Abstract

Human 5-Lipoxygenase (5-LOX) is an enzyme that responds to the call for inflammation by initiating the synthesis of leukotrienes involved in asthmatic and allergic reactions. Acting at the nuclear membrane in leukocytes, 5-LOX oxygenates its lipid substrate arachidonic acid to produce leukotriene $A_4$. The structure of 5-LOX was solved in a closed conformation with the typically-elongated helix-$n_2$ shortened and two bulky hydrophobic residues from the helix coring the active site. In most other animal lipoxigenase structures, helix-$n_2$ rims a solvent-accessible active site. We asked whether this “open” conformation might be available to 5-LOX as well and sought to investigate the molecular determinants important for the transition from a closed to an open structure. We designed site-directed mutants of helix-$n_2$ residues to favor either the open or closed conformations. We evaluated these mutants in limited proteolysis and thermal denaturation assays. We found that mutations designed to favor a closed state were more resistant to limited proteolysis. Those mutations introduced to favor the open state increased protease susceptibility. Finally, we used stopped-flow kinetics to observe changes in enzymatic activity. By excluding activators of 5-LOX in kinetic assays, we observed differences in pre-steady-state kinetics between open and closed mutants. We also saw changes in the $k_\text{cat}$ due to mutations favoring an open conformation. By enhancing the presence of a closed or open conformation by mutagenesis, our results suggest that the commonly observed elongated helix-$n_2$ may be accessible to 5-LOX. Determination of a structure of 5-LOX in an open conformation will enable robust models for substrate acquisition and inhibitor binding applicable to structure-based drug design.

Methods

- **Site-Directed Mutagenesis** was used to create the following sets of mutations in Stable 5-LOX: G174A, D176A and F193D, F197D.
- **Limited Proteolysis** of Stable 5-LOX mutants by pepsin was used to evaluate the relative flexibility of the polypeptide backbones.
- **Differential Scanning Fluorimetry** was used to determine the thermodynamic effects of the mutations. Melting temperatures were obtained by heating the samples from 25°C to 85°C, increasing temperature every 30 seconds.
- **Stopped-Flow Kinetics** was used to monitor 5-HETE production by measuring $A_{235}$ versus time (s). Plots of velocity vs. substrate concentration were clearly sigmoidal, and the Hill equation was used to fit the production of 5-HETE as a function of arachidonic acid concentration.

Results

- **Proteolysis assays** showed that Stable 5-LOX was fairly resistant to proteolysis by pepsin in the reaction conditions. The Closed Model showed a similar resistance to proteolysis, while the Open Model showed the least resistance.
- **Thermal shift assays** showed that Stable 5-LOX malted at 57.8 ± 0.3°C. The Closed Model ($T_m = 55.6 ± 0.0°C$) and the Open Model ($T_m = 55.6 ± 0.3°C$) showed slightly lowered $T_m$ values compared to Stable 5-LOX.
- **Kinetic assays** showed that Stable 5-LOX produced a $k_\text{cat}$ value of 0.43 ± 0.02 s$^{-1}$ and $K_m$ of 19.0 ± 0.8 mM. By comparison, the Closed Model produced a $k_\text{cat}$ value of 0.46 ± 0.01 s$^{-1}$ and $K_m$ of 15.3 ± 0.8 mM. The $k_\text{cat}$ of the Open Model was increased to 1.67 ± 0.01 s$^{-1}$ and the $K_m$ decreased to 5.5 ± 0.2 mM.

Conclusions

- H$\text{2}_2$ of human 5-LOX plays a critical role in enzymatic activity. The sigmoidal fit of initial velocity as a function of AA concentration supports a model with multiple conformational states. When the active site of 5-LOX is closed to the environment, it must overcome conformational barriers in order to reach steady-state catalysis.
- The data support increased access to the catalytic site in our Open Model, which is consistent with our hypothesis for an open 5-LOX. A conformational change event may occur as part of the regulation of 5-LOX catalytic activity, membrane association, or both.