Demystifying the Activation and Inhibition of 5-Lipoxygenase through X-ray Crystallography

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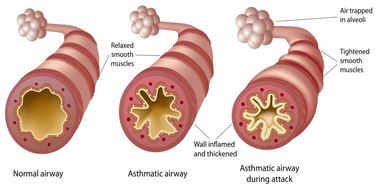
Abstract

Human 5-Lipoxygenase (5-LOX) is a peripheral membrane-binding protein that initiates the biosynthesis of pro-inflamatory leukotrienes (LTs). These LTs have been implicated in asthma, atherosclerosis, neurodegenative, and allergic disorders. The structure of stable-5-LOX has been solved at cryogenic temperatures. However the conformational changes of the enzyme required for catalysis are still unknown. To elucidate these changes, we aim to solve the structure 5-LOX at room temperature where the protein is more flexible. To achieve this we need to grow small, uniform crystals of 5-LOX that are amenable to jetting at LCLS. One way we are attempting to do this is by co-crystallizing 5-LOX in the presence of its inhibitors. Since 5-LOX is most stable in its closed conformation, we hope that the inhibitors act by encouraging 5-LOX into a closed conformation . Altogether, our efforts in understanding the conformational changes of the protein structure in different environments such as at the membrane or bound to a small molecule inhibitor will support structure-based drug design efforts for the discovery of novel anti-LT therapies

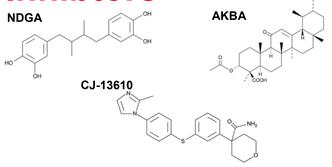
Background

Asthma



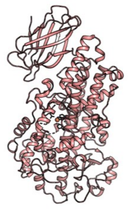
- Asthma is a disease that causes difficulty breathing due to inflammation and narrowing of the airway passage.
- When the inner lining of the airways are inflamed, they can swell and produce mucous.
- This makes the makes the airways more sensitive to certain triggers that induce asthma attacks.

Inhibtors

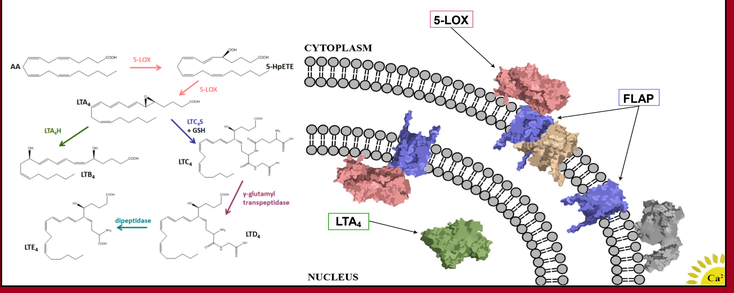


- These molecules inhibit the activity of 5-LOX, blocking leukotriene formation
- NDGA and AKBA natural product inhibitors. NDGA is a competitive inhibitor and AKBA is an allosteric inhibitor.
- CJ-13610 is a synthetic inhibitor.

5-Lipoxygenase



- 5-LOX is a peripheral membrane binding protein that initiates the biosynthesis of leukotrienes.
- 5-LOX first adds molecular oxygen to arachidonic acid (AA) to produce 5-hydroperoxyeicosatetraenoic acid, then can perform a second step to modify the intermediate to leukotriene A_4 (LTA_4).
- LTA_4 hydrolase often converts LTA_4 to leukotriene B4, a chemoattractant that activates inflammatory cells.
- Or LTC_4 synthase can conjugate LTA_4 to reduced glutathione, forming CysLTs implicated in the response to aeroallergens.



Results

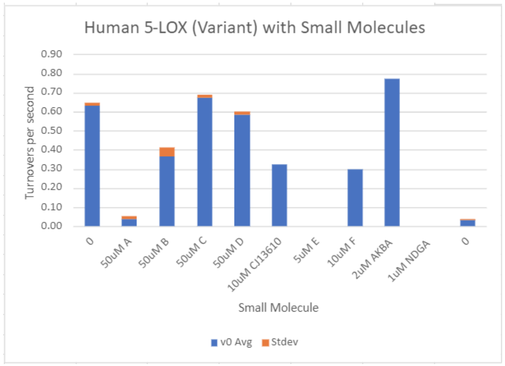


Figure 1.

Stopped-flow spectrometers are used to conduct enzyme kinetics assays. Turnovers per second represents the activity of the enzyme under when it is combined with various small molecules. Novel, significant 5-LOX inhibition was exhibited by molecules A, B, E, and F, making them promising candidates for co-crystallization attempts.

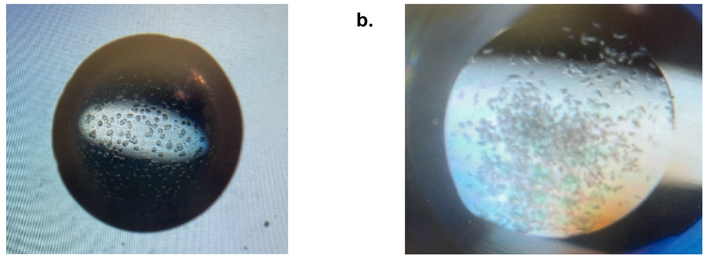


Figure 2. Vapor Diffusion Crystallization

a. Crystals grown from hanging drop vapor diffusion with a kinetically faster variant of 5-LOX and molecule B. The crystals are small and uniform with seemingly defined edges. **b**. Stable-5-LOX crystals grown using sitting-drop vapor diffusion. Although these crystals are densely packed, they appear to be uniform.

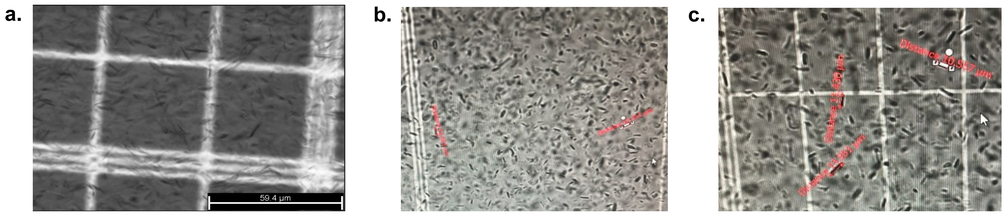


Figure 3. Stable-5-LOX Batch Crystallizations

a. Stable-5-LOX microcrystals measuring between 15 and 20 microns **b.** Microcrystals grown using a higher protein concentration than in Figure 3a. Overall these crystals were smaller with the largest ones being about 13 microns. **c.** 10-13 micron sized microcrystals of stable-5-LOX using the same protein concentration as in Figure 3a. but a higher concentration of Tacsimate.

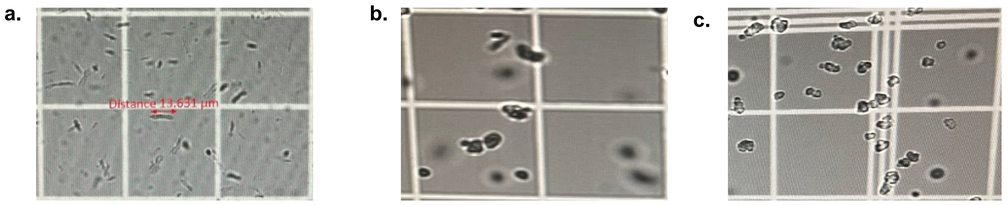
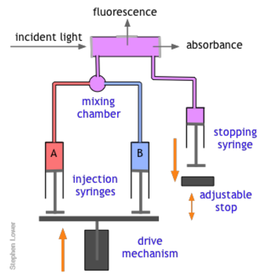


Figure 3. Batch Crystallizations using a Kinetically Faster variant of 5-LOX (Trip)

a. Microcrystals of Trip measuring average of 13 microns. **b.** Trip microcrystals grown at the same concentration of protein and Tacsimate as in Figure3a but in the presence of molecule B. The addition of the small molecule yielded crystals with a drastically different morphology. **c.** Microcrystals grown with molecule A in similar conditions to the crystals in Figure 3b. Although the crystals in Figures 3b and 3c were grown in the presence of different inhibitors they have similar morphologies.

Methods and Materials

Inhibitor Detection



- Purified protein stored at -80 degrees Celsius was thawed and used. Protein was diluted to 250 nM in buffer consisting of 20 mM TrisHCl (pH 7.5), 150 mM NaCl, 0.5 mM EDTA.
- A mix of the substrate, Arachidonic Acid (AA), with the aforementioned buffer was prepared. AA was diluted to 40uM.
- An Applied Photophysics SX20 stopped-flow spectrometer was used to measure absorbance at 238 nm. The intermediate of the 5-LOX reaction, 5-HPETE, is known to absorb at this wavelength.
- Each concentration of inhibitor was measured in triplicate, with a reaction time of 40 seconds. All measurements were taken at room-temperature.

Crystallization

- Crystals trays were set up with 4 -20% Tacsimate pH 6.0.
- The methods of crystallization used were hanging and sitting drop vapor diffusion and batch crystallization.
- The vapor diffusion trays were evaluated periodically over the course of 2 weeks.
- The batch crystallization samples were continuously oscillated and evaluated daily.

Sitting Drop Vapor Diffusion

Hanging Drop Vapor Diffusion

Next Steps

- Continue working to develop a protocol for developing small, uniform crystals of 5-LOX in and out of the presence of inhibitors.
- Conducting addition kinetics assay's to detect possible inhibitors of 5-LOX that encourage a stable conformation of the enzyme.
- Shooting small, uniform crystals with the X-ray free electron laser at LCLS to generate an adequate diffraction pattern of the enzyme that can be used to solve the structure at high resolution.

References

- Gilbert, Nathaniel C et al. "The structure of human 5-lipoxygenase." *Science (New York, N.Y.)* vol. 331,6014 (2011): 217-9. doi:10.1126/science.1197203
- Gilbert, Nathaniel C et al. "Structural and mechanistic insights into 5-lipoxygenase inhibition by natural products." Nature chemical biology vol. 16,7 (2020): 783-790. doi:10.1038/s41589-020-0544-7
- Pace, Simona et al. "Anti-inflammatory celastrol promotes a switch from leukotriene biosynthesis to formation of specialized pro-resolving lipid mediators." Pharmacological research vol. 167 (2021): 105556. doi:10.1016/j.phrs.2021.105556
- Gilbert, Nathaniel C et al. "Untangling the web of 5-lipoxygenase-derived products from a molecular and structural perspective: The battle between pro- and anti-inflammatory lipid mediators." Biochemical pharmacology vol. 193 (2021): 114759. doi:10.1016/j.bcp.2021.114759
- Gallegos, Eden M et al. "Helical remodeling augments 5-lipoxygenase activity in the synthesis of proinflammatory mediators." The Journal of biological chemistry vol. 298,9 (2022): 102282. doi:10.1016/j.jbc.2022.102282