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Cryo-EM structures of human arachidonate 12S-Lipoxygenase (12-LOX) bound to endogenous and exogenous inhibitors

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Key Points

1) The first full-length structures of human arachidonate 12S-Lipoxygenase (12-LOX) reveal mechanisms of oligomeric and conformational states
2) The structures uncover natural inhibitor of 12S-Lipoxygenase (12-lox) and reveal a binding site of inhibitor ML355

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Abstract

Human 12-lipoxygenase (12-LOX) is an enzyme involved in platelet activation and is a promising target for antiplatelet therapies. Despite the clinical importance of 12-LOX, the exact mechanisms of how it affects platelet activation are unclear, and the lack of structural information has limited drug discovery efforts. In this study, we used single-particle cryo-electron microscopy to determine the high-resolution structures (1.7 Å - 2.8 Å) of human 12-LOX for the first time. Our results showed that 12-LOX can exist in multiple oligomeric states, from monomer to hexamer, which may impact its catalytic activity and membrane association. We also identified different conformations within a 12-LOX dimer, likely representing different time points in its catalytic cycle. Furthermore, we were able to identify small molecules bound to the 12-LOX structures. The active site of the 12-LOX tetramer is occupied by an endogenous 12-LOX inhibitor, a long-chain acyl-Coenzyme A. Additionally, we found that the 12-LOX hexamer can simultaneously bind to arachidonic acid and ML355, a selective 12-LOX inhibitor that has passed a phase I clinical trial for treating heparin-induced thrombocytopenia and has received fast-track designation by the FDA. Overall, our findings provide novel insights into the assembly of 12-LOX oligomers, its catalytic mechanism, and small molecule binding, paving the way for further drug development targeting the 12-LOX enzyme.

Introduction

Platelet activation is essential for maintaining haemostasis. However, uncontrolled platelet activation leads to abnormal clot formation and an increased risk of thrombosis and cardiovascular disease [1,2]. Inhibition of platelet activation has been shown as an effective treatment that reduces the morbidity and mortality of cardiovascular ischemic events, such as myocardial infarction and stroke. Despite the use of antiplatelet therapies such as aspirin and $P2Y_{12}$ receptor antagonists to reduce thrombotic risk, a high prevalence of ischemic events leading to unacceptable levels of morbidity and mortality remain. Due to this continued risk for thrombosis, the development of alternative therapies to further limit occlusive thrombotic events is warranted.

The enzyme, human 12-lipoxygenase (12-LOX, ALOX12), is highly expressed in platelets [3], and its activation leads to the production of 12-hydroperoxyeicosatetraenoic acid (12-HpETE), a prothrombotic oxylipin [4,5]. The inhibition of 12-LOX prevents platelet activation [6,7], has a minimal effect on haemostasis, and does not promote increased bleeding, a common side effect of other antiplatelet therapies [6,8,9]. The selective 12-LOX inhibitor, ML355 [10], has passed phase I clinical trials for the treatment of heparin-induced thrombocytopenia and has received fast-track designation by the FDA. Despite 12-LOX being a promising target for antiplatelet therapies there are no experimentally determined structures of the entire human 12-LOX; thus, limiting our understanding of the mechanism and regulation of 12-LOX activity. Although structures of other LOX isozymes have been determined by x-ray crystallography [11-15], they do not provide enough information to fully comprehend the mechanism of 12-LOX oligomerization and inhibitor binding.

Here we present the first high-resolution structures (1.7–2.8 Å) of human 12-LOX determined using cryo-electron microscopy (cryo-EM). We show that 12-LOX possesses a similar protein fold compared to other lipoxygenases[11-15]. From a single sample of 12-LOX, we were able to determine cryo-EM structures in multiple oligomeric forms from the monomer up to a hexamer. This observation is consistent with prior studies that demonstrated the existence of multiple oligomeric forms of lipoxygenases, including monomers, dimers and tetramers [16-18]. Similar to human 15-LOX, we also captured 12-LOX in different conformational states that likely reflect the different parts of the catalytic cycle for this enzyme – the “open” and “closed” conformation [11]. Due to the high-resolution features of the cryo-EM map, we were able to identify an endogenous 12-LOX inhibitor, a long-chain acyl-Coenzyme A, that co-purifies with the enzyme from mammalian cells. Finally, we were able to elucidate a putative allosteric binding site for the phase II inhibitor ML355. Collectively, we anticipate these structures guiding further research into the function of 12-LOX for platelet activation and promote further drug discovery efforts at this clinically relevant enzyme.

Materials and Methods

A more detailed methods section is provided in the Supplemental Data.

Expression and purification

The human 12-LOX was expressed in Expi293 cells. Following cell lysis, the protein was purified using Ni-affinity and size-exclusion chromatography (SEC).

Cryo-electron microscopy

Vitrification was performed at 1 mg/ml using UltrAufoil R1.2/1.3 300 mesh holey grids and a Vitrobot Mark IV. Movies were collected at 0.82 Å/pix on a G1 Titan Krios microscope with the K3 detector. Data processing was performed following standard pipelines in RELION v3.1[19,20] and cryoSPARC[21] with the aid of UCSF MotionCor2 [22] and GCTF[23]. The 3D variability analysis (3DVA)[24] was used to assess sample variability. Modelling was performed with the aid of the AlphaFold Protein Structure Database[25,26], UCSF ChimeraX[27], COOT[28], PHENIX[29], MolProbity[30] and the GRADE webserver[31].

Steady-State Kinetics and IC_{50} Determination

The 12-LOX enzyme kinetics was performed as described previously[32].

Liposome binding

A lipid suspension was made with the following molar ratios: 99.9:0.1 DOPC: DSPE-PEG (i.e. DOPC). Liposomes were created using the literature protocol[33] using a 100 nm filter at a final concentration of 10 mg/mL.

Data availability. Atomic coordinates and the cryo-EM density maps have been deposited in the Protein Data Bank and the Electron Microscopy Data Bank. The accession codes are 8GHB and EMD-40039 for 12-LOX monomers; 8GHC and EMD-40040 for dimers; 8GHE and EMD-40042 for tetramers and 8GHD and EMD-40041 for hexamers.

Results

High-resolution structures of 12S-Lipoxygenase

The purification of 12-LOX from mammalian Expi293 cells yielded an oligomeric mixture of 12-LOX forms that were separated by size using size-exclusion chromatography (SEC) **(Fig. 1** and **Supp. Fig. 1)**. The two main peak fractions primarily corresponded to a dimer and a tetramer based on their size, however, the populations were heterogeneous and contained small amounts of other oligomeric forms **(Supp. Fig. 1E).** Both SEC fractions displayed similar enzyme kinetics with a k_{cat} of 12 ± 0.9 s^{-1} for the dimer and 4.8 ± 0.2 s^{-1} for the tetramer. Both had a k_{cat}/K_M value of 1.4 ± 0.1 $s^{-1}\mu M^{-1}$. The enzyme activities of both SEC fractions were inhibited by ML355 with IC_{50}'s of 1.6 ± 0.3 μM and 1.4 ± 0.3 μM, for the dimer and tetramer, respectively **(Supp. Fig. 1C,D and Table S2)**. Prior efforts to determine structures of 12-LOX using x-ray crystallography likely failed due to sample heterogeneity. Thus, we turned to cryo-EM to determine the structure of 12-LOX from each SEC fractions. To understand the binding mechanism of ML355 binding site, we added the inhibitor during protein expression and purification used in cryo-EM.

Unexpectedly, the dimer peak from the SEC 12-LOX purification gave rise to multiple high-resolution cryo-EM structures of 12-LOX in different oligomeric forms including monomers (2.8 Å), dimers (2.5 Å), tetramers (2.3 Å), and hexamers (2.6 Å), all from the same imaged grid **(Fig. 1**, **Supp. Fig. 2, 4** and **Table S1)**. In contrast, the tetramer peak yielded a structure of only the 12-LOX tetramer **(Fig. 1**, **Supp. Fig. 3,4** and **Table S1)** with an overall resolution of 1.8 Å. All oligomeric forms of 12-LOX exhibited significant intermolecular flexibility determined by 3D variability analysis (3DVA) in cryoSPARC **(Supp. Movies 1-3)**[24]. Thus, we employed local refinement (cryoSPARC) to improve the map resolution and quality for the individual subunits (**Supp. Fig. 2-5)** within each oligomer. For the tetramer peak sample, this improved the resolution to 1.7 Å allowing for accurate model building of the full-length 12-LOX (residues G2 to I663) **(Fig. 2A-B)**.

The structural architecture of 12-LOX is typical of lipoxygenases with the N-terminal β-barrel PLAT (polycystin-1-lipoxygenase α-toxin) domain and a C-terminal α-helical catalytic domain (**Fig. 2A**). Structural alignments with other LOX structures **(Supp. Fig. 6A)** revealed markedly similar folds with root mean square deviations (RMSDs) <1Å with the largest variation occurring in the PLAT domain (**Supp. Fig. 6B**). The active site of 12-LOX is located in the catalytic domain, where a catalytic iron atom is coordinated by three conserved histidine residues (H360, H365 and H540) as well as N544 and the carboxyl C-terminus of I663 **(Supp. Fig. 6C-D)**. Next to the catalytic iron is the typical LOX U-shaped lipid-binding pocket that is lined with hydrophobic residues**.** The entrance to the active site is bordered by an arched helix and an α2-helix in an extended conformation (**Fig. 2A** and **Supp. Fig. 6C-D**).

Oligomeric states of 12S-lipoxygenase

The biological unit of the 12-LOX oligomers appears to be a dimer arranged "head-to-toe". The tetramer and hexamer are made of a dimer of dimers and a trimer of dimers, respectively **(Fig. 1** and **2)**. The overall dimer substructure in each oligomeric form is maintained mainly through Van der Waals interactions between the α2-α4 helixes and the α3-α4 loop **(Fig. 2B-D)**. The dimer substructures of the 12-LOX tetramer and hexamer were virtually identical (RMSDs 0.78Å), while the individual subunits of the dimer were rotated by 30°, due to change in the conformation of the α2-helix (described below) (**Supp.Fig. 7)**. Higher-level

oligomerisation in 12-LOX tetramers was maintained through additional Van der Waals interactions of the α2-helix and hydrogen bond interactions of the arched helix and β9-β10 loop between neighbouring subunits (**Fig. 2C)**. The architecture of the hexamers was supported by an additional hydrogen bonding network and a disulphide bond (C89-C89) between neighbouring PLAT domains (**Fig. 2D).**

The oligomerisation of 12-LOX affects the accessibility of the active site to bulk solvent. The entrance to the 12-LOX catalytic site is defined by the α2- and arched helixes and is in the same plane as the predicted membrane-binding residues W70/L71/A180[34]. The entrance is accessible to solvent in the 12-LOX monomer, dimer (the "open" subunit, see below), and hexamer, but is obscured when the two dimers associate to form a tetramer due to the adjacent subunit. (**Supp. Fig. 8)**. Conversely, in the "closed" subunit of a 12-LOX dimer, the occlusion is a result of a conformational change (explained further below).

To investigate whether the oligomerisation affects 12-LOX membrane binding we tested the dimer and the tetramer SEC fractions **(Fig. 1)** for their ability to bind artificial DOPC liposomes. Both 12-LOX preparations bind liposomes with similar extent (21+/-6% and 36+/-6% for dimers and tetramer peak, respectively) **(Supp Fig. 1B)**.

Conformational changes of 12S-Lipoxygenase

The structure of the 12-LOX monomer in all oligomeric forms were similar, except for the 12-LOX dimer. Similar to the arrangement within the 12-LOX tetramer and the hexamer, the monomers in the dimer are arranged "head-to-toe", with most of the contacts mediated via hydrophobic interactions between the α2-helixes (**Fig. 2B**), previously determined by HDX-MS[35]. However, contrary to the protein chains in the tetramer and hexamer, the individual subunits in a dimer are not equivalent. Instead, they adopt either an "open" conformation (as observed in the 12-LOX monomer, tetramer, and a hexamer) or a "closed" conformation, predominantly facilitated by a large-scale motion of the α2-helix and corresponding rearrangements of the neighbouring loops **(Fig. 3)**. In the open conformation, the α2-helix forms a long single helix that lies at the edge of the active site. Conversely, in the closed conformation, the α2-helix undergoes a rigid 23° pivot and rotation that blocks the entrance to the active site reducing its internal volume **(Fig. 3B**). The conformational change of the α2-helix also leads to a 30° rotation of the two monomers relative to each other and relative to the dimer substructure observed in the 12-LOX tetramers and hexamers **(Supp. Fig. 7**).

Natural inhibition of 12S-Lipoxygenase by long chain fatty acid acyl-CoAs

In all of our 12-LOX structures the active site of the 12-LOX subunits in the open conformation was occupied by extra density in the cryo-EM maps **(Supp. Fig. 9**), suggesting the presence of a bound ligand. The shape of the density varied between oligomeric forms suggesting different ligands. The high-resolution of the tetramer (1.7 Å) and the hexamer (2.3 Å) cryo-EM maps allowed us to model ligands into these densities with high confidence. However, due to the lower resolution of the monomer and dimer cryo-EM maps, we were not able to confidently identify the bound molecules. We hypothesized the observed densities were either ML355 or an endogenous lipid(s) co-purified from Expi293 cells.

The 12-LOX tetramer is made of a dimer of dimers. The subunits at the inter-dimer interface face each other with their lipid binding sites **(Fig. 4A)**. Within each of the U-shaped pockets, we observed a density that resembled a lipid tail. The lipid density extended out of the binding site, spanning the gap between two neighbouring subunits **(Supp. Fig. 10A)**. This density was

also present in the apo 12-LOX tetramer samples that were expressed and purified in the absence of ML355 suggesting the ligand was co-purified from the HEK293 cells (data not shown). To improve the resolution of the cryo-EM maps further, we performed a 3D variability analysis (3DVA) on individual subunits within a tetramer **(Sup Fig. 10B, Supp. Movie 2).** Using the cluster mode of the 3DVA, we were able to separate the protein chains that were fully occupied with the molecule and reconstruct the corresponding 12-LOX subunits and a full tetramer to a resolution of 1.9 Å and 2.05 Å, respectively **(Fig. 4A-C)**. Furthermore, the 3DVA revealed that the lipid is only bound to one of the subunits at the inter-dimer interface at a time (thus averaging to ½ lipid occupancy in the entire 12-LOX tetramer) **(Supp. Fig. 10C, Supp. Movie 4)**. In contrast, the opposite subunit was mostly empty with some weak non-continuous density in the active site that could represent another unidentified lipid or incomplete separation of the occupied vs. unoccupied subunits during 3DVA.

Because of the high resolution and quality of the density map, we were able to identify the lipid as a fatty acid acyl-CoA ester with a tail approximately 18 carbons long and unknown saturation (oleoyl-CoA was used for modelling purposes) **(Fig. 4B)**. The CoA headgroup is positioned at the inter-dimer interface at the entrance to the catalytic site, between the α2-helix and the arched helix with the fatty acid tail extending into the U-shaped hydrophobic cavity **(Fig. 4C)**. The purine group of CoA forms CH-π interactions with I413, a hydrogen bond with Q406 of the arched helix, and cation-π interactions with the neighbouring molecule's R290 **(Fig. 4D)**. The carbonyl of the oleic acid forms a hydrogen bond with H596. The three phosphate groups form electrostatic interactions with R189, R290, and R585 of the bound 12-LOX, as well as R189, R290, K416 and R585 of the neighbouring 12-LOX subunit. Overlay of these two subunits reveals that the polar residues at the dimeric interface undergo significant rearrangement to better accommodate the interaction with oleoyl-CoA **(Fig. 4F).** The fatty acid tail extends into the catalytic site, forming extensive hydrophobic contacts **(Fig. 4E)**.

Due to the chemical lability of the acyl-CoA's thioester and hence difficulty in detection by mass spectrometry, we set out to confirm our structural findings by determining whether fatty acid acyl-CoAs inhibit 12-LOX. We tested a panel of long chain acyl-CoAs with different lipid tail length and saturation to determine their ability to inhibit 12-LOX catalysis **(Table S3)**. The 12-LOX inhibition by acyl-CoAs depends on both their length and saturation status, with oleoyl-CoA (18:1) being the most potent inhibitor with an IC_{50} of 32 ± 4 μM. None of the tested acyl-CoAs were substrates for 12-LOX. These data confirm that oleyl-CoA is the most potent inhibitor of 12-LOX, although the exact nature of the bound acyl-CoA in the structure is unconfirmed.

ML355 binding of 12-LOX

In contrast to the tetramer structure, cryo-EM density within the active site of the 12-LOX hexamer was identical across subunits and was distinct from the acyl-CoA. Moreover, the density was of two independent molecules that could be perfectly fit with AA and ML355 **(Fig. 5A-C)**. The AA molecule occupies the U-shaped hydrophobic cavity that was occupied by the fatty acid tail of the acyl-CoA in the 12-LOX tetramer. The carboxyl group of AA interacts with 12-LOX via a H-bond with H596, as predicted [36], positioning the C11-C12 double bond in the vicinity of the catalytic iron. The remainder of the contacts are from Van der Waals interactions with hydrophobic residues lining the channel of the active site (**Fig. 5E**). The position of AA is nearly identical to that of the anaerobic structure of coral 8R-LOX [37].

Docking and mutagenesis studies predicted ML355 to bind deep in the 12-LOX active site [32], but our cryo-EM density maps showed no evidence of ML355 occupying that region. Unexpectedly, however, a molecule of ML355 perfectly fit into the EM density found at the entrance to the active site in the hexamer. Interactions of ML355 with 12-LOX include the hydroxyl group of the 2-hydroxy-3-methoxyphenyl moiety forming H-bonds with the backbone carbonyl of L178 and amide of A182 (**Fig. 5D**). The sulphur of benzothiazole ring forms a H-bond with R189 with the sulphonyl group within H-bonding distance to R290 and R585. The sulphonyl interactions of ML355 mimic the interactions observed with the phosphates from oleoyl-CoA and residues R189, R290, and R585. The rest of the molecule forms Van der Waals interactions with M185, I413, L589 and I 5993 **(Fig. 5D).** To validate the ML355 binding pose, we generated four 12-LOX mutants: L589A, L589F, 4A (R189A/R290A/R585A/K416A), and DLQN (R189D/R290L/K416Q/R585H) **(Fig. 5D)**. Although all mutants folded correctly (based on their thermal unfolding profiles), L589F, 4A, and DLQN were catalytically inactive. Notably, the L589A mutation decoupled catalytic activity from ML355 inhibition **(Supp. Fig. 11 And B)**. This mutant displayed similar kinetics as the wt 12-LOX but remained unresponsive to ML355 inhibition. Mass photometry demonstrated that L589A also impaired the formation of higher-order oligomers associated with ML355 or acyl-CoA binding **(Supp. Fig. 11C)**. While these results support the identified ML355 binding site, a more rigorous investigation into the mechanism of ML355 binding and inhibition is required in the future.

Discussion

To our knowledge, this is the first study to use cryo-EM to determine high-resolution structures of lipoxygenases. Compared to x-ray crystallography, the ability of cryo-EM to separate heterogeneous samples into discrete populations revealed distinct 12-LOX oligomeric states. Human LOXs display oligomeric diversity: while 5-LOX and 15-LOX primarily function as monomers, they dimerize under high protein and salt concentrations[17]. On the contrary, 12-LOX is primarily dimeric[38], but can form larger aggregates[16]. Other studies suggested that most human LOXs can form high-order oligomers in solution[39]. Our structures provide the first high-resolution insights into the diversity of 12-LOX oligomeric forms that can likely be extended to other LOXs.

SAXS experiments predicted that all LOX dimers (12-LOX[16], 15-LOX[18] and 5-LOX[17]) have a similar organisation, including the “head-to-toe” arrangement of individual monomers that are interacting through their α2-helixes. Prior to our structures, such an arrangement was only directly observed in x-ray structures of rabbit 15-LOX-1[11,13] and human 15-LOX-2[40]. While the overall dimer organisation is similar between all 3 enzymes, the relative position of individual subunits varies, owing to differences in specific interacting residues.

Cryo-EM allowed us for the first time to observe structures of 12-LOX tetramers and hexamers. Interestingly, both are made from the dimer building blocks that further oligomerise either into the dimer of dimers or trimer of dimers. Prior studies suggested that reducing agents might prevent the oligomerisation of 12-LOX [16], proposing that higher-order oligomers form through intramolecular disulphide bonds upon protein oxidation. Our 12-LOX hexamer structure is consistent with this observation as it is stabilised by an intermolecular disulphide bond (C89-C89). Other interactions in the dimer, tetramer and a hexamer included an extensive network of hydrophobic interactions and hydrogen bonds. As such, the assembly of 12-LOX into dimers and tetramers is independent of the oxidation state of the enzyme, while higher-molecular oligomers could represent a change to the oxidative environment of the cell.

The oligomerisation of 12-LOX might be a regulatory mechanism for enzyme activity and/or membrane binding as the accessibility of the active site varies between different oligomeric forms. The predicted membrane-binding residues for the 12-LOX are located in the same plane as the entrance to its binding site. Interestingly, the membrane binding surface within the 12-LOX dimer building block (present in dimers, tetramers and hexamers) is located within the same surface plane. However, in the tetramer the membrane-binding surface and active site entrance are further sequestered by interdimer contacts. As such, they might represent inactive states or storage pools for the enzyme. While we did not observe significant differences in AA oxidation rates or DOPC liposome binding between the dimer and tetramer SEC peaks used for cryo-EM data collection, the data could possibly be explained by our heterogeneous preparations containing a mixture of the 12-LOX oligomeric forms. Thus, further analysis using isolated 12-LOX oligomeric forms is necessary to better understand their role in membrane binding and catalysis.

Intriguingly, the higher-order oligomers of 15-LOX-1 were found to induce pore formation in the lead to organelle clearance during erythrocyte maturation[41]. The two-ring arrangement of the 12-LOX hexamer creates a channel with a diameter of ~30 Å. While the physiological role of this oligomeric species of 12-LOX requires further investigation, it is tempting to speculate that similar conformations might exist in other LOXs.

The protein chains in the 12-LOX monomer, tetramer, and hexamer adopt "open" conformation characterised by an extended α2-helix that pack along the entrance to the active site. Such an α2 conformation is seen in many of the LOX structures, including coral 8R-LOX [37], human 15-LOX-2 [14] and porcine 12-LOX (ALOX15)[15]. In the 12-LOX dimer, one subunit adopts an "open" conformation while another undergoes significant conformational change involving a large-scale α2 movement. The alterations to the extended α2 conformation were observed previously in crystal structures of stable 5-LOX [12,42-44] (broken or disordered α2) and 15-LOX-1 [11,13] (large-scale α2 movement). The 15-LOX-1 and now the 12-LOX are the only LOXs that were captured forming non-symmetrical dimers with one subunit in the "open" and one in the "closed" conformations. While the conformational change leading to the formation of the "closed" conformation differs in the degree of the α2 movement and the subunit rotation relative to each other, both result in the closure of the entrance to the active site.

The conformational change between the "open" and "closed" subunits in LOX dimers might be linked to their catalytic cycle[18] or be involved in inhibitor binding[11]. Similar to the 15-LOX-1 structure[11], some of our 12-LOX oligomers demonstrate half occupancy of their active sites. In the 12-LOX dimer, only the active site of the "open" subunit is occupied by what appears to be a lipid density. This suggests that only half of the oligomeric subunits may be active at any given time, while the other subunit serves a regulatory role. This mechanism could be responsible for differences in inhibitor binding observed previously between the dimeric and monomeric 12-LOX (converted by introducing L183E/L187E mutations). Only the dimer showed inhibition by ML355 (Ki = 0.43 μM), while monomeric 12-LOX was unaffected[35]. Unfortunately, our dimer 12-LOX structure cannot distinguish between part-of-the-site reactivity mechanism, where only one subunit is capable of catalysis, as described for cyclooxygenases (COX)[45,46], and the flip-flop mechanism, where the subunits are taking turns at the catalysis as is the case for biotin carboxylase and transketolase [47,48,49]. Additional analysis is needed to understand the 12-LOX catalytic mechanism further.

One of the unexpected findings was the presence of the fatty acid acyl-CoA molecule in the 12-LOX tetramer that co-purified with our enzyme from Expi293 cells. Fatty acid acyl-CoA derivatives have long been known to inhibit platelet aggregation[50,51] in a chain, length, and saturation-dependent manner. Specifically, the medium-chain acyl-CoA (palmitoyl, stearoyl, oleoyl and linoleoyl) inhibit lipoxygenase activity in platelets at concentrations ranging from 10 to 50 μM[52]. We have confirmed that oleoyl-CoA inhibits 12-LOX at micromolar concentrations. Thus, the presence of acyl-CoA in the binding site is intriguing, particularly as the purified enzyme remains catalytically active. This paradox may relate to the 12-LOX reactivity mechanism and potential half-occupancy of active sites (described above). However, the cause for the half-site occupancy of the tetramer is different from that of a dimer, as the neighbouring subunits create steric hindrance preventing acyl-CoA binding to opposing subunit. Considering that the levels of acyl-CoAs within the cell could reach micromolar concentration [53], the long chain acyl-CoAs could be physiologically important regulators of 12-LOX function in the cell. The effect of acyl-CoA on platelet aggregation is thought to be mediated through P2Y1 and P2Y12 receptors [54]. However, with the discovery that fatty acid acyl-CoA directly binds and inhibits 12-LOX, it might be possible that the inhibition of 12-LOX could also contribute to this process.

Despite the presence of ML355 during the expression and purification of 12-LOX, ML355 was only bound in the hexameric form of 12-LOX. It is likely, that ML355 was competed out in the other oligomeric forms due to the presence of endogenous lipids. The observed pose of ML355 is in contradiction to previously published docking/ mutagenesis studies that predicted ML355 binding deep in the 12-LOX active site [32]. However, the simultaneous binding of ML355 and AA observed in our structure could explain the “mixed” mode of ML355 inhibition described previously[55]. Nevertheless, future studies are needed delineate the mechanism of ML355 inhibition with respect to different oligomeric forms of the enzyme along with the role of endogenous inhibitors that may or may not be present in platelets.

In conclusion, this study presents the first high-resolution cryo-EM structures of 12-LOX in multiple oligomeric forms, provides the first structural information on the clinically relevant 12-LOX inhibitor ML355, shows evidence for conformational changes that might accompany the 12-LOX catalytic cycle, and demonstrates that acyl-CoA can serve as endogenous 12-LOX inhibitor. This structural information will aid future studies of 12-LOX biology and its contribution to platelet activation and facilitate structure-based drug discovery efforts on a therapeutically validated enzyme.

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Author contributions A.G. developed protein purification strategy, performed protein expression and negative stain transmission EM. A.G. and J.I.M purified the protein. H.V. vitrified the sample and performed image acquisition within the Monash EM facility. J.I.M., K.A.B and A.G. performed cloning, cryo-EM data processing, model building, refinement and validation. M.T. performed enzyme kinetics, inhibition and liposome binding. W.A.C.B. performed mass photometry and 12-LOX mutant purification, characterization, and kinetics. M.H., T.D., participated in experimental design and result interpretation. A.G., D.M.T, J.I.M. and K.A.B wrote the manuscript with contributions from all authors. A.G. and D.M.T. supervised the project.

Competing interests. M. Holinstat is an equity holder and serves on the scientific advisory board for Veralox Therapeutics and Cereno Scientific. M. Holinstat and T. R. Holman are co-inventors for the patented compound ML355.

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Figure Legends

Figure 1. Different oligomeric forms of 12-LOX. (A) Size exclusion chromatography (SEC) UV absorbance trace (280 nm absorbance) from 12-LOX purification. 12-LOX separated as two distinct peaks, one corresponding to a tetramer (red box) and the other a dimer (blue box). (B) Cryo-EM map of a 12-LOX tetramer from "tetrameric" SEC peak. (C) Cryo-EM maps of different 12-LOX oligomers resolved from "dimeric" SEC peak.

Figure 2. Oligomeric structures of 12-LOX. Models of 12-LOX as a (A) monomer, (B) dimer, (C) tetramer and (D) hexamer. Each subunit represented in a different colour and the α2-helix coloured in pink and arched helix in cyan. Graphical representation of each oligomeric state in bottom left and insets details the oligomeric interface. Interacting amino acids are shown as sticks. (D) Cys39 (in red) contributes a disulphide bridge to the interface of the hexamer. Fe atom is shown as a red sphere.

Figure 3. Conformational changes in the 12-LOX dimer. (A) Surface representation of 12-LOX in the "open" (left) and "closed" states (right) showing the active site cavity of the dimer 12-LOX subunits. In the open conformation this cavity is occupied by a small molecule. (B) An alignment of "open" and "closed" states shows a 23°C rotation and unwinding of the N-terminal residues of the α2-helix. The inset shows zoomed-in view of the active site entrance. The α2-helix is in cyan.

Figure 4. Acetyl-CoA binding site in the 12-LOX tetramer. (A) Model of a 12-LOX tetramer, with density in the catalytic site shown as cyan volume. Graphical representation is in right corner. (B) Acyl-CoA model and the density. Density is shown as wire mesh, the model is in sticks coloured by heteroatoms. (C) Model of acetyl-CoA within the catalytic site of 12-LOX. (D-E) 12-LOX residues that contact the acetyl-CoA (orange) shown as sticks. (D) Contacts of the adenosine tri-phosphate group. (E) Contacts of the acetyl tail. (F) Conformational change of residues in contact with the acetyl-CoA. Acetyl-CoA bound subunit in purple and unbound in pink. Fe atom is shown as a red sphere.

Figure 5. ML355 and arachidonic acid (AA) binding sites in the 12-LOX hexamer. (A) Model of the 12-LOX hexamer with density in the catalytic site shown as grey volume. Graphical representation in the bottom left. (B) Density for AA and ML355. Density is shown as wire mesh, the models is in sticks (orange for ML355 and pink for AA) coloured by heteroatoms. (C) Model of 12-LOX bound to ML355 and AA. (D-E) 12-LOX residues that contact (D) ML355 (orange) and (E) AA (pink) shown as sticks. Fe atom is shown as a red sphere.