which coincide with the regiospecificity determinants described for the boot-shaped channel in 12/15-LOX (7); Leu431, also a regiospecificity determinant of some LOXs (8, 12); and Met606, a position claimed to be relevant in the binding orientation of the fatty acid substrate (49).

On one side of the arched helix, the entrance to the active site chamber is blocked by Phe185 that sits on a loop between helices 9 (310-helix) and a2. On the other side, a gap between Leu374, Ile412, and Leu420 leads to a pair of cavities (95 and 113 Å³) that also reach toward the protein surface. These cavities are lined perpendicularly to the largest one, and are separated from each other by a constriction formed by Leu379, Ile421, and a conserved salt bridge Glu382–Arg417, that participates in lodging the arched helix. Access on this side is obstructed by Tyr154 and the loop where that residue is situated.

Let us call the orifices blocked by Phe185 and Tyr154 entrances A and B, respectively. Both of these entrances have a positively charged residue in the vicinity, which could neutralize the carboxylate group of a fatty acid substrate. These cavities are lined perpendicularly to the largest one, and are separated from each other by a constriction formed by Leu379, Ile421, and a conserved salt bridge Glu382–Arg417, that participates in lodging the arched helix. Access on this side is obstructed by Tyr154 and the loop where that residue is situated.

Let us call the orifices blocked by Phe185 and Tyr154 entrances A and B, respectively. Both of these entrances have a positively charged residue in the vicinity, which could neutralize the carboxylate group of a fatty acid substrate. These cavities are lined perpendicularly to the largest one, and are separated from each other by a constriction formed by Leu379, Ile421, and a conserved salt bridge Glu382–Arg417, that participates in lodging the arched helix. Access on this side is obstructed by Tyr154 and the loop where that residue is situated.

Let us call the orifices blocked by Phe185 and Tyr154 entrances A and B, respectively. Both of these entrances have a positively charged residue in the vicinity, which could neutralize the carboxylate group of a fatty acid substrate. These cavities are lined perpendicularly to the largest one, and are separated from each other by a constriction formed by Leu379, Ile421, and a conserved salt bridge Glu382–Arg417, that participates in lodging the arched helix. Access on this side is obstructed by Tyr154 and the loop where that residue is situated.

Let us call the orifices blocked by Phe185 and Tyr154 entrances A and B, respectively. Both of these entrances have a positively charged residue in the vicinity, which could neutralize the carboxylate group of a fatty acid substrate. These cavities are lined perpendicularly to the largest one, and are separated from each other by a constriction formed by Leu379, Ile421, and a conserved salt bridge Glu382–Arg417, that participates in lodging the arched helix. Access on this side is obstructed by Tyr154 and the loop where that residue is situated.

Let us call the orifices blocked by Phe185 and Tyr154 entrances A and B, respectively. Both of these entrances have a positively charged residue in the vicinity, which could neutralize the carboxylate group of a fatty acid substrate. These cavities are lined perpendicularly to the largest one, and are separated from each other by a constriction formed by Leu379, Ile421, and a conserved salt bridge Glu382–Arg417, that participates in lodging the arched helix. Access on this side is obstructed by Tyr154 and the loop where that residue is situated.

Let us call the orifices blocked by Phe185 and Tyr154 entrances A and B, respectively. Both of these entrances have a positively charged residue in the vicinity, which could neutralize the carboxylate group of a fatty acid substrate. These cavities are lined perpendicularly to the largest one, and are separated from each other by a constriction formed by Leu379, Ile421, and a conserved salt bridge Glu382–Arg417, that participates in lodging the arched helix. Access on this side is obstructed by Tyr154 and the loop where that residue is situated.

Let us call the orifices blocked by Phe185 and Tyr154 entrances A and B, respectively. Both of these entrances have a positively charged residue in the vicinity, which could neutralize the carboxylate group of a fatty acid substrate. These cavities are lined perpendicularly to the largest one, and are separated from each other by a constriction formed by Leu379, Ile421, and a conserved salt bridge Glu382–Arg417, that participates in lodging the arched helix. Access on this side is obstructed by Tyr154 and the loop where that residue is situated.

Let us call the orifices blocked by Phe185 and Tyr154 entrances A and B, respectively. Both of these entrances have a positively charged residue in the vicinity, which could neutralize the carboxylate group of a fatty acid substrate. These cavities are lined perpendicularly to the largest one, and are separated from each other by a constriction formed by Leu379, Ile421, and a conserved salt bridge Glu382–Arg417, that participates in lodging the arched helix. Access on this side is obstructed by Tyr154 and the loop where that residue is situated.

Let us call the orifices blocked by Phe185 and Tyr154 entrances A and B, respectively. Both of these entrances have a positively charged residue in the vicinity, which could neutralize the carboxylate group of a fatty acid substrate. These cavities are lined perpendicularly to the largest one, and are separated from each other by a constriction formed by Leu379, Ile421, and a conserved salt bridge Glu382–Arg417, that participates in lodging the arched helix. Access on this side is obstructed by Tyr154 and the loop where that residue is situated.
to be conserved as it is present in all published lipoxygenase crystal structures (18, 20, 22, 50–53) (Fig. 3A). The PLAT domain Trp107 is invariant among studied LOXs and is a part of the conserved sequence FPCYRW on the /H9252 strand of animal LOX (28). The cationic residue of the catalytic domain (Lys172 in 11R-LOX) is more variable, but can still be found in either of the two positions shown on the alignment (Fig. 3B).

**Ca\(^{2+}\)**-binding Sites—The 11R-LOX crystals were obtained in calcium-free conditions, but for catalytic activity, the presence of Ca\(^{2+}\) is a must. When compared with available structures of Ca\(^{2+}\)-PLAT complexes, the Ca\(^{2+}\)-binding loops of the apo-domain in 11R-LOX differ significantly. The PLAT domains of coral 8R-LOX (21) and gangrene \(\alpha\)-toxin (54) both contain three occupied binding sites, these are formed by three adjacent loops and are well conserved both in sequence and structure (21). All three sites are also preserved in the 11R-LOX sequence, yet are absent in the tertiary structure (Fig. 4). The invariant Trp is situated on the \(\beta\)-strand right next to site \(\gamma\). Conservation of binding sites, conserved residues are in red, 11R-LOX, 8R-LOX, and gangrene \(\alpha\)-toxin are aligned according to a structural superposition; human 5-LOX is aligned according to sequence. Residues that constitute sites I (blue), II (red), and III (green) in 8R-LOX and \(\alpha\)-toxin are denoted by circles (empty if participates by main chain, filled if by side chain atom). The invariant Trp is indicated by a star. 

**FIGURE 3.** The conserved \(\pi\)-cation bridge binds the regulatory PLAT and the catalytic domains. A, superposition of 11R-LOX (white), rabbit 12/15-LOX (gray), human 5-LOX (orange), and soybean LOX-1 (green) reveals the common interface. B, partial sequence alignments of the PLAT domain and the lid that establish the \(\pi\)-cation bridge. The PLAT domain Trp107 is invariant, the cationic residue can be found in either of the two positions (blue arrows).

**FIGURE 4.** The PLAT domain and putative Ca\(^{2+}\)-binding sites. A, superposition of the coral 8R-LOX Ca\(^{2+}\)-PLAT complex (green) and the 11R-LOX apo-PLAT (orange). The invariant Trp is situated on the \(\beta\)-strand right next to site \(\gamma\). B, conservation of binding sites, conserved residues are in red, 11R-LOX, 8R-LOX, and gangrene \(\alpha\)-toxin are aligned according to a structural superposition; human 5-LOX is aligned according to sequence. Residues that constitute sites I (blue), II (red), and III (green) in 8R-LOX and \(\alpha\)-toxin are denoted by circles (empty if participates by main chain, filled if by side chain atom). The invariant Trp is indicated by a star.
TABLE 2
Positional specificity of 11R-LOX (%)

| Enzyme | 15-HpETE | 11-HpETE | 8(12)-HpETE | 5-HpETE |
|--------|----------|----------|-------------|---------|
| WT     | 2        | 98       | ND          | ND      |
| V430A  | 1        | 99       | ND          | ND      |
| V609A  | 8        | 91       | 1           | ND      |
| L431A  | 3        | 87       | 10          | ND      |
| M606A  | 2        | 89       | 9           | ND      |
| V609W  | 23       | 69       | 3b          | 5       |

ND, not detected.

Although the data display a trend, these studies do not rule out the use of the alternative U-shaped channel (Fig. 2C). To find more substantial evidence to differentiate between the channels, Val430, which lies near the proximal side of the hydrophobic pocket, was substituted with a large tryptophan to block the distal end of the cavity, and propagate the usage of the U-shaped channel. As a consequence, the catalytic efficiency of the enzyme plunged 50-fold, but surprisingly, the $k_{cat}$ remained unaffected despite the dramatic reduction in binding space that such a mutation should have caused. Apparently, the loss of activity was entirely due to the diminished turnover rate (Table 3). Reaction specificity suffered greatly, as well, as the share of 11-HpETE dropped down to 69% and a multitude of by-products (15/5/8/12-HpETE in descending order by proportion) was formed (Table 2). The chirality of the three major products of V609W mutant, 11/15/5-HpETE, was determined using chiral phase HPLC, to confirm the substrate orientation in the active site. Practically pure 11R and 5S products were detected, oxygenation at C15 created an R/S (35:65) mixture (data not shown). The formation of 5S-HpETE intimates a head-first binding if entrance A is considered as the point of entry, whereas for 11R-HpETE, tail-first orientation has been suggested (25).

The interpretation of these data is not straightforward. If one invokes the use of the boot-shaped cavity, the residual activity of the V609W mutant may be a result of incomplete closure of the cavity and a motional flexibility to allow room for substrate entry despite the bulky tryptophan (Fig. 2C). On the other hand, if the fatty acid binds into the U-shaped channel, the role of the hydrophobic pocket may be to provide the flexibility necessary for the substrate to product transition. This would easily explain the unchanged $K_m$. Nevertheless, the various regiospecificities of distinct LOXs must somehow be reflected by their binding sites (e.g. different cavity volumes). The U-shaped channel is highly conserved, as emphasized by Neau et al. (18), but the invariant amino acids alone cannot explain the distinct products among lipoxygenases. Those side chains that impart specificity would be expected to lie outside the cluster of conserved amino acids. The hydrophobic pocket fulfills that criterion, and binding of AA in that cavity is supported by product shifts in L431A and M606A mutants.

**DISCUSSION**

**Allosteric Lid Segment**—The substrate channel entrance A of 11R-LOX is blocked by Phe185. Interestingly, a similar element, a Phe-Tyr “cork,” has been described in human 5-LOX (22). This cork is situated where the other LOX crystal structure models are open to allow access to the catalytic site. In these

**Regulation of 11R-Lipoxygenase Catalysis**

**TABLE 3**

Kinetic properties of wild-type 11R-LOX and selected mutants

| Enzyme | $k_{cat}$ | $K_m$ | $k_{cat}/K_m$ |
|--------|----------|-------|--------------|
| WT     | $310.7 \pm 24.6$ | $12.3 \pm 2.2$ | 25.2 |
| L431A  | $50.5 \pm 3.3$ | $7.8 \pm 1.4$ | 6.5 |
| M606A  | $398.0 \pm 20.8$ | $13.7 \pm 1.5$ | 29.0 |
| V609W  | $5.8 \pm 0.6$ | $12.4 \pm 3.0$ | 0.5 |

$K_m$ values were obtained via Lineweaver-Burk plots. All enzymes were readily purified and analyzed as described under “Experimental Procedures.”
two structures, however, the α2 helix is considerably shorter and is flanked by loops and 3_10-helices forming a lid that covers entrance A (Fig. 5). In the N-terminal end of this motif is the conserved π-cation bridge (Trp107–Lys172) that connects the PLAT and the catalytic domain. The π-cation bridge is preceded in turn by the lid of entrance B, with Tyr154 blocking the access. The corresponding cork in human 5-LOX is Trp147, and again, the similarity between the coral and human enzyme lids is remarkable. Notably, this is the entrance Gilbert et al. (22) have suggested to be utilized in 5-LOX, as opening it requires only a rotamer flip, and this way the substrate can enter the channel “tail first.” The stereochemistry of the products of 5- and 11-LOX suggests that A is bound in inverse orientations in these enzymes: may it be a result of the substrate entering the active site in inverse orientation using the same orifice or the utilization of different entrances. In either case, access to the catalytic site in both enzymes requires a conformational change, an opening of the lid. The structure of 11-LOX can provide a stable framework for understanding the relationship between Ca^{2+}-dependent membrane binding and lid opening.

It has been speculated that the interface of PLAT and catalytic domains, including the conserved FPCYRW fragment on the β7 strand, might be involved in an allosteric regulatory mechanism, transmitting a conformational change in the PLAT domain induced by calcium and membrane binding to the lid component in the catalytic domain (28). We propose, that the conserved π-cation bond could be mediating this interaction, as it stands right between the Ca^{2+}-binding sites and the allosteric lid. The different regulatory properties of various lipoxygenases (e.g. the necessity of calcium and membranes for 11-LOX catalysis) can be explained by differing conditions that are needed to set off the cascade of conformational changes, which among other contributors depend strongly on the structure of the Ca^{2+}-binding loops (55). The activity that is present without any inducing factors in most LOXs can be attributed to a semi-open orifice, or possibly allosteric binding of the substrate itself. Additionally, in human 5-LOX the FPCYRW fragment has been shown to be involved in the binding of the coactosin-like protein, which promotes leukotriene formation (56). Although the exact mechanism of the latter is unknown, it further substantiates the regulatory role of the bridge.

In 11-LOX, entrance A seems the more plausible access route for several reasons. First, the same orifice is used in rabbit 15S-LOX according to docking studies (14). For 11- and 15S-specificity, the same hydrogen must be abstracted in the initial step of catalysis; thus, substrate binding should also be identical. Second, coral 8R-LOX, the binding of which should differ from 11- and 15S-LOX only by “frameshift,” has been suggested to employ entrance A as well (18). Another detail in favor of entrance A is that there are several bulky, hydrophobic residues like Phe192, Phe205, and Trp204 on the C-terminal end of its lid fragment, distal to the PLAT domain interface (Fig. 5). With slight conformational changes, these residues could readily anchor the catalytic domain to the lipid membrane, and facilitate an additional mechanism of lid removal. Analogous residues are present in other lipoxygenases, too, including human 5-LOX. Furthermore, the entrance A lid is followed by the putative PDZ domain, which might also contribute to allosteric regulation, making this opening the more likely candidate not only for 11-LOX, but for other LOXs as well.

Additional experimental data are essential to elucidate the possible access portals in this family of enzymes. In a recent experiment, removing the entrance A cork of olive LOX1 by site-directed mutagenesis augmented the activity of the enzyme remarkably (57). A similar approach could be used in further study to determine the true substrate entrance of 11-LOX, but also of other LOXs.

T-shaped Substrate Channel—In general, the 11-LOX model contains a closed, roughly T-shaped system of cavities, wherein the active site iron is located at the junction of perpendicular channels, and the potential entrances for the substrate are situated at both ends of the “T-bar” (Fig. 2C). Although the channel system seems to be segmented in the model, minimal side chain movements are necessary to connect the neighboring pockets. In this system of cavities, the so called boot-shaped channel described by Kühn et al. (7, 12) would constitute the passage from entrance A to the bottom of the hydrophobic pocket. The alternative U-shaped channel proposed by Neau et al. (18), on the other hand, would consist of a culvert stretching below the arch helix and connecting both entrances, and thus, disregarding the pocket altogether.

The results obtained by site-directed mutagenesis of 11-LOX suggest that the integrity of the T-shaped channel is required for proper positioning of the substrate. The fact that L431A and M606A substitutions resulted in an 8/11-LOX, albeit with modest amounts of the 8-product, suggests that AA enters the hydrophobic pocket tail-first. This model is also supported by the dramatic reduction of catalytic activity and specificity when the pocket was blocked by the V609W substitution, even though the kinetic parameters for that mutant have left room for alternative interpretations. It is likely that in regard to substrate binding, 11R-LOX is analogous to the enzymes described to have a boot-shaped channel (e.g. rabbit 12/15-LOX).

The presence of cavities that connect the hypothetical entrance B with the active site still makes one question their potential role. The U-shaped channel is lined with highly con-
served Leu and Ile residues that imply a structure-functional importance. However, the highly conserved amino acids alone cannot define the different catalytic properties of lipoxygenases. And whereas the cavity that forms the B side of the T-site may provide an entry way for molecular oxygen access as suggested for soybean LOX-1 (58, 59), it is not clear whether leucines, as opposed to any hydrophobic amino acids, are necessary for an O₂ channel. It just might be that distinct lipoxygenases each utilize the central core of the binding site, but regiospecificity is defined by the access to that core. One could imagine a theory that merges the boot- and U-shaped passages, yielding a T-shaped substrate channel. Depending on the catalytic specificity of a particular LOX, the substrate could enter tail-first utilizing either one T-bar entrances. Additional mechanisms like positively charged residues could further induce and stabilize the substrate binding. Yet, for specificity, the aliphatic tail requires the internal hydrophobic pocket. Further studies, especially co-crystallization of the enzyme with the substrate could bring more definitive answers to these matters.

Acknowledgments—We thank Cory LaCrous for expert assistance in protein expression and purification, and Dr. David Neau in data collection. Preliminary work was performed at the Center for Advanced Microstructures and Devices (Baton Rouge), funded in part by the Louisiana Governors’ Biotechnology Initiative. X-ray data were collected at Beam Line 24-ID-E of NE-CAT at the Advanced Photon Source, supported by Award RR-15301 from the National Center for Research Resources at the National Institutes of Health. Use of the Advanced Photon Source, an Office of Science User Facility operated for the United States Department of Energy (DOE) Office of Science by Argonne National Laboratory, was supported by the United States DOE under Contract DE-AC02-06CH11357.

REFERENCES

1. Brash, A. R. (1999) Lipoxygenases. Occurrence, functions, catalysis, and acquisition of substrate. J. Biol. Chem. 274, 23679–23682
2. Pidgeon, G. P., Lyons, J., Krishnamoorthy, S., Reynolds, J. V., O’Byrne, K., Nie, D., and Honn, K. V. (2007) Lipoxygenase metabolism. Roles in tumor progression and survival. Cancer Metastasis Rev. 26, 503–524
3. Yoshida, H., and Kisugi, R. (2010) Mechanisms of LDL oxidation. Clin. Chim. Acta 411, 1875–1882
4. Duroudier, N. P., Tulah, A. S., and Sayers, I. (2009) Leukotriene pathway genetics and pharmacogenetics in allergy. Allergy 64, 823–839
5. Ivanov, I., Heydeck, D., Hofheinz, K., Roffeis, J., O’Donnell, V. B., Kuhn, H., and Walther, M. (2010) Molecular enzymology of lipoxygenases. Arch. Biochem. Biophys. 503, 161–174
6. Schneider, C., Pratt, D. A., Porter, N. A., and Brash, A. R. (2007) Control of oxygenation in lipoxygenase and cyclooxygenase catalysis. Chem. Biol. 14, 473–488
7. Kuhn, H., Saam, J., Ebach, S., Holzhüter, H. G., Ivanov, I., and Walther, M. (2005) Structural biology of mammalian lipoxygenases. Enzymatic consequences of targeted alterations of the protein structure. Biochem. Biophys. Res. Commun. 338, 93–101
8. Sloane, D. L., Leung, R., Craik, C. S., and Sigal, E. (1991) A primary determinant for lipoxygenase positional specificity. Nature 354, 149–152
9. Chen, X. S., and Funk, C. D. (1993) Structure-function properties of human platelet 12-lipoxygenase. Chimeric enzyme and in vitro mutagenesis studies. FASEB J 7, 694–701
10. Borngräber, S., Kuban, R. J., Anton, M., and Kühn, H. (1996) Phenylalanine 353 is a primary determinant for the positional specificity of mammalian 15-lipoxygenases. J. Mol. Biol. 264, 1145–1153
11. Borngräber, S., Browner, M., Gillmor, S., Gerth, C., Anton, M., Fletterick, R., and Kühn, H. (1999) Shape and specificity in mammalian 15-lipoxygenase active site. The functional interplay of sequence determinants for the reaction specificity. J. Biol. Chem. 274, 37345–37350
12. Vogel, R., Jansen, C., Roffeis, I., Reddanna, P., Forsell, P., Claesson, H. E., Kuhn, H., and Walther, M. (2010) Applicability of the triad concept for the positional specificity of mammalian lipoxygenases. J. Biol. Chem. 285, 5369–5376
13. Gan, Q. F., Browner, M. F., Sloane, D. L., and Sigal, E. (1996) Defining the arachidonic acid binding site of human 15-lipoxygenase. Molecular modeling and mutagenesis. J. Biol. Chem. 271, 25412–25418
14. Toledo, L., Masgrau, L., Maréchal, J. D., Lluch, J. M., and González-Lafont, A. (2010) Insights into the mechanism of binding of arachidonic acid to mammalian 15-lipoxygenases. J. Phys. Chem. B 114, 7037–7046
15. Toledo, L., Masgrau, L., Lluch, J. M., and González-Lafont, A. (2011) Substrate binding to mammalian 15-lipoxygenase. J. Comput. Aided Mol. Des. 25, 825–835
16. Boeglin, W. E., Itoh, A., Zheng, Y., Coffa, G., Howe, G. A., and Brash, A. R. (2008) Investigation of substrate binding and product stereochemistry issues in two linoleate 9-lipoxygenases. Lipids 43, 979–987
17. Jisaka, M., Iwanaga, C., Takahashi, N., Goto, T., Kawada, T., Yamamoto, T., Ikeda, I., Nishimura, K., Nagaya, T., Fushiki, T., and Yokota, K. (2005) Double dioxygenation by mouse 85-lipoxygenase. Specific formation of a potent pepsin-preformed activator-receptor α agonist. Biochem. Biophys. Res. Commun. 338, 136–143
18. Neau, D. B., Gilbert, N. C., Bartlett, S. G., Boeglin, W., Brash, A. R., and Newcomer, M. E. (2009) The 1.85 Å structure of an 8R-lipoxygenase suggests a general model for lipoxygenase product specificity. Biochemistry 48, 7906–7915
19. Gillmor, S. A., Villaseñor, A., Fletterick, R., Sigal, E., and Browner, M. F. (1997) The structure of mammalian 15-lipoxygenase reveals similarity to the lipases and the determinants of substrate specificity. Nat. Struct. Biol. 4, 1003–1009
20. Choi, J., Chon, I. K., Kim, S., and Shin, W. (2008) Conformational flexibility in mammalian 15S-lipoxygenase. Reinterpretation of the crystallographic data. Proteins 70, 1023–1032
21. Oldham, M. L., Brash, A. R., and Newcomer, M. E. (2005) Insights from the X-ray crystal structure of coral 8R-lipoxygenase. Calcium activation via a C2-like domain and a structural basis of product chirality. J. Biol. Chem. 280, 39545–39552
22. Gilbert, N. C., Bartlett, S. G., Waight, M. T., Neau, D. B., Boeglin, W. E., Brash, A. R., and Newcomer, M. E. (2011) The structure of human 5-lipoxygenase. Science 331, 217–219
23. Walther, M., Wiesner, R., and Kuhn, H. (2004) Investigations into calcium-dependent membrane association of 15-lipoxygenase-1. Mechanistic roles of surface-exposed hydrophobic amino acids and calcium. J. Biol. Chem. 279, 3717–3725
24. Kulkarni, S., Das, S., Funk, C. D., Murray, D., and Cho, W. (2002) Molecular basis of the specific subcellular localization of the C2-like domain of 5-lipoxygenase. J. Biol. Chem. 277, 13167–13174
25. Mortimer, M., Järving, R., Brash, A. R., Samel, N., and Järving, I. (2006) Identification and characterization of an arachidonate 11R-lipoxygenase. Arch. Biochem. Biophys. 445, 147–155
26. Walther, M., Hofheinz, K., Vogel, R., Roffeis, J., and Kühn, H. (2011) The N-terminal β-barrel domain of mammalian lipoxygenases including mouse 5-lipoxygenase is not essential for catalytic activity and membrane binding but exhibits regulatory functions. Arch. Biochem. Biophys. 516, 1–9
27. Ivanov, I., Di Venere, A., Horn, T., Scheerer, P., Nicolai, E., Stebling, S., Richter, C., Skrzypczak-Jankun, E., Mei, G., Maccarrone, M., and Kühn, H. (2011) Tight association of N-terminal and catalytic subunits of rabbit 12/15-lipoxygenase is important for protein stability and catalytic activity. Biochim. Biophys. Acta 1811, 1001–1010
28. Allard, J. B., and Brock, T. G. (2005) Structural organization of the regulatory domain of human 5-lipoxygenase. Curr. Protein Pept. Sci. 6, 125–131
29. Jankun, J., Doerks, T., Aleem, A. M., Lysiak-Szydlowska, W., and Skrzypczak-Jankun, E. (2008) Do human lipoxygenases have a PDZ regulatory domain? Curr. Mol. Med. 8, 768–773
Regulation of 11R-Lipoxygenase Catalysis

30. Aleem, A. M., Jankun, I., Dignam, J. D., Walther, M., Kühn, H., Svergun, D. I., and Skrzypczak-Jankun, E. (2008) Human platelet 12-lipoxygenase, new findings about its activity, membrane binding, and low-resolution structure. J. Mol. Biol. 376, 193–209

31. Wang, W., Ivanov, I., Svergun, D. I., Borbulevych, O. Y., Aleem, A. M., Stehling, S., Jankun, J., Kühn, H., and Skrzypczak-Jankun, E. (2011) Probing dimerization and structural flexibility of mammalian lipoxygenases by small-angle x-ray scattering. J. Mol. Biol. 409, 654–668

32. Koljak, R., Boutaud, O., Sheib, B. H., Samel, N., and Brash, A. R. (1997) Identification of a naturally occurring peroxidase-lipoxygenase fusion protein. Science 277, 1994–1996

33. Lõhelaid, H., Järving, R., Valmsen, K., Varvas, K., Kreen, M., Järving, I., and Coffa, G., Schneider, C., and Brash, A. R. (2005) A comprehensive model of positional and stereo control in lipoxygenases. Biochim. Biophys. Acta 1780, 315–321

34. Coffa, G., Schneider, C., and Brash, A. R. (2005) A comprehensive model of positional and stereo control in lipoxygenases. Biochim. Biophys. Acta 1780, 315–321

35. Studier, F. W. (2005) Protein production by autoinduction in high density shaking cultures. Protein Expr. Purif. 41, 207–234

36. Winter, G. (2010) Xia2. An expert system for macromolecular crystallography. J. Appl. Crystallogr. 43, 186–190

37. Keegan, R. M., and Winn, M. D. (2007) Automated search-model discovery and preparation for structure solution by molecular replacement. Acta Crystallogr. D Biol. Crystallogr. 63, 447–457

38. McCoy, A. J., Grosse-Kunstleve, R. W., Adams, P. D., Winn, M. D., Storoni, L. C., and Read, R. J. (2007) Phaser crystallographic software. J. Appl. Crystallogr. 40, 658–674

39. Vagin, A. A., Steiner, R. A., Lebedev, A. A., Potterton, L., McNicholas, S., Long, F., and Murshudov, G. N. (2004) REFMAC5 dictionary. Organization of prior chemical knowledge and guidelines for its use. Acta Crystallogr. D Biol. Crystallogr. 60, 2184–2195

40. Collaborative Computational Project, Number 4 (1994) The CCP4 suite. Programs for protein crystallography. Acta Crystallogr. D Biol. Crystallogr. 50, 760–763

41. Emsley, P., Lohkamp, B., Scott, W. G., and Cowtan, K. (2010) Features and development of Coot. Acta Crystallogr. D Biol. Crystallogr. 66, 486–501

42. Adams, P. D., Afonine, P. V., Bunkóczi, G., Chen, V. B., Davis, I. W., Echols, N., Headd, J. J., Hung, L. W., Kapral, G. J., Grosse-Kunstleve, R. W., McCoy, A. J., Moriarty, N. W., Oeffner, R., Read, R. I., Richardson, D. C., Richardson, J. S., Terwilliger, T. C., and Zwart, P. H. (2010) PHENIX. A comprehensive Python-based system for macromolecular structure solution. Acta Crystallogr. D Biol. Crystallogr. 66, 213–221

43. Pettersen, E. F., Goddard, T. D., Huang, C. C., Couch, G. S., Greenblatt, D. M., Meng, E. C., and Ferrin, T. E. (2004) UCSF Chimera. A visualization system for exploratory research and analysis. J. Comput. Chem. 25, 1605–1612

44. Sanner, M. F., Olson, A. J., and Spehner, J. C. (1996) Reduced surface. An efficient way to compute molecular surfaces. Biopolymers 38, 305–320

45. Krissinel, E., and Henrick, K. (2007) Inference of macromolecular assemblies from crystalline state. J. Mol. Biol. 372, 774–797

46. Chenna, R., Sugawara, H., Koike, T., Lopez, R., Gibson, T. J., Higgins, D. G., and Thompson, J. D. (2003) Multiple sequence alignment with the Clustal series of programs. Nucleic Acids Res. 31, 3497–3500

47. Gouet, P., Courcelle, E., Stuart, D. I., and Métoz, F. (1999) ESPript. Analysis of multiple sequence alignments in PostScript. Bioinformatics 15, 305–308

48. Chen, V. B., Arendall, W. B., 3rd, Headd, J. J., Keedy, D. A., Immormino, R. M., Kapral, G. J., Murray, L. W., Richardson, J. S., and Richardson, D. C. (2010) MolProbity. All-atom structure validation for macromolecular crystallography. Acta Crystallogr. D Biol. Crystallogr. 66, 12–21

49. Isisaka, M., Kim, R. B., and Boeglin, W. E. (2000) Identification of amino acid determinants of the positional specificity of mouse 8-lipoxygenase and human 15-lipoxygenase-2. J. Biol. Chem. 275, 1287–1293

50. Youn, B., Sellhorn, G. E., Mirchel, R. J., Gaffney, B. J., Grimes, H. D., and Kang, C. (2006) Crystal structures of vegetative soybean lipoxygenase VLY-B and VLY-D, and comparisons with seed isoforms LOX-1 and LOX-3. Proteins 65, 1008–1020

51. Minor, W., Steczko, J., Sto, B., Otwinowski, Z., Bolin, J. T., Walter, R., and Axelrod, B. (1996) Crystal structure of soybean lipoygenase L-1 at 1.4-Å resolution. Biochemistry 35, 10687–10701

52. Chruszcz, M., Wlodawer, A., and Minor, W. (2008) Determination of protein structures. A series of fortunate events. Biophys. J. 95, 1–9

53. Skrzypczak-Jankun, E., Borbulevych, O. Y., Zavodszyk, M. I., Baranski, M. R., Padmanabhan, K., Petricek, V., and Jankun, J. (2006) Effect of crystal freezing and small-molecule binding on internal cavity size in a large protein. X-ray and docking studies of lipoygenase at ambient and low temperature at 2.0-Å resolution. Acta Crystallogr. D Biol. Crystallogr. 62, 766–775

54. Naylor, C. E., Jepson, M., Crane, D. T., Titball, R. W., Miller, J, Basak, A. K., and Bolgiano, B. (1999) Characterization of the calcium-binding C-terminal domain of Clostridium perfringens α-toxin. J. Mol. Biol. 294, 757–770

55. Hammarberg, T., Provost, P., Persson, B., and Rådmark, O. (2000) The N-terminal domain of 5-lipoxygenase binds calcium and mediates calcium stimulation of enzyme activity. J. Biol. Chem. 275, 38787–38793

56. Esser, J., Rakonjac, M., Hofmann, B., Fischer, L., Provost, P., Schneider, G., Steinhalber, D., Samuelsson, B., and Rådmark, O. (2010) Coacyslin-like protein functions as a stabilizing chaperone for 5-lipoxygenase. Role of tropothan 102. Biochim. J. 425, 265–274

57. Palmieri-Thiers, C., Alberti, J. C., Canaan, S., Brunini, V., Gambotti, C., Tomi, F., Oliw, E. H., Berti, L., and Maury, J. (2011) Identification of putative residues involved in the accessibility of the substrate-binding site of lipoygenase by site-directed mutagenesis studies. Arch. Biochem. Biophys. 509, 82–89

58. Knapp, M. J., Seebeck, F. P., and Kliman, J. P. (2001) Steric control of oxygenation regiochemistry in soybean lipoygenase-1. J. Am. Chem. Soc. 123, 2931–2932

59. Knapp, M. J., and Kliman, J. P. (2003) Kinetic studies of oxygen reactivity in soybean lipoygenase-1. Biochemistry 42, 11466–11475