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The Structure of Human 5-Lipoxygenase

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The synthesis of both proinflammatory leukotrienes and anti-inflammatory lipoxins requires the enzyme 5-lipoxygenase (5-LOX). 5-LOX activity is short-lived, apparently in part because of an intrinsic instability of the enzyme. We identified a 5-LOX–specific destabilizing sequence that is involved in orienting the carboxyl terminus, which binds the catalytic iron. Here, we report the crystal structure at 2.4 angstrom resolution of human 5-LOX stabilized by replacement of this sequence.

Leukotrienes and lipoxins are potent mediators of the inflammatory response derived from arachidonic acid (AA). When leukocytes are activated, AA is released from the nuclear membrane by the action of cytosolic phospholipase A_2 and binds 5-lipoxygenase–activating protein (FLAP). The increased Ca^{2+} concentration of the activated cells simultaneously promotes translocation of 5-LOX to the nuclear membrane, where it acquires its substrate from FLAP (*1*, *2*). AA is converted to leukotriene A_4 in a two-step reaction that produces the 5*S*- isomer of hydroperoxyeicosatetraenoic acid (5*S*-HPETE) as an intermediate (*3*, *4*).

Autoinactivation of 5-LOX activity has been described, and this loss of activity is perhaps important in limiting the synthesis of its pro- and anti-inflammatory products (*5*). Previous reports indicate that non–turnover-based inactivation is a consequence of an O_2 sensitivity linked to the oxidation state of the catalytic iron (*6*). However, not all LOXs display this hypersensitivity to O_2. For example, 8*R*-LOX activity is stable despite a solvent-exposed iron coordination sphere equivalent to that in 5-LOX (*7*). In similar conditions, 50% of 5-LOX activity is lost in 10 hours (*8*). We reasoned that 5-LOX–specific destabilizing features may confer susceptibility to non–turnover-based inactivation. Regulatory mechanisms that facilitate transient activation include targeted degradation, phosphorylation, and allosteric control of enzyme activities. Autoinactivation as a consequence of intrinsic protein instability may play a similar role. For example, the instability of the tumor suppressor protein p53, relative to its orthologs such as p73, has been proposed to have a functional role (*9*).

On the basis of the crystal structures of two AA-metabolizing lipoxygenases [an 8*R*-LOX from *Plexaura homomalla* (*7*, *10*) and a 15-LOX from rabbit reticulocyte (*11*, *12*)], each with ~40% sequence identity to 5-LOX, we identified a 5-LOX–specific lysine-rich region near the C terminus of the enzyme that might confer instability (*13*). In the 8*R*- and 15-LOX structures, a turn centered on amino acid 655 (5-LOX numbering) leads from the C-terminal helix to the C-terminal segment and allows the terminal carboxylate to penetrate the LOX body and bind the catalytic iron (Fig. 1, A and B). In most LOXs, amino acid 655 is a highly conserved Leu, with its side chain pointing toward an invariant Arg (Arg^{651}). A striking 5-LOX–specific feature is Lys in place of Leu at this position as part of a di- or tri-Lys peptide (fig. S1). Although numerous salt links anchor the C-terminal helix to the

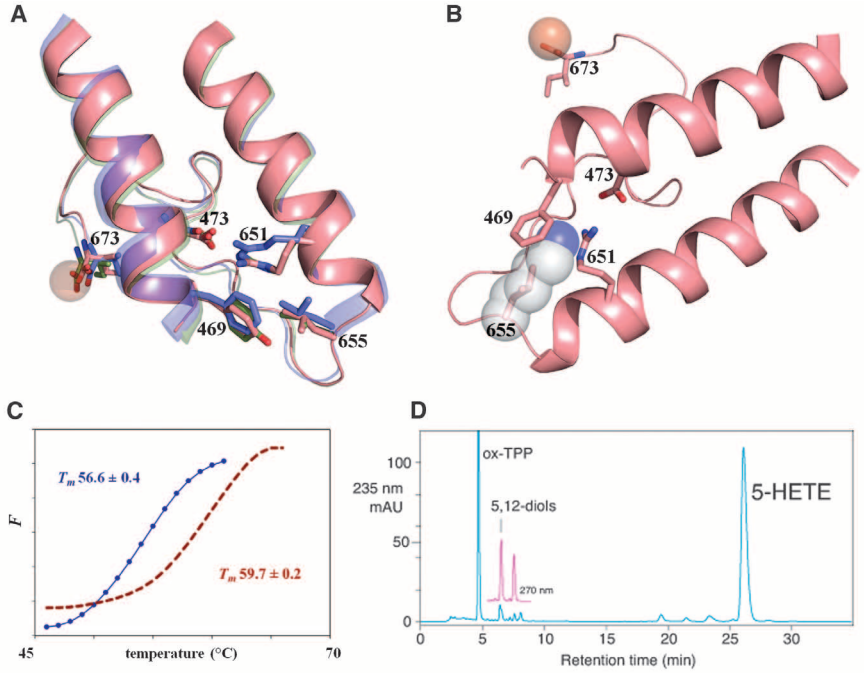

Fig. 1. Stabilization of human 5-LOX. (**A**) Superposition of the C-terminal regions of the structures of 15-, 8*R*-, and Stable-5-LOX. The C-terminal segment that leads to the catalytic Fe emanates from the helix that terminates at amino acid 655 (5-LOX numbering; Stable-5-LOX, pink; 8*R*-LOX green; 15-LOX, blue). Highly conserved amino acids (Leu and Phe/Tyr) and an invariant salt link (Asp-Arg) are depicted in stick rendering. (**B**) Detail of the turn at the end of the terminal helix. The 5-LOX–specific Lys (replaced in Stable-5-LOX with Leu) is modeled at position 655 as its most common rotamer (transparent sphere rendering). As positioned, it would interfere with the invariant salt-link and cation-π interactions. All figures were generated with Pymol (*31*). (**C**) Thermal denaturation of Stable-5-LOX (red) and the parent enzyme Sol-5-LOX (blue). Fluorescence (*F*) is monitored as a function of temperature. T_m (with SD) 56.6°C (±0.4°) and 59.7°C (±0.2°) for Sol-5-LOX and Stable-5-LOX, respectively. (**D**) High-performance liquid chromatography chromatogram. Product analysis of Stable-5-LOX reveals both 5-HETE (5-HPETE reduced by the addition of triphenylphosphine, TPP) and leukotriene A_4 hydrolysis products (5,12-diols).

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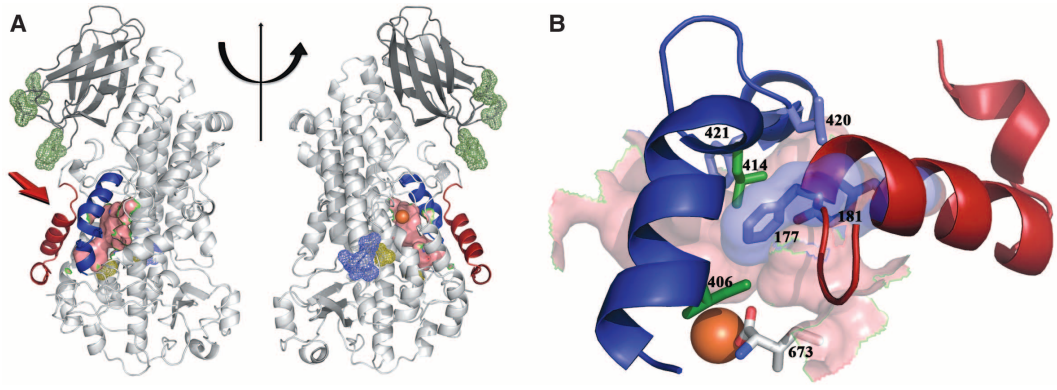

Fig. 2. The structure of Stable-5-LOX. (**A**) A cartoon rendering of 5-LOX. The two views differ by a 180° rotation about the vertical line. The N-terminal C2-like domain is in dark gray, and the catalytic domain in light gray. The distinctive arched helix is in blue, and helix α2 in red. The internal cavity, generated with CastP (*32*), is in pink, and the Fe is an orange sphere. The positions of the mutated amino acids are indicated in mesh rendering: green, putative membrane insertion residues; yellow, proximal cysteines; and blue, the KKK→ENL substitution. (**B**) Detail of the relation of the arched helix and helix α2 to the active site as viewed from the perspective indicated by the red arrow in (A). Shown in stick rendering are amino acids 406, 414, 420, and 421 of the arched helix and Phe^{177} and Tyr^{181} from helix α2 (with transparent surface rendering). The latter two bulky amino acids obstruct access to the cavity. The proximity of the C-terminal Ile^{673} to the corked portal is apparent.

body of the protein in the structures of the two homologs noted above, none of these salt links is conserved in the 5-LOX sequence. As a consequence of the lysine-rich sequence and the absence of helix-anchoring salt links, the orientation of the terminal helix is less favorable and the C-terminal ligand to the active site Fe is likely to be tenuously restrained. Conservative mutations in the C-terminal helix have been noted to reduce enzyme expression levels and activity (*14*). These observations led us to replace 5-LOX K^{653}KK^{655} with the corresponding sequence from 8*R*-LOX (ENL) in an effort to stabilize the enzyme for crystallographic studies (*15*).

The mutant human enzyme (herein referred to as Stable-5-LOX) was prepared in the context of a soluble 5-LOX (Sol-5-LOX) that lacks putative membrane-insertion amino acids (Δ40to 44GS, W13E, F14H, W75G, and L76S), as well as a pair of cysteines (C240A and C561A) predicted to be proximal in the 5-LOX structure. Substitution of the membrane insertion loops was based on a similar approach with the *P. homomalla* enzyme, which shares both these amino acids and Ca^{2+}-binding residues with 5-LOX in the N-terminal membrane-binding domain (*16*). The replacement of KKK with ENL in this context led to a ~3°C increase in the melting temperature (T_m) of the enzyme (Fig. 1C). Moreover, Stable-5-LOX has a longer half-life at 37°C (~16 hours versus ~7 hours) (fig. S2). Furthermore, Stable-5-LOX produces both the intermediate 5*S*-HPETE and the product leukotriene A_4 (Fig. 1D), as does its progenitor protein Sol-5-LOX (fig. S3). In addition, we measured an apparent dissociation constant (K_m) for AA of ~11 μM (fig. S4), equivalent to that of the wild-type enzyme (*17*). These observations are consistent with the proposal that the KKK sequence is destabilizing and that its substitution does not affect catalytic fidelity. The structure of Stable-5-LOX was determined to 2.4 Å resolution (Fig. 2A, fig. S5, and table S1).

The canonical LOX framework contains two distinct domains: the N-terminal "C2-like" domain (~120 amino acids), which in 5-LOX confers Ca^{2+}-dependent membrane binding (*18*–*21*), and the larger catalytic domain. The latter is primarily α-helical and harbors the nonheme catalytic iron. The iron is coordinated by three conserved histidines (histidines 367, 372, and 550), as well as the main-chain carboxylate of the C terminus (Ile^{673}). Another structurally distinct conserved feature in this domain, previously described in detail by Minor *et al* (*22*) for soybean LOX L-1, is an arched helix that shields access to the catalytic iron. At the vertex of the Stable-5-LOX arched helix is Leu^{414} (Fig. 2B), an invariant amino acid that in other lipoxygenases has been proposed to control access of O_2 to the substrate (*23*, *24*) or to position the substrate pentadiene for attack (*7*). Additional amino acids from the arched helix that help define the catalytic site are Leu^{420} and Phe^{421}. The crystal structure of Stable-5-LOX reveals a striking variation on the classic lipoxygenase fold in helix α2, which defines one edge of the active site. In the structures of 8*R*- and 15-LOX, helix α2 is six to seven turns, whereas in Stable-5-LOX, it is a short three-turn helix flanked by extended loops. The shortened helix is positioned at ~45° to its counterparts in the 8*R*- and 15*S*- enzymes (Fig. 3, A and B). The unique orientation of helix α2 in Stable-5-LOX greatly limits access to the catalytic iron and yields a distinctive active site cavity. Specifically, the side chains of Phe^{177} and Tyr^{181} are positioned inward and close off an access channel to the catalytic iron that is observed in both the 8*R*- and 15-LOX structures (Figs. 2B and 4A). The remainder of the secondary structural elements and their relative orientations are maintained (Fig. 3B). In addition, the structural context of the Lys-rich peptide also appears conserved as the C-terminal helices superimpose (Fig. 1A). However, it is apparent that a Lys at position 655 would interfere with invariant salt link and cation-π interactions (Fig. 1B).

In Stable-5-LOX, the active site is an elongated cavity, with no clear access to bulk solvent,

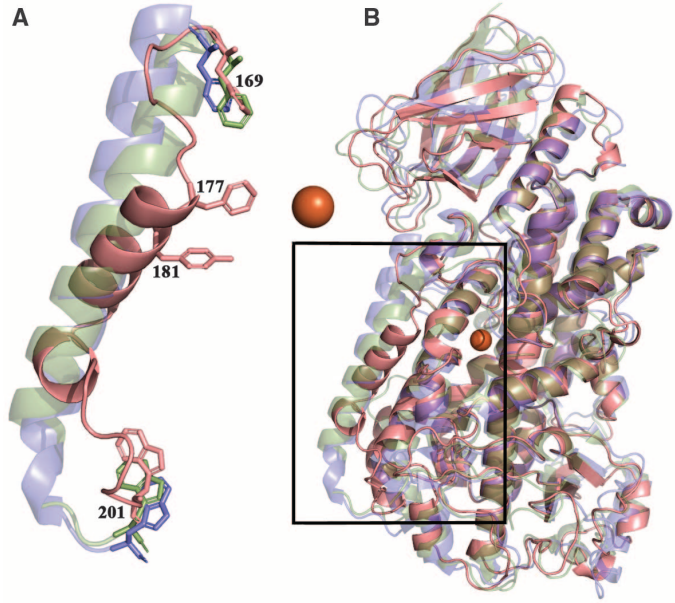

Fig. 3. The positioning of helix α2 is unique in 5-LOX. (**A**) A 5-LOX cartoon is rendered in pink, 15-LOX in blue and 8*R*-LOX in green. Conserved aromatic amino acids (Phe^{169} and Trp^{201}) that flank the region are in stick rendering. Phe^{177} and Tyr^{181}, which make up the cork that helps define the active site, are in stick. The catalytic iron is an orange sphere. (**B**) A full overlay of the three structures in which it is apparent that, with the exception of α2, the secondary structural elements in the enzymes are conserved. The box indicates the region amplified in (A).

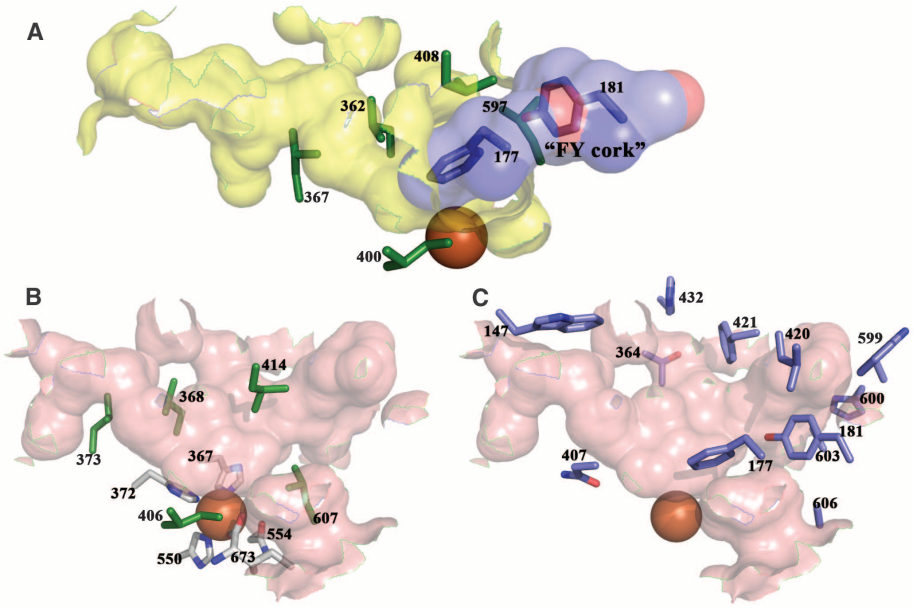

Fig. 4. The 5-LOX active site. Internal cavities calculated with CastP (*32*). (**A**) The active site cavity of 15-LOX (2P0M) is in yellow. Invariant Leu and Ile side chains are in green stick rendering. The 5-LOX FY cork is superposed on the 15-LOX cavity and plugs the entrance. (**B**) The equivalent orientation of the active site cavity of Stable-5-LOX in pink; invariant Leu and Ile side chains in green sticks. Note the similarity of the positions of these amino acids to their counterparts in 15-LOX (A). Iron coordination sphere amino acids (C, white) are in stick rendering, and the iron an orange sphere. (**C**) 5-LOX amino acids that contribute to the active site cavity. Entry into this cavity requires a conformational change.

lined with both invariant and 5-LOX–specific amino acids. Leucines 368, 373, 414, and 607 and Ile^{406} are conserved in all AA-metabolizing lipoxygenases (*7*) and form a structurally similar constellation of branched hydrophobic side chains that envelops the region where the pentadiene must be positioned for catalysis (Fig. 4, A and B). Tyr^{181}, Ala^{603}, Ala^{606}, His^{600}, and Thr^{364} are specific to 5-LOX sequences, and the small side chains of Ala^{603} and Ala^{606} appear to be required for the conformation of Tyr^{181}, which, along with Phe^{177}, "corks" the cavity at one end. Tyr^{181} is in van der Waals contact with Ala^{603}, and the small side chains of both 603 and 606 allow both bulky aromatics (Phe^{177} and Tyr^{181}) to point into the cavity where they can be shielded from solvent (Fig. 4C). An additional 5-LOX–specific amino acid, Trp^{599}, appears to buttress the FY cork from one side. Amino acids Asn^{407} and His^{432} also help define the active site.

The closed cavity (volume, 663 Å^3) raises the question of how substrate gains access to the catalytic iron. Two possibilities can be envisioned: (i) removal of the FY cork at one end of the cavity and/or movement of Trp^{599} that secures it, or (ii) a rotamer shift of Trp^{147} at the opposite end. A rotamer shift in Trp^{147} would require only rotation of the side chain, whereas the former may require both side-chain and main-chain movements in two amino acids. This observation suggests that AA may enter 5-LOX from the opposite direction as it does in the 15*S*- or 8*R*- enzymes, which lack the FY cork. This site of entry fits well with what is known about the catalytic mechanism: H abstraction and peroxidation occur on opposite sides of the pentadiene (*25*). The *S*-stereochemistry of the 5-LOX product is consistent with an "inverse" orientation of AA relative to that for the 15*S*- and 8*R*- enzymes (*26*, *27*). An opening at the Trp^{147} end would allow the AA to enter methyl end first and position the substrate for the production of the *S* isomer of 5-HPETE. While the above model is attractive, the structure does not rule out the alternative: that the AA enters the same portal it does in 8*R*- and 15*S*-enzymes. Carboxylate-first entry in the latter mode achieves the same binding orientation and reaction specificity.

The 2.4 Å structure of Stable-5-LOX reveals an active site which, despite a conserved constellation of five invariant amino acids, is clearly distinct from the active sites of the AA metabolizing lipoxygenases for which structures are available. The structure provides a context for the development of 5-LOX–specific inhibitors and, together with the crystal structures of FLAP (*28*) and the downstream enzyme leukotriene C_4 synthase (*29*, *30*), a molecular model for early events in leukotriene biosynthesis.

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Supporting Online Material

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