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RESEARCH ARTICLES

Structure Conservation in Lipoxygenases: Structural Analysis of Soybean Lipoxygenase-1 and Modeling of Human Lipoxygenases

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ABSTRACT **Lipoxygenases are a class of non-heme iron dioxygenases which catalyze the hydroperoxidation of fatty acids for the biosynthesis of leukotrienes and lipoxins. The structure of the 839-residue soybean lipoxygenase-1 was used as a template to model human 5-, 12-, and 15-lipoxygenases. A distance-based algorithm for placing side chains in a low homology environment (only the four iron ligands were fixed during side chain placement) was devised. Twenty-six of the 56 conserved lipoxygenase residues were grouped in four distinct regions of the enzyme. These regions were analyzed to discern whether the side chain interactions could be duplicated in the models or whether alternate conformers should be considered. The effects of site directed mutagenesis variants were rationalized using the models of the human lipoxygenases. In particular, variants which shifted positional specificity between 12- and 15-lipoxygenase activity were analyzed. Analysis of active site residues produced a model which accounts for observed lipoxygenase positional specificity and stereospecificity.**

Key words: homology modeling, 5-lipoxygenases, 12-lipoxygenases, 15-lipoxygenases, side chain placement, arachidonic acid metabolism, leukotrienes, lipoxins

INTRODUCTION

Lipoxygenases are a class of non-heme iron dioxygenases that catalyze the hydroperoxidation of polyunsaturated fatty acids in plants, animals, and microorganisms. In addition to the natural substrates—linoleic acid and arachidonic acid—a wide variety of polyunsaturated fatty acids can act as substrates of these enzymes. Plant lipoxygenases catalyze the hydroperoxidation of linoleic acid (18:2) as the first step in the biosynthesis of the growth regulatory substance jasmonic acid and of factors involved in wound healing—traumatin and traumatic acid.[1–4] Mammalian lipoxygenases catalyze the hydroperoxidation of arachidonic acid (20:4), initiating the synthesis of two families of potent physiological effectors: leukotrienes and lipoxins.[5–8]

Pairwise sequence identity between plant and mammalian lipoxygenases is 21–27%, while identity among pairs of plant sequences is 43–86%, and identity among pairs of mammalian sequences is 39–93%. The sequence identity between plant and mammalian lipoxygenases is highest in the portions of the catalytic domain near the iron atom. The 61 residues from W479 to N539 in soybean lipoxygenase-1 include 16 residues that are conserved in all lipoxygenases. The amino-terminal 200 residues from the plant lipoxygenases have little homology to the mammalian sequences (≤ 15% pairwise sequence identity). Since the mammalian enzymes (662–674 residues in length, including the amino-terminal methionine) are 165 to 261 residues shorter than the plant enzymes (839–923 residues in length), it is likely that the amino-terminal domain in plant lipoxygenases is not present in the mammalian enzymes.

The structure of soybean lipoxygenase-1 (SB1) has been determined to 2.6Å using X-ray crystallography.[9] In SB1, the amino-terminal 146 amino acids form an eight-stranded antiparallel β-barrel, while the carboxy-terminal 693 residues form the catalytic domain, consisting of 23 α-helices and two antiparellel β-sheets. The catalytic site iron atom is liganded with octahedral coordination by one oxygen of the carboxy-terminus and three histidine epsilon nitrogens, leaving two iron coordination positions that appear to be unoccupied in the present structure.

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The three iron-liganded histidines are located on two adjacent π-helices, each situated in the middle of an α-helix. In the area around the iron atom, there are two large cavities: one cavity is thought to be involved in substrate binding and the second—a funnel-shaped channel leading to the surface of the catalytic domain—is thought to be a means of entry for the dioxygen substrate. One of the unoccupied iron coordination positions (opposite iron ligand H690) is accessible to the putative substrate binding cavity and the other (opposite iron ligand H504) is located near the terminus of the funnel-shaped channel.

Lipoxygenases require fatty acids containing a 1,4-diene with at least one of the olefins in the Z geometry.[10] The lipoxygenase reaction proceeds with hydrogen abstraction from the methylene carbon (carbon 3) of substrates containing a 1,4-diene followed by addition of dioxygen to one of the olefinic carbons (either carbon 1 or 5). The major product of reactions with fatty acids containing a 1Z,4Z-diene is the (S)-hydroperoxy fatty acid, and abstraction of one of the methylene pentadienyl hydrogens occurs antarafacial to addition of dioxygen. The nomenclature of mammalian lipoxygenases is based on positional specificity and includes the carbon atom (5th, 8th, 12th, or 15th carbon) to which dioxygen binds in products of (5Z,8Z,11Z,14Z)-eicosatetraenoic acid (arachidonic acid).

To further our understanding of structure conservation in lipoxygenases—especially the human enzymes—we analyzed the structure of soybean lipoxygenase-1 and modeled human 5-, 12-, and 15-lipoxygenases using the soybean enzyme as the template structure. (Plant lipoxygenases were not modeled due to high sequence identity with soybean lipoxygenase-1 and almost certain structural similarity.) We used these models to examine the steric constraints responsible for positional specificity in the human lipoxygenases, as well as to investigate the roles of residues that are highly conserved in all lipoxygenase sequences. In this article, lipoxygenase sequence numbers refer to the residue number in soybean lipoxygenase-1 as aligned in the results and discussion section (Fig. 1), unless noted otherwise. The abbreviations *hl5lo, hp12lo,* and *hr15lo* refer to the three human lipoxygenases modeled; human leukocyte 5-lipoxygenase, human platelet 12-lipoxygenase, and human reticulocyte 15-lipoxygenase; respectively.

MATERIALS AND METHODS

Equipment and Model Building Procedure

The three-dimensional structure of soybean lipoxygenase-1 was used as a template to model human 5-, 12-, and 15-lipoxygenases. A Convex 220 was used for computationally intensive phases of model building and results were analyzed on a Silicon Graphics Indigo. The following four-step scheme was used to produce the models:

I. Define and regularize main chain atoms
   1. Align sequences of the template protein and the model protein
   2. Define atom positions in the model by copying coordinates from the template protein (Quanta 3.3, Molecular Simulations Inc., Cambridge, MA)
   3. Shift additions and deletions based on structural considerations (Quanta 3.3)

II. Define and regularize side chain atoms
   1. Build undefined side chain atoms
   2. Regularize side chains (250 steps of conjugate gradient minimization, excluding VDW and ELEC energy terms, in XPLOR[11] with iron ligands and main chain atoms frozen)

III. Minimize structure to relax main chain
   1. Place side chains (see Side Chain Placement)
   2. Minimize side chains (250 steps of conjugate gradient minimization in XPLOR with iron ligands and main chain atoms frozen)
   3. Minimize whole structure (250 steps of conjugate gradient minimization in XPLOR with iron ligands and main chain termini frozen)

IV. Minimize structure
   1. Place side chains
   2. Minimize side chains (250 steps of conjugate gradient minimization in XPLOR with iron ligands and main chain atoms frozen)
   3. Minimize complete structure (250 steps of conjugate gradient minimization in XPLOR with iron ligands and main chain termini frozen)

Side Chain Placement

The sequence identity between plant and human lipoxygenases is not high enough to assume that conserved residues have identical side chain conformations in SB1 and the human enzymes. Therefore, the conformations of all side chains were considered unknown, regardless of whether or not the residues are conserved, with the exception of the three histidine iron ligands, that were constrained to the conformations found in the SB1 structure. Monte Carlo approaches to this problem are hindered by the large size of lipoxygenases, so a combinatorial approach was devised, using early pruning of side chain conformers based on the rejection of hard sphere clashes between atoms. For the search, side chain dihedral angles were initially rotated to the *trans* configuration (180°). A complete set of rotamers was generated by rotating side chain dihedrals of all residues in discrete intervals, avoiding *cis* conformations. In-

tervals of 30° were used for residues with one or two side chain dihedral angles, and larger intervals of 60° or 120° were used for χ-2 through χ-5 of residues with three or more side chain dihedral angles. The side chain placement program (program available upon request) uses three pruning algorithms to reduce the number of acceptable rotamers:

Algorithm I—Prune rotamers on the basis of clashes with the ablated* structure
- Generate rotamers by rotating side chain dihedrals in discrete intervals
- Discard rotamers which clash with the ablated structure

Algorithm II—Prune rotamers on the basis of the sum of pairwise clashes with all rotamers of all other side chains in the dependency set†
- Generate new dependency sets for all residues which have side chains
- Score each rotamer by summing pairwise clashes with all rotamers of all other residues in the dependency set
- Discard the worst rotamer (worst cumulative clash score) of each residue

Algorithm III—Prune combinations of dependency set rotamers on the basis of the sum of the clashes within the combination of rotamers
- Calculate side chain clashes for each combination of rotamers in a dependency set
- Score each combination by summing the side chain clashes (precalculated main chain clashes, if any, are added)
- Discard combinations that have a score greater than the average of the highest and the lowest score
- Determine which rotamers are always discarded
- Discard rotamers from other dependency sets which rely on discarded rotamers

In the first algorithm, cases in which too few or too many rotamers are pruned are avoided by modulating the cutoff distance between atoms (initially 3.0Å). Reduction of the cutoff distance is also used to avoid rejecting possible hydrogen bonding or salt bridging rotamers. The second and third algorithms involve pruning rotamers or combinations of rotamers based on the summed scores of their individual clashes. The score for an individual clash is an empirical function which penalizes for interatomic distances below the cutoff distance, D_{CO}. [Score = $(D_{CO}^2 - D^2)^6$ for $D < D_{CO}$; score = 0 for $D \geq D_{CO}$ where the cutoff distance, D_{CO}, is 2.7Å for pairs of possible hydrogen bonding atoms and otherwise 3.0Å].

These algorithms were used in the following order to produce rough models.

Algorithm used	Rotamers remaining
	144 (121 with no *cis* dihedrals)
I	10–50
II × 40	10 (one rotamer discarded with each iteration)
4 × (III, 2 × II)	8–10, 6–10, 4–10, 2–10
III, II	1–10

The right-hand column is an example of how many rotamers remain after each step for a residue with two side chain dihedral angles such as histidine. If more than one rotamer remains after the last step, the rotamer with the best score is chosen.

RESULTS AND DISCUSSION

Structure-Based Sequence Alignment

Several alignments of plant and animal lipoxygenase sequences have been published[12–14] and our structure-based alignment agrees at the majority of residues, but there are four areas which differ significantly:

Gap 1

There is a large gap in mammalian lipoxygenases in the 32-residue loop region between Strand 12 and Helix 7 in soybean lipoxygenase-1 (363–394). Much of this region—about 40 residues including Helix 7—is on the surface of the protein where the conformation of the residues is less influenced by neighboring regions of the protein. The main chain folds back on itself twice in this region, providing good sites for gaps to be placed. The distance between positions 385 and 395 is 6.6Å, allowing a gap to be introduced here; and the distance between positions 371 and 377 is 6.5Å, allowing a smaller gap to be introduced here. Both sites were used to place the 13-residue gap in *hp*12*lo* and *hr*15*lo*, whereas only the former site was used in *hl*5*lo* due to the smaller gap size (9 residues). A small shift in the position of Helix 7 helped to close the gaps.

Gap 2

The region between Helix 8 and Helix 9 (424–473) contains three β-strands connected by small loops. The loop between Strand 15 and Strand 16 is the largest and allows up to six residues to be cut without affecting the β-strands (the distance between positions 454 and 461 is 5.4Å). A second gap of three residues (472–474) shortens Helix 9, but

*The **ABLATED** structure has all movable side chain atoms removed so that all residues other than Glycine and Proline are truncated to Alanine.

†A **DEPENDENCY SET** is the set of all residues that can interact directly with a particular residue.

optimizes the alignment of conserved residues—especially **P469** (conserved residues in **bold**). It is possible that the residues comprising Strand 16 (between the two gaps) are not part of the β-sheet in the mammalian lipoxygenases since prolines—especially adjacent prolines—tend to disrupt the hydrogen bonding pattern of β-sheet structures.

Gap 3

The region between Helix 18 and Helix 21 (702–740) contains a seven-residue gap (in *hr15lo* the gap is eight residues). There is a long loop after Helix 18 followed by Helix 19 and Helix 20—both short, eight-residue α-helices on the surface of the protein. It is difficult to cut residues from the loop for three reasons: most of the loop is buried in the protein core, the loop has a linear conformation (residues separated by seven positions are >15Å apart), and the sequence homology to SB1 is high. Removal of the most peripheral helix, Helix 19, allows Helix 20 to remain in place, whereas the removal of Helix 20 requires drastic relocation of Helix 19. Since the position of Helix 19 is not conserved in either case, the gap was finally placed in the models of the human lipoxygenases at Helix 19. Due to uncertainty in this region, several residues on either side of Helix 19 (residues 717–729) were not built in the final models, creating a 13.7Å gap in the main chain.

Gap 4

The region from Helix 22 to the carboxy-terminus (801–839) is 18 residues shorter in the human lipoxygenases. The main chain folds back on itself twice in this region, providing sites for two gaps to be placed: the distance between positions 800 and 816 is 7.6Å, allowing a large gap to be introduced here; and the distance between positions 823 and 832 is 4.4Å, allowing an eight-residue gap to be introduced here. If residues 801 to 815 are removed from the first site, conserved residues **N800, P816,** and **Y817** shift positions to reconnect the main chain. In the models, only 10 residues were removed from this site, allowing the conserved residues to remain unperturbed.

Amino-Terminal Domain

The amino-terminal residues of the human lipoxygenases (138 residues in *hl5lo* and 140 residues in *hp12lo* and *hr15lo*) are between 11 and 15.5% identical to the first 254 residues of SB1. The poor homology and large size difference suggest that major structural differences exist between the amino-termini of plant and mammalian lipoxygenases. One way to account for this large size difference is to assume that the β-barrel domain of SB1 (145 residues in size) is not present in the mammalian enzymes. Without the β-barrel, lipoxygenases would begin with a short loop (146–155) and Helix 1 (156–170) followed by a long 84-residue loop. The residues which appear to be important in organizing the 84-residue loop are rarely conserved between plant and mammalian lipoxygenases. This leads one to guess that these residues either form a small amino-terminal domain while foreshortening the catalytic domain, or that these residues fold onto the catalytic domain in some novel way. Regardless of which is true, this region could not be modeled through homology and was left out of the human lipoxygenase models.

The unmodeled amino-terminal region could be of importance to understanding substrate-binding in the human lipoxygenases. The unmodeled region forms part of the putative substrate binding cavity (Cα-Fe distance is 11.6Å for A254 in SB1), and the absence of these residues connects one end of the cavity to the protein surface in the models. Even the residues at the very amino-terminus of the mammalian lipoxygenases could be critical in some way since the truncation of only nine residues from human platelet 12-lipoxygenase produces an inactive enzyme.[15] Addition of a polyhistidyl tail, however, does not significantly affect activity.[15]

The structure-based sequence alignment of SB1, *hl5lo, hp12lo,* and *hr15lo* from the beginning of Helix 2 (position 255) to the carboxy-terminus (position 839) is shown in Figure 1. In this region, the sequence identity between SB1 and the three human lipoxygenases is 26.0, 26.1, and 25.8% for *hl5lo, hp12lo,* and *hr15lo,* respectively. There are 87 residues common to all four enzymes in this modeled region (positions 255–839), representing 14.9% of the SB1 sequence in this region and 16.4–16.6% of the human lipoxygenase sequences in this region. *Hl5lo* is 42.5% identical to *hp12lo* and 40.8% identical to *hr15lo* whereas *hp12lo* and *hr15lo* share 66.6% identical residues.

Test of Side Chain Placement Program

The side chain placement program was run on the catalytic domain (694 residues in length) of the soybean lipoxygenase-1 crystal structure as a test of the program, and the resulting SB1 model was compared with the crystal structure. Side chain dihedral angles were catagorized as one of three conformers (*gauche+*, *gauche−*, or *trans*) in both the model and the crystal structure. Over 74% of the χ-1 dihedrals in the model were in the correct conformation (80% of core residues and 70% of surface residues). When all side chain dihedrals were considered (χ-1 through χ-5), 67% were in the correct conformation (73% of core residues and 64% of surface residues). The χ-1 dihedrals of bulky aromatic residues (F, H, Y, and W) were more often correct than those of small residues (C, S, T, and V)—87% compared with 59%. The three histidine iron ligands were frozen during side chain placement and are not included in the statistics.

```
SB1       255 LEIGTKSLSQIVQPAFESAFDLKSTPIEFHSFQDVHDLYEGGIKLPRDVISTIIPLPVIK 314
hl5lo     142 YRWMEWNPGFPLSIDAKCHKDLP*RDIQFDSEKGVDFVLNYSKAMENLFINRFMHMFQSS 200
hp12lo    139 YCWATWKEGLPLTIAADRKDDLP*PNMRFHEEKRLDFEWTLKAGALEMALKRVYTLL*SS 196
hr15lo    139 YRWGNWKDGLILNMAGAKLYDLP*VDERFLEDKRVDFEVSLAKGLADLAIKDSLNVL*TC 196
 Identity     3232232 3223 232    443  2224222323332  2 222  22322   22 22

                 ..-β10  -β11-              ...α6.....      -β12
SB1       315 ELYRTDGQHILKFPQPHVVQVSQSAWMTDEEFAREMIAGVNPCVIRGLEEFPPKSNLDPA 374
hl5lo     201 WNDFADFEKIFVKISNTISERVMNHWQEDLMFGYQFLNGCNPVLIRRCTELPEKLPVTTE 260
hp12lo    197 WNCLEDFDQIFWGQKSALAEKVRQCWQDDELFSYQFLNGANPMLLRRSTSLPSRLVL*** 253
hr15lo    197 WKDLDDFNRIFWCGQSKLAERVRDSWKEDALFGYQFLNGANPVVLRRSAHLPARLVF*** 253
 Identity     3222 43  432  22 223232  42242242333334244222432234 2322

                              ..α7... --β13- ......α8.....      --β14
SB1       375 IYGDQSSKITADSLDLDGYTMDEALGSRRLFMLDYHDIFMPYVRQINQLNSAKTYATRTI 434
hl5lo     261 MVECSLERQLS*********LEQEVQQGNIFIVDFELLDGIDANKTDPCTLQFLAAPICL 311
hp12lo    254 **PSGMEELQA********QLEKELQNGSLFEADFILLDGIPANVIRGEK*QYLAAPLVM 302
hr15lo    254 **PPGMEELQA********QLEKELEGGTLFEADFSLLDGIKANVILCSQ*QHLAAPLVM 302
 Identity      2 2232223        2332332 3 342243 33333 3323     3 3343222

                 ---    -----β15----      ---β16--     ....................
SB1       435 LFLREDGTLKPVAIELSLPHSAGDLSAAVSQVVLPAKEGVESTIWLLAKAYVIVNDSCYH 494
hl5lo     312 LYKNLANKIVPIAIQLNQIP******GDENPIFLPSD***AKYDWLLAKIWVRSSDFHVH 362
hp12lo    303 LKMEPNGKLQPMVIQIQPPSP****SSPTPTLFLPSD***PPLAWLLAKSWVRNSDFQLH 355
hr15lo    303 LKLQPDGKLLPMVIQLQLPRT****GSPPPPLFLPTD***PPMAWLLAKCWVRSSDFQLH 355
 Identity     422 22333 422433223      222 22234423   22 244444 3432343224

                 α9.....................  TT ..α10..TT TT.....α11....     α12
SB1       495 QLMSHWLNTHAAMEPFVIATHRHLSVLHPIYKLLTPHYRNNMNINALARQSLINANGIIE 554
hl5lo     363 QTITHLLRTHLVSEVFGIAMYRQLPAVHPIFKLLVAHVRFTIAINTKAREQLICECGLFD 422
hp12lo    356 EIQYHLLNTHLVAEVIAVATMRCLPGLHPIFKFLIPHIRYTMEINTRARTQLISDGGIFD 415
hr15lo    356 ELQSHLLRGHLMAEVIVVATMRCLPSIHPIFKLIIPHLRYTLEINVRARTGLVSDMGIFD 415
 Identity     22224342343224322243242 43 24443433234 423224422442243 22 4333

                 ..    .....α13.....      ...α14..                    ....
SB1       555 TTFLP*SKYSVEMSSAVYKNWVFTDQALPADLIKRGVAIKDPSTPHGVRLLIEDYPYAAD 613
hl5lo     423 KANATGGGGHVQMVQRAMKDLTYASLCFPEAIKARGMES*********KEDIPYYFYRDD 473
hp12lo    416 KAVSTGGGGHVQLLRRAAAQLTYCSLCPPDDLADRGL***********LGLPGALYAHD 463
hr15lo    416 QIMSTGGGGHVQLLKQAGAFLTYSSFCPPDDLADRGL***********LGVKSSFYAQD 463
 Identity     22 23333334322 23 2 333 3232423322442            3222 2243 4

                 .......α15.........  ..α16. .....α17.....                ..
SB1       614 GLEIWAAIKTWVQEYVPLYYARDDDVKNDSELQHWWKEAVEKGHGDLKDKPWWPKLQTLE 673
hl5lo     474 GLLVWEAIRTFTAEVVDIYYEGDQVVEEDPELQDFVNDVYVYGMRGRKSSGFPKSVKSRE 533
hp12lo    464 ALRLWEIIARYVEGIVHLFYQRDDIVKGDPELQAWCREITEVGLCQAQDRGFPVSFQSQS 523
hr15lo    464 ALRLWEIIYRYVEGIVSLHYKTDVAVKDDPELQTWCREITEIGLQGAQDRGFPVSLQARD 523
 Identity     24224324 2232224 324 242 43 43444 3223223 42 222323332323222

                 ............α18.............                ...α19.. .α20
SB1       674 DLVEVCLIIIWIASALHAAVNFGQYPYGGLIMNRPTASRRLLPEKGTPEYEEMINNHEKA 733
hl5lo     534 QLSEYLTVVIFTASAQHAAVNFGQYDWCSWIPNAPPTMRAPPPtak*******gvvTIEQ 586
hp12lo    524 QLCHFLTMCVFTCTAQHAAINQGQLDWYAWVPNAPCTMRMPPPttk*******edvTMAT 576
hr15lo    524 QVCHFVTMCIFTCTGQHASVHLGQLDWYSWVPNAPCTMRLPPPttk********daTLET 575
 Identity     3322223223332233443332442332232342342334 334323        223 22

                 ....   ......α21......                  ..........α22....
SB1       734 YLRTITSKLPTLISLSVIEILSTHASDEVYLGQRDNPHWTSDSKALQAFQKFGNKLKEIE 793
hl5lo     587 IVDTLPDRGRSCWHLGAVWALSQFQENELFLGMYPEEHF*IEKPVKEAMARFRKNLEAIV 645
hp12lo    577 VMGSLPDVRQACLQMAISWHLSRRQPDMVPLGHHKEKYF*SGPKPKAVLNQFRTDLEKLE 635
hr15lo    576 VMATLPNFHQASLQMSITWQLGRRQPVMVAVGQHEEEYF*SGPEPKAVLKKFREELAALD 634
 Identity     22 3332  22222222 3 432232223 3422 3223 322223222 243  42222

                 .......  .α23.
SB1       794 EKLVRRNNDPSLQGNRLGPVQLPYTLLYPSSEEGLTFRGIPNSISI               839
hl5lo     646 SVIAERNKKK**********QLPYYYLSPD********RIPNSVAI               673
hp12lo    636 KEITARNEQL**********DWPYEYLKPS********CIENSVTI               663
hr15lo    635 KEIEIRNAKL**********DMPYEYLRPS********VVENSVAI               662
 Identity     223  44 22          2244234 43         3244324
```

Fig. 1. The structurally-based sequence alignment of SB1, *hl5lo*, *hp12lo*, and *hr15lo* is shown. Lower case refers to undetermined coordinates in the models (717–729 inclusive). Residues in **bold** type are conserved in all sequenced lipoxygenases (P213, R216, and G221 may also be conserved, but are not shown in this figure). The three turns shown in Table I (V520-L521, P530-H531, and R533-N543) are denoted with "T."

TABLE I. Salt Bridges and Hydrogen Bonds in Soybean Lipoxygenase-1 and the Human Lipoxygenase Models

	Location of	
Salt bridges	First residue	Second residue
D343-K483	α6	α9
D508-R543	α9	α11
H522-D642	α9–α10 loop	α16–α17 loop
R162-**E644**	α1	α17
D490-R707 or **D490**-R487 (models only)	α9	α18–α19 loop or α9
	Location of	
Hydrogen bonds	First residue	Second residue
M341-**K483**	β11–α6 loop	α9
D343-W479	α6	α9
E508-N539	α9	α11
G218-**E508**	α1–α2 loop	α9
H504-N539 (2 hydrogen bonds)	α9	α11
S519-**H522** (type-I turn)	α9–α10 loop	α9–α10 loop
Y633-D642	α15	α16–α17 loop
D642-L645	α16–α17 loop	α17
Q646-L669	α17	α17–α18 loop
H522-E644 (weak)	α9–α10 loop	α17
T529-Y532 (type-I turn)	α10	α10–α11 loop
Y532-N535 (type-II turn)	α10–α11 loop	α11
P530-R533	α10–α11 loop	α10–α11 loop
H531-N835	α10–α11 loop	C-terminal loop
R533-**S836**	α10–α11 loop	C-terminal loop
R767-**N835**	α21–α22 loop	C-terminal loop
H690-wat841	α18	Water
S836-wat841	C-terminal loop	Water
N355-D490	α6-β12 loop	α9
H494-V693	α9	α18
N694-**Q697**	α18	α18
N694-**L754**	α18	α21

Four Regions of Conserved Residues

Since all residues in the human enzymes—except for the three histidine iron ligands—were modeled in an unbiased manner, analysis of the conformations of conserved residues in the human lipoxygenase models was used to evaluate the reliability of the models. Among the 22 lipoxygenase sequences published to date,[12,13,16–38] there are 56 conserved residues (including the four iron ligands). Twenty-six of these conserved residues are grouped in four regions which play structural roles in lipoxygenases (residues conserved in all lipoxygenase sequences will be in **bold** type in the following discussion). Three of these regions are involved in anchoring the two longest helices: the 43-residue Helix 9, containing two iron ligands (**H499** and **H504**), and the 30-residue Helix 18, containing one iron ligand (**H690**). The fourth region is involved in stabilizing the conformation of the loop containing the fourth iron ligand—the carboxylate of carboxy-terminal **I839.**

The agreement between the SB1 structure and the three human lipoxygenase models was good for the 26 conserved residues. In most cases, polar interactions observed in the SB1 structure were duplicated in the models (Hydrogen bonds and salt bridges found in these regions are included in Table I), confirming the reliability of the models. Major differences in side chain orientation existed only for a surface residue (**W479**) and residues which become surface-exposed in the models (**E644, Q646,** and **R543**) due to the absence of the amino-terminal residues.

Region I. Beginning of Helix 9 (D343, F346, I448, P469, W479, A482, K483, V486)

Near the amino-terminal end of Helix 9 there is a sequence of five highly conserved residues (**W479,** L480, L481, **A482,** and **K483**) that appears to anchor the end of Helix 9 to Helix 6 and to the β-sheet region involving Strands 15 and 16. In addition to the polar interactions listed in Table I, the hydrophobic residues in this region strengthen the interaction between these secondary structural elements. Modeling this region in the human lipoxygenases was complicated by a three-residue gap at the beginning of Helix 9, between the conserved residues **P469** and **W479.** These three residues were assigned to positions 472-4 without altering the positions of the conserved residues (discussed above in Structure-based sequence alignment).

Region II. Middle of Helix 9 (H504, E508, I538, N539, R543)

In SB1, interactions between Helix 9 and Helix 11 involve conserved residues found on the same side of the helices at intervals of i and i + 4 (**H504** and **E508** from Helix 9; **I538, N539,** A542 and **R543** from Helix 11). The network of interactions between these residues not only anchors Helix 11 to Helix 9, but also fixes the side chain of iron ligand **H504** in a specific conformation. The negative charge of **E508** may be necessary for lipoxygenase function since substitution with glutamine in *hl5lo* (E376Q *hl5lo*) yields an inactive enzyme[39,40] while substitution with asparatate (E376Q *hl5lo*) yields an enzyme with partial activity.[41]

Region III. End of Helix 9 (L518, H522, P523, Y633, D642, E644, L645, Q646)

In the SB1 structure, a group of residues stabilizes the interactions between Helix 16, Helix 17, and the loop between Helices 9 and 10 (H517 to **H522**). Results of mutagenesis experiments which substitute **H522** with uncharged amino acids (low activities or low yields were found)[15,17,39,42–44] support the con-

clusion that **H522** has a structural role, forming a conserved salt bridge with **D642.** Mutagenesis of **D642** further supports this conclusion; substitution of **D642** with negatively-charged glutamate (D502E *hl5lo*)[40] preserves activity while substitution with uncharged asparagine (D502N *hl5lo*)[41] greatly reduces the activity.

Region IV. Carboxy-terminal loop (H531, H690, N835, S836, I839)

In SB1, there are several conserved interactions between carboxy-terminal residues **N835** and **S836**[‡] and the five-residue loop between Helix 10 and Helix 11 (P530 to N534) that play a role in organizing the two loops into the specific geometry necessary to orient iron ligand **I839.** In addition, a buried water forms hydrogen bonds with the carbonyl oxygen of **S836** and the Nδ of iron ligand **H690.** The mammalian lipoxygenases are missing 18 residues in the carboxy-terminal region between positions 800 and 839. These 18 residues were assigned to two poorly conserved segments in the SB1 structure (10 and eight residues in length) and were removed without altering main chain geometries in regions containing highly conserved residues (discussed above in Structure-based sequence alignment). The three human lipoxygenase models show that the orientation of **H531** is almost identical to that in the SB1 structure, preserving the hydrogen bond between the Nδ of **H531** and the carbonyl oxygen of **N835.** Mutagenesis experiments on **H531** support these findings. Substitutions of **H531** with asparagine and with glutamine (H399Q *hl5lo,*[42] H399N *hl5lo,*[39] and H392Q *hp12lo*[15]) have the highest activities, perhaps due to the ability of side chain amides to duplicate the hydrogen bond formed by **H531.**

Conservation of the D490-R707 Salt Bridge

In the SB1 structure, a buried salt bridge between R707 and **D490** forms a division in the large internal cavity lined by highly conserved residues. It has been noted that if these two residues shifted, the resulting 40Å cavity would connect the iron site to within a few Ångstroms of the surface of the protein, making it possible for the cavity to be the substrate entry route and binding site.[9] **D490** is conserved in all lipoxygenases, but in mammalian lipoxygenases the position of R707 is occupied by an alanine.

The absence of R707 in mammalian lipoxygenases, would appear to leave the negative charge of **D490** uncompensated and deeply buried in the protein, but the models show that the R707–**D490** salt bridge is effectively conserved by concerted changes of R707A and I487R. In the human lipoxygenase models, the arginine at position 487 is oriented so that the guanidinium moiety occupies the same region as the guanidinium of R707 from the SB1 structure (Fig. 2). This alternative salt bridge allows the carboxylate of **D490** to remain in roughly the same position and still maintain a hydrogen bond with the amide of conserved residue **N355.** Thus, the subdivision of the large internal cavity by an ion pair in the SB1 structure seems to also be present in the mammalian lipoxygenases. The salt bridge is not necessary for lipoxygenase activity, however, since substitution of **D490** with asparagine (D358N *hl5lo*) in *hl5lo* does not abolish activity.[39]

The Role of Residue H494

The side chain of histidine 494 lies on the wall of the proposed substrate binding cavity only 8Å (Fe–Cε distance) from the iron atom. Its orientation allows the Nε of the imidazole ring to form a hydrogen bond with the carbonyl oxygen of residue V693, and exposes the Nδ side of the ring to the cavity. The proximity of H494 to the iron atom raises the possibility that it could be a positive counter ion involved in stabilizing the negatively charged carboxylate of fatty acid substrates.

Analysis of H494 in the human lipoxygenase models and the results of mutagenesis studies suggest that H494 has a structural role. The models give H494 an orientation almost identical to that in the SB1 structure, preserving the hydrogen bond between the Nε and the carbonyl oxygen of residue 693. This interaction helps to anchor Helix 9 (containing iron ligands **H499** and **H504**) to Helix 18 (containing iron ligand **H690**) and may have a role in initiating the π-helical section of Helix 9, which begins at H494 and ends at residue 506. Analysis of the models of *hp12lo* and *hr15lo* raises the possibility that the carboxylate of an adjacent glutamate (position 495 is a glutamate in many mammalian lipoxygenases and in pea lipoxygenase-3) could be oriented back towards H494 to form a salt bridge. Mutagenesis experiments in *hp12lo* and other lipoxygenases, however, do not support the conclusion that H494 is a positively-charged salt bridge partner in these cases. Substitutions of H494 with glutamine (H494Q SB1,[17] H362Q *hl5lo,*[42] and H355Q *hp12lo*[15]) show that an uncharged residue at this position does not abolish enzyme activity. In fact, the recently published sequence of murine platelet 12-lipoxygenase[38] has a glutamine in this position

[‡]N835, S836, and iron ligand I839 are not conserved in the sequence of *rl5lo.* Comparison of the gene from *rl5lo*[32] and the gene from *hl5lo*[30,31] (*hl5lo* and *rl5lo* share 93% amino acid sequence identity) indicates a possible frame shift error in the six carboxy-terminal residues of *rl5lo.*

hl5lo gene	cca	gac	cgg	att	ccg	aac	agt	gtg	gcc	atc	tga
Sequence	P	D	R	I	P	N	S	V	A	I	Stop
rl5lo gene	cca	gac	ag?	**att**	**cca**	**aac**	**agt**	**gta**	**gcc**	atc	taa
Sequence	P	D	R	**F**	**Q**	**T**	V	**Stop**			
Possible Sequence	P	D	R?	I	P	N	S	V	A	I	Stop

Due to the uncertainty in the carboxy-terminal six residues of *rl5lo,* the three underlined residues—N835, S836, and I839—are considered to be conserved in all lipoxygenases.

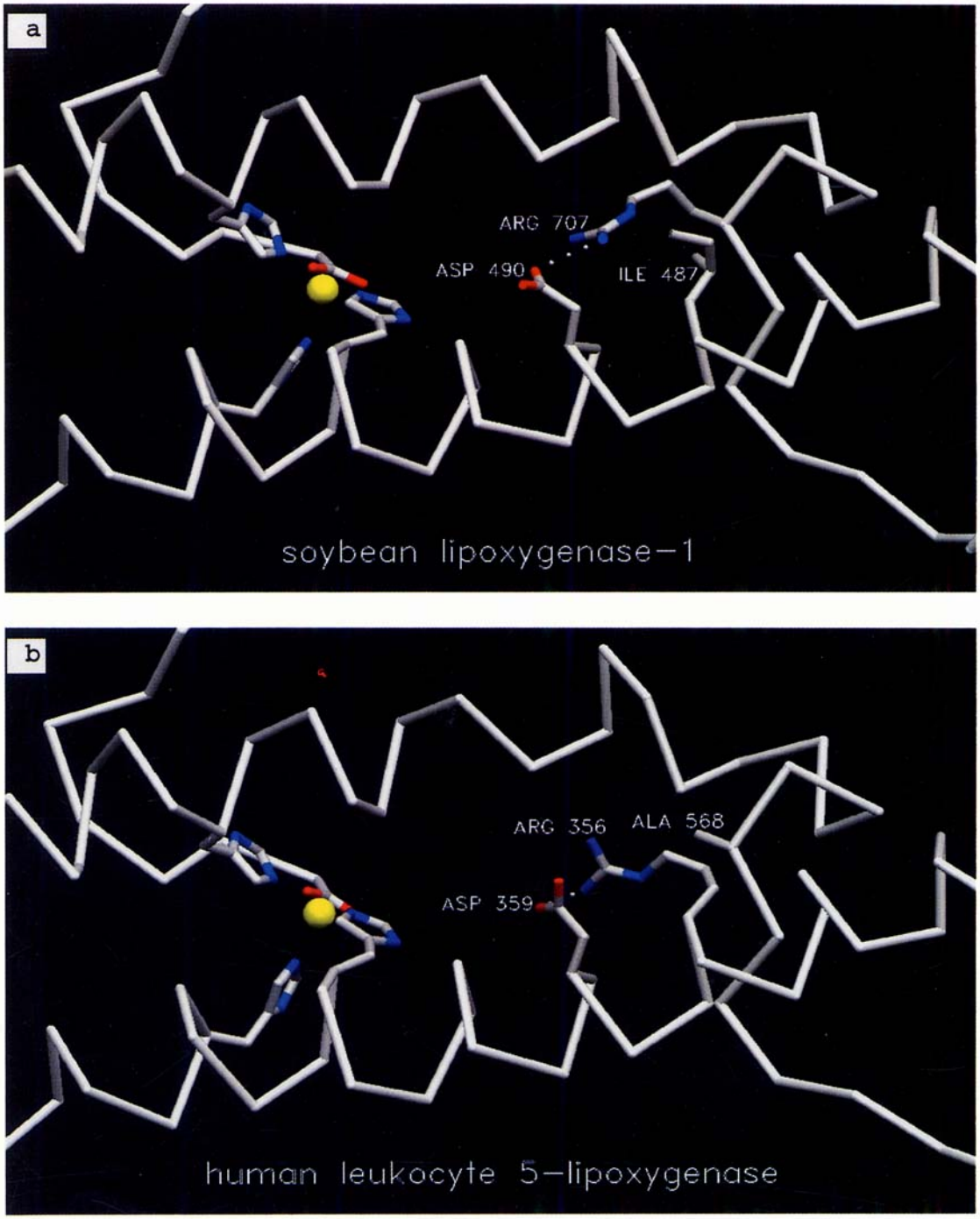

Fig. 2. The backbone of Helix 9 and Helix 18 are shown with a conserved salt bridge and the active site iron ligands. **a:** The structure of soybean lipoxygenase-1 in which residue 487 is an isoleucine and a salt bridge exists between D490 and R707. **b:** A model of human leukocyte 5-lipoxygenase in which residue 707 is an alanine (A568 *hl5lo*) and the salt bridge now exists between D490 (D359 *hl5lo*) and R487 (R356 *hl5lo*).

(and a glutamate at position 495), making H494 no longer a conserved residue in lipoxygenase sequences. The loss of activity in the H494K (H363K *hl5lo*) variant in *hl5lo*[43] and the activity of the H494L (H356L *pl12lo*) variant in *pl12lo*[44] further demonstrate that a positive charge (lysine) at position 494 is deleterious to enzyme function and an uncharged residue (leucine) is not—even in lipoxy-

genases with a glutamate at position 495. Thus, it appears that H494 is uncharged in lipoxygenases and does not interact ionically with the carboxylates of fatty acid substrates.

The Role of Residue N694

Sequence alignments show that position 694 is occupied by an asparagine in plant lipoxygenases, mammalian 5-lipoxygenases and human platelet 12-lipoxygenase, but by a histidine in other mammalian 12-lipoxygenases and all mammalian 15-lipoxygenases. The orientation of this side chain is critical due to its proximity to the unoccupied iron coordination position (opposite **H504**) at the terminus of the putative dioxygen entry channel. Small changes in this area could affect iron coordination and the size of the channel, which is only 2.5Å wide in this region. In the soybean lipoxygenase-1 structure, the amide oxygen of N694 is 2.8Å from the amide of conserved **Q697,** while its amide nitrogen is 2.6Å from the carbonyl oxygen of conserved **L754.** These hydrogen bonds orient the N694 side chain, making the distance between the amide oxygen and the iron atom 3.3Å. (In another structural study on SB1, N694 was reported to be an iron ligand).[45]

Two possibilities for the orientation of a histidine in position 694 arose during modeling of human reticuloctye 15-lipoxygenase. The two histidine conformers differ in χ-1 by about 90°; one could be an iron ligand, while the other forms a salt bridge (Fig. 3). The first conformer is oriented in the same direction as the asparagine in the SB1 structure and may be capable of duplicating one or both of the hydrogen bonds formed by N694 in the SB1 structure. Small manual adjustments of this conformer show that the histidine Nδ could be an iron ligand, but the Nε cannot be oriented to be an iron ligand. Ideal orientation of the histidine ring for iron coordination geometry creates several severe clashes with other residues: one between the histidine Nε and the carbonyl oxygen of **L754** and others between the histidine Cε and atoms of **I839.** Attempts to alleviate these clashes tilt the histidine ring with respect to the Fe–Nδ bond and force the histidine to adopt a non-ideal iron coordination geometry. The second histidine conformer is oriented away from the vacant iron coordination position (opposite **H504**) and towards the glutamate which replaces P834 in all mammalian 12- and 15-lipoxygenases. Substitution of F695 with leucine seems to generate some of the space necessary to orient the histidine side chain in this direction. All lipoxygenases that have a histidine at position 694 also have a glutamate at position 834 and a leucine at position 695, making the salt bridge possible.

It is not clear from the models which conformer of H694 is most likely to be found in lipoxygenases. Mutagenesis of N694 indicates that residue 694 is important for lipoxygenase function, but not for iron binding. Mutant lipoxygenases in which N694 was substituted with aspartate, glutamine, histidine, alanine, or serine (N554D *hl5lo* and N554Q *hl5lo*[40]; N713H SB3, N713A SB3, and N713S SB3[46]) all incorporated iron (the lowest iron content found was 51% of wild type), however, only the N694H variant had activity comparable to the wild type enzyme (the N694D variant had barely detectable activity). The possibility has been raised that N694 in soybean lipoxygenase-1 may shift position during dioxygen binding to interact with the distal oxygen atom of iron-bound dioxygen, helping to orient and stabilize the oxygen addition step of the lipoxygenase reaction.[9] The conserved residue **Q697** (as discussed above, the side chains of **Q697** and N694 form a hydrogen bond in the SB1 structure) may also be involved in some aspect of dioxygen binding—either alone or in conjunction with N/H694. Substitution of **Q697** with glutamate (Q557E *hl5lo*) in *hl5lo* results in very low activity (~3%).[43]

Positional Specificity in 12- and 15-Lipoxygenases

A series of mutagenesis studies in *hp*12*lo*,[15] *be*12*lo* (bovine epithelial 12-lipoxygenase),[47] *pl*12*lo* (porcine leukocyte 12-lipoxygenase),[44] *rb*12*lo* (rat brain 12-lipoxygenase)[48] and *hr*15*lo*[49] attempted to shift the site of dioxygen addition from the 15th carbon to the 12th carbon of arachidonic acid and vice versa. Several residues were identified as possible determinants of 12/15 positional specificity from sequence comparisons of mammalian 12-lipoxygenases and mammalian 15-lipoxygenases, however, only substitutions at residues 556 and 557 (corresponding to positions 417 and 418 in *hp*12*lo* and *hr*15*lo*) affected the positional specificities of the enzymes (Table II). Residues 556 and 557 seemed to shift positional specificity in mammalian 12- and 15-lipoxygenases by affecting where the methyl terminus of substrate fatty acid binds. Bulky amino acids at positions 556 and 557 would not permit substrate to bind as deeply, exposing C15 of arachidonic acid to oxygenation instead of C12.[49]

Of the sites chosen in the mutagenesis studies aimed at shifting positional specificity, only four (545, 547, 556, and 557) are close to the active site iron (Cβ–Fe distances <15Å) and have side chains facing towards the iron (Cα–Cβ–Fe angles >90°). In the models of *hp*12*lo* and *hr*15*lo,* the beta carbon to iron distances for residues 545, 547, 556, and 557 are 14Å, 12Å, 15Å, and 15Å, respectively; the Cα–Cβ–Fe angles are 117°, 118°, 145°, and 140°, respectively. Positions 545 and 547 are separated from positions 556 and 557 by more than 10Å (Cβ–Cβ distance), making it impossible for both sites to interact with the methyl terminus of bound substrate. Analysis of the Connolly surface of the SB1 putative substrate binding cavity superimposed on the models of *hp*12*lo* and *hr*15*lo* indicates that positions 545

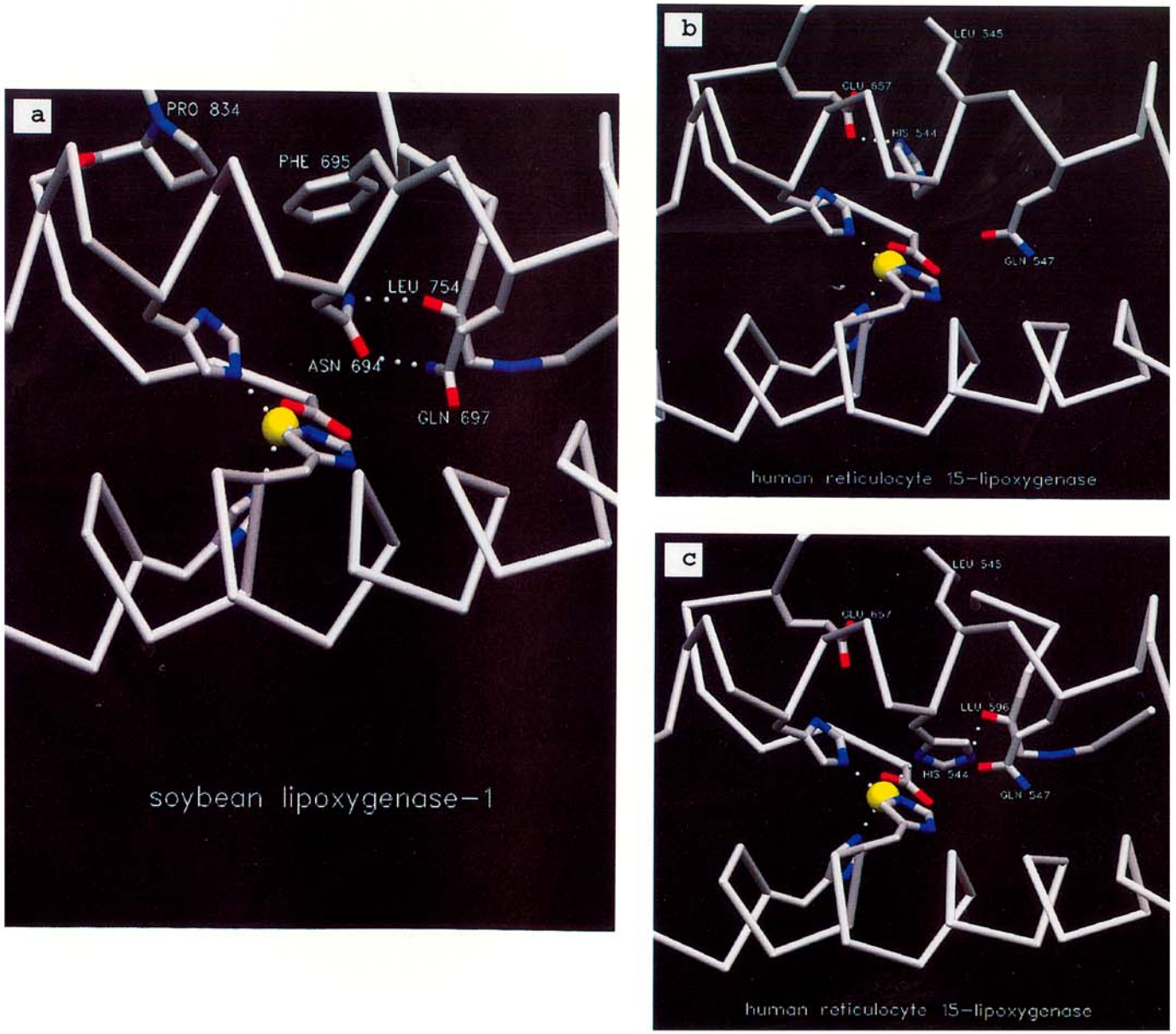
a
PRO 834
PHE 695
LEU 754
ASN 694
GLN 697
soybean lipoxygenase-1
b
LEU 545
GLU 657
HIS 544
GLN 547
human reticulocyte 15-lipoxygenase
c
LEU 545
LEU 596
HIS 544
GLN 547
human reticulocyte 15-lipoxygenase

TABLE II. Summary of Reported Effects of Mutagenesis on Residues 556 and 557 in Mammalian 12- and 15-Lipoxygenases

Enzyme	Residues at positions 556-7		Shift in positional specificity	Total activity
	556	557		
*hp*12*lo**	A417	V418		
	A	M	15-Specificity 0→0%	94%
	I	V	15-Specificity 0→10%	74%
	I	M	15-Specificity 0→18%	67%
*be*12*lo*[47]	V418	V419		
	V	M	15-Specificity 6→6%	n.a.
	I	M	15-Specificity 6→25%	n.a.
*pl*12*lo*†	V418	V419		
	V	M	15-Specificity 9→33%	n.a.
	I	V	15-Specificity 9→68%	n.a.
	I	M	15-Specificity 9→85%	n.a.
*rb*12*lo*[48]	A418	M419		
	A	V	12-Specificity 86→71%	8%
	I	M	15-Specificity 14→10%	150%
*mp*12*lo*	V417	V418		
*ml*12*lo*	V418	M419		
*hr*15*lo*[49]	I417	M418		
	I	V	12-Specificity 10→50%	112%
	A	V	12-Specificity 10→94%	n.a.
*rr*15*lo*	I416	M417		

*Chen and Funk.[15] A six amino acid substitution including V557M (T540I/Q545G/I547V/G550M/K555Q/V557M) increased 15-specificity to 33%, however, the total activity of this variant was low (25%). Three substitutions including A556I/V557M were made: the 15-activities of K555Q/A556I/V557M, T540V/K555Q/A556I/V557M, and I547V/G550M/K555Q/A556I/V557M are 18, 21, and 20%, respectively; and the total activities are 67, 61, and 29%, respectively.

†Suzuki et al.[44] Reported reverse-phase HPLC data indicates that all three variants have total activities similar to the wild type.

and 547 and positions 556 and 557 have different roles in substrate binding. Positions 545 and 547 lie on the wall of the putative substrate binding cavity opposite the iron atom, where they would be most likely to be in the vicinity of the pentadienyl moiety of bound substrate (position 545 especially), whereas positions 556 and 557 lie towards one end of the substrate binding cavity, where they would be most likely to interact with the methyl terminus of bound substrate.[§]

[§]The Connolly surface [50] was calculated using a 1.2 Å probe radius.[51]

Fig. 3. The backbone of Helix 9 and Helix 18 are shown with residues in the vicinity of N694 and active site iron ligands. **a:** The structure of soybean lipoxygenase-1 in which N694 forms hydrogen bonds with Q697 and the carbonyl oxygen of L754. **b,c:** Two alternate conformers of H694 (H544 *hr*15*lo*) in human reticulocyte 15-lipoxygenase. The first conformer of H694 (b) forms a salt bridge with E834 (E657 *hr*15*lo*) while the second conformer of H694 (c) has been manually oriented to be an iron ligand (poor geometry).

The ratio of 15-activity to 12-activity increases with increased bulkiness of residues 556 and 557, and decreases with decreased bulkiness of these residues in all of the mutants except for the rat brain 12-lipoxygenase variants (Fig. 4). In *rb*12*lo*, the M557V variant (M418V *rb*12*lo*) decreased 12-specificity from 86 to 71% and the A556I variant (A417I *rb*12*lo*) increased 12-specificity from 86 to 90% (sequence numbers are those used by Watanabe and Haeggstrom).[48] Analysis of residues in the putative substrate binding pocket, especially residue 491, provides a rationale for why these mutations had different effects in *rb*12*lo* than they did in other lipoxygenases. In comparison to other 12-lipoxygenases, wild type *rb*12*lo* contains a more bulky residue at position 557 (methionine instead of valine) and a less bulky residue at position 491 (leucine instead of phenylalanine) (Table III). The methyl terminus of arachidonic acid may bind more in the vicinity of residue L491 in rat brain 12-lipoxygenase due to the shift in bulk between residues 491 and 557. The M557V variant was designed to increase 12-specificity (as was done in *hr*15*lo*), however, in *rb*12*lo* the mutation created a loose binding pocket, with positions 491, 556, and 557 occupied by leucine, alanine, and valine, respectively. As a result, the positional specificity decreased along with the total activity (8% of wild type). The A556I variant in *rb*12*lo* was designed to increase 15-activity (as was done in *hp*12*lo*), however, with the reduced bulk of L491 taken into consideration, this mutation may have optimized substrate binding. As a result, the 12-specificity increased along with the total activity (150% of wild type). Since the recently sequenced murine leukocyte 12-lipoxygenase[38] (93% sequence identity to *rb*12*lo*) contains identical residues at positions 491 (leucine) and 557 (methionine), it could bind the methyl terminus of arachidonic acid in a manner similar to that of rat brain 12-lipoxygenase.

Further analysis of the residues around positions 556 and 557 indicates that four residues (residues 552, 701, 750, and 751) are common to murine leukocyte and rat brain 12-lipoxygenase, but differ in other mammalian 12-lipoxygenases (Table III). These four residues, in conjunction with residues 491, 556, and 557, may affect the nature of the substrate binding sites of 12-lipoxygenases. Residues 552 and 750 face each other on one wall of the putative substrate binding site, maintaining a hydrophobic interaction. Residues 701 and 751 are hydrophobic in *ml*12*lo* and *rb*12*lo*, and hydrophilic in the other 12-lipoxygenases (possibly forming a hydrogen bond).

Model of Lipoxygenase Positional Specificity

Mutagenesis studies suggest that binding of the methyl end of fatty acid substrate is responsible for positional specificity by aligning the substrate relative to the catalytic site. Other evidence supports

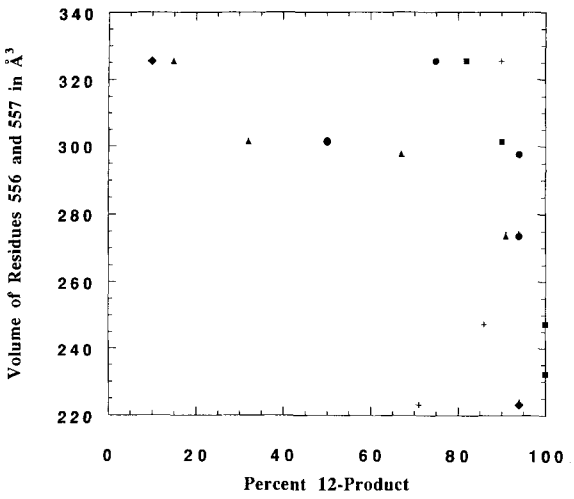

Fig. 4. Human platelet 12-lipoxygenase (■) (Chen and Funk[15]), bovine epithelial 12-lipoxygenase (●) (Sloane and Sigal, 1994),[47] porcine leukocyte 12-lipoxygenase (▲) (Suzuki et al.[44]) and human reticulocyte 15-lipoxygenase (◆) (Sloane et al.[49]) produced a higher ratio of 15-product to 12-product with increased bulk at residues 556 and 557. Rat brain 12-lipoxygenase (+) was the only lipoxygenase which did not follow this trend. Values for amino acid bulk were taken from a table of amino acid partial specific volumes in solution.[78]

TABLE III. Residues Near Positions 556 and 557 Not Conserved in Wild Type Mammalian Lipoxygenases*

Enzyme	491	552	556	557	701	747	750	751
SB1	S	I	T	F	G	S	V	I
hl5lo	F	L	A	N	C	H	A	V
rl5lo	F	L	A	N	C	H	A	V
hp12lo	F	I	A	V	Y	Q	I	S
be12lo	F	V	V	V	Y	Q	I	T
pl12lo	F	I	V	V	Y	Q	I	T
rb12lo	**L**	**F**	A	**M**	**F**	Q	**V**	**V**
mp12lo	F	I	V	V	Y	Q	I	T
ml12lo	**L**	**F**	V	**M**	**F**	Q	**V**	**V**
hr15lo	F	I	I	M	Y	Q	I	T
rr15lo	F	I	I	M	F	Q	I	V

*References to the above sequences can be found in the footnote in the beginning of the Results and Discussion section. Residues in **bold** type are common to murine leukocyte and rat brain 12-lipoxygenase.

this model of positional specificity in 12- and 15-lipoxygenases. Several experiments have shown that 12- and 15-lipoxygenases from plants and mammals are sensitive to the location of the reactive 1,4-diene relative to the methyl terminus, but not the carboxylate of fatty acid substrates.[52–54] Fatty acids with modified carboxy termini (methylcarboxylate,[55] sulfate,[56] cholesterol esters and phospholipids[57]) are 15-lipoxygenase substrates while fatty acids with altered methyl termini (19-methylarachidonic acid[53]) are not substrates. In addition, human reticulocyte 15-lipoxygenase can catalyze oxygenation of low-density-lipoproteins in addition to biological membranes,[57] and rabbit reticulocyte 15-lipoxygenase can catalyze the oxygenation of fatty acids in the membranes of organelles[58]—reactions thought to be important in the degradation of mitochondrial membranes in reticulocyte maturation and the processing of low-density-lipoprotein in atherogenesis.[¶] Together, these results are consistent with the model of positional specificity in 12- and 15-lipoxygenases based on positioning of the substrate methyl terminus. One final piece of evidence is the stereospecificity of dioxygen addition (S)** and hydrogen abstraction (ProS) in 12- and 15-lipoxygenase reactions. In all well characterized lipoxygenases, the chirality of 12- and 15-lipoxygenase reactions is identical, requiring only a 2.7Å shift in the alignment of arachidonic acid substrate to change positional specificity (Fig. 5).

The above model of positional specificity in 12- and 15-lipoxygenase involves a translational shift of arachidonic acid in the substrate binding cavity. Further translation of the fatty acid will not produce 5- or 8-lipoxygenase products with the correct stereospecificity of hydrogen abstraction and dioxygen addition. However, if arachidonic acid binds in the opposite direction (carboxylate and methyl positions exchanged), the 5-lipoxygenase reaction becomes spacially identical to the 15-lipoxygenase reaction and the 8-lipoxygenase reaction becomes spacially identical to the 12-lipoxygenase reaction (Fig. 6). This model explains the observed stereospecificity of 5-, 12-, and 15-lipoxygenase reactions and predicts the stereospecificity of the 8-lipoxygenase reaction (abstraction of the C10 ProR-H and insertion of dioxygen, forming (8S)-hydroperoxy product) by fusing two paradigms: the first pertaining to translation of substrate in the active site,[47] and the second pertaining to the orientation of substrate in the active site.[55,63]

The general model of lipoxygenase positional specificity and stereospecificity requires the substrate carboxylate to bind in 5- and 8-lipoxygenases in the same pocket as the substrate methyl terminus binds in 12- and 15-lipoxygenases. The carboxylate binding pocket of 5- and 8-lipoxygenases is expected to position the substrate based on the binding of the substrate carboxylate instead of the substrate

[¶]An article by Sparrow et al.[59] contradicts the results regarding LDL oxygenation by lipoxygenases.

**When substrate containing a (E-Z)-1,4-diene moiety is used, the major product can be the (R) hydroperoxy fatty acid, however, this is the expected chirality for antarafacial addition to an olefin in the (E)-geometry.[10] Partially purified extracts from several tissue sources catalyze oxygenation of arachidonic acid to produce (8R)-, (11R)-, or (12R)- hydro(pero)xyeicosatetraenoic acids,[60–62] however, these enzymes have not been well characterized and some of them may be cytochromes or other oxygenases.

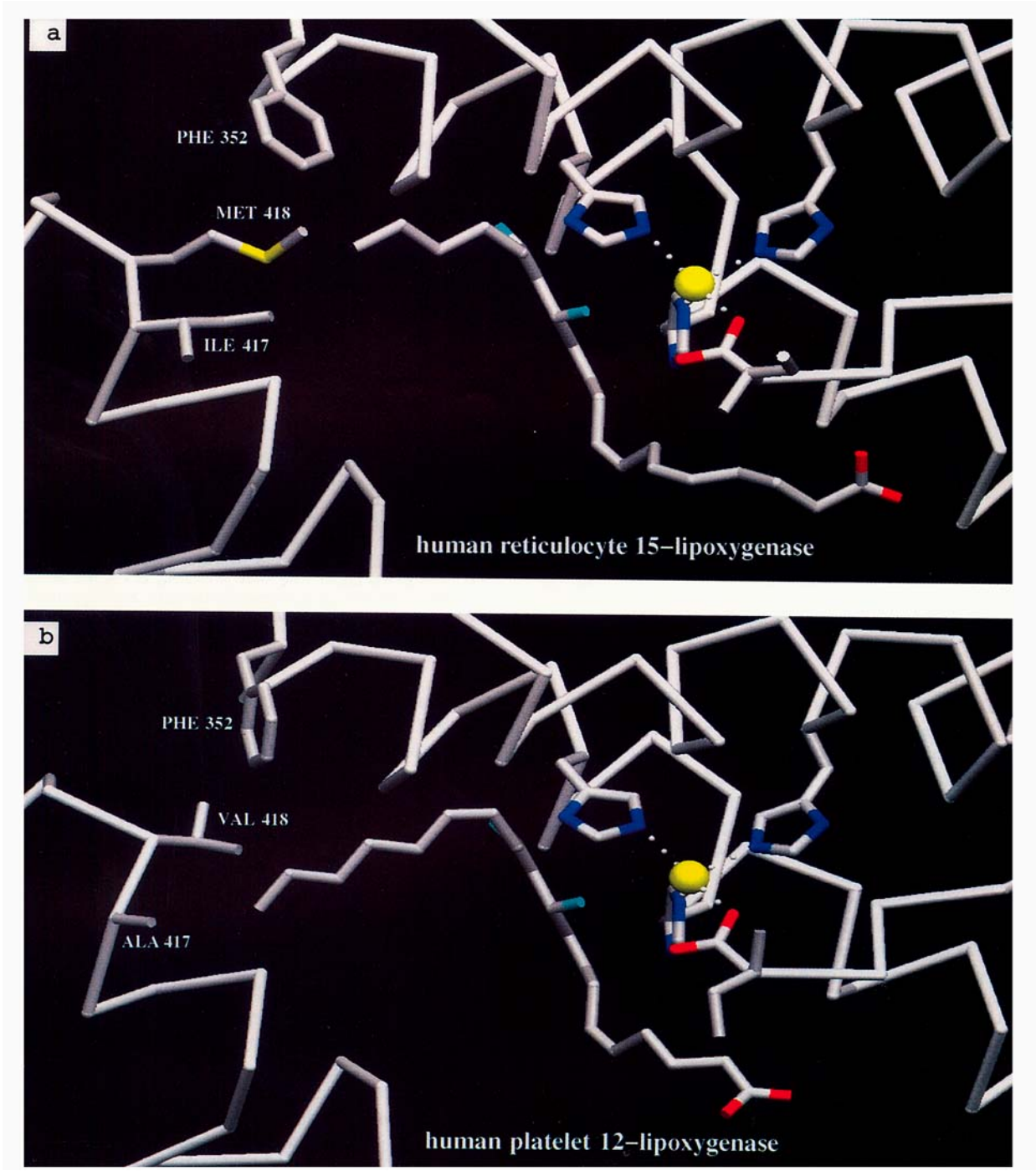

Fig. 5. The side chains of active site iron ligands are shown with substrate arachidonic acid and three residues which interact with the methyl terminus of the arachidonic acid. **a:** A model of human reticulocyte 15-lipoxygenase in which F491 (F352 *hr*15*lo*), I556 (I417 *hr*15*lo*), and M557 (M418 *hr*15*lo*) interact with the methyl terminus of arachidonic acid. **b:** A model of human platelet 12-lipoxygenase in which F491 (F352 *hp*12*lo*), A556 (A417 *hp*12*lo*), and V557 (V418 *hp*12*lo*) interact with the methyl terminus of arachidonic acid. The arachidonic acid was placed manually based on hypothetical hydrogen abstraction by an iron bound hydroxyl. The turquoise atoms on the arachidonic acid mark the sites of oxygen addition and hydrogen abstraction (C15 and C13, respectively, in a and C12 and C10, respectively, in b).

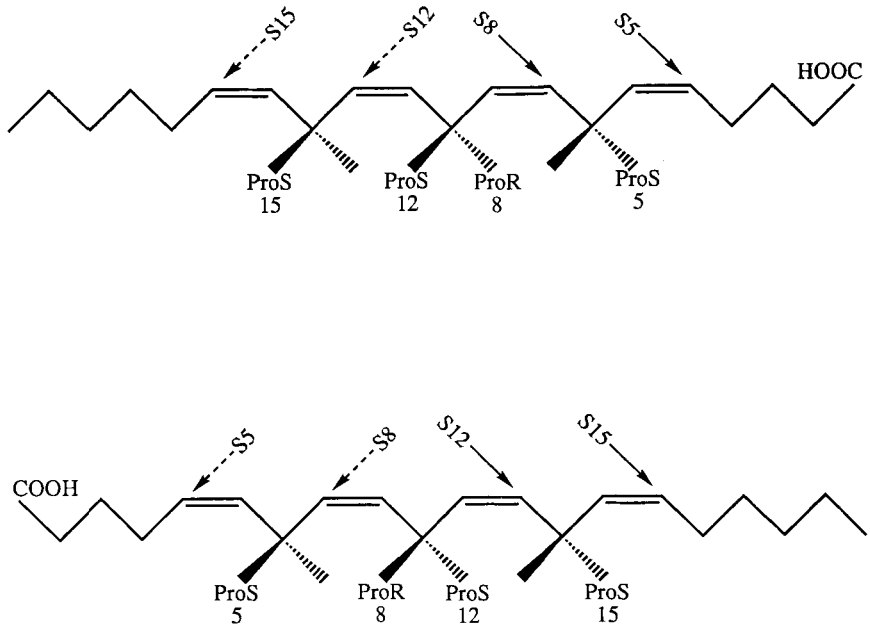

Fig. 6. When arachidonic acid is arranged head to tail in opposite orientations, the 5- and 8-lipoxygenase reactions become spacially identical to the 15- and 12-lipoxygenase reactions. Arrows denote the four locations of dioxygen addition (dashed arrows are below the plane of the page and solid arrows are above the plane of the page). The stereospecificity of oxygen addition and stereoselectivity of hydrogen abstraction in 8-lipoxygenase is presumed from the model and is not currently known.

methyl terminus. This idea is supported experimentally by results which show that, unlike 12- and 15-lipoxygenases, 5- and 8-lipoxygenases are sensitive to the location of the reactive 1,4-diene relative to the carboxylate of fatty acid substrate.[64–66] Analysis of the residues in the binding cavity shows that a histidine is located at position 747 (H600 *hl5lo*) and that an asparagine occupies position 557 (N425 *hl5lo*—this position was thought to be a determinant of 15-specificity in the mutagenesis studies) in both of the sequenced 5-lipoxygenases (Table III). In the model of *hl5lo,* the histidine and asparagine side chains are oriented towards the iron atom. When sequence information becomes available, it will be interesting to see if hydrophilic residues occupy nearby positions in 8-lipoxygenases.

Lipoxygenases often exhibit dual positional specificity—most commonly 12/15 or 5/8 dual positional specificity. Dual positional specificity may have arisen as a means of generating two products without expressing two independent enzymes. Lipoxygenases could produce the ratio of products required by the tissues in which they are expressed. For example, the four "leukocyte type" 12-lipoxygenases produce 15-product, whereas the two "platelet type" 12-lipoxygenases do not produce any detectable 15-product. Dual positional specificity in some lipoxygenases is also a means of synthesizing the epoxide moiety of leukotriene compounds from arachidonic acid. Mammalian 5-lipoxygenases and potato 5-lipoxygenase produce a minor 8-product and are capable of synthesizing 5,6-LTA_4 (5S,6S-oxido-7E,9E,11Z,14Z-eicosatetraenoic acid)[67–69] through successive 5- and 8-lipoxygenase reactions. Similarly, porcine leukocyte 12-lipoxygenase exhibits dual 12/15 specificity and is capable of synthesizing 14,15-LTA_4 (14S,15S-oxido-5Z,8Z,10E,12E-eicosatetraenoic acid)[70] through successive 15- and 12-lipoxygenase reactions. Like porcine leukocyte 12-lipoxygenase, human and rabbit reticulocyte 15-lipoxygenase produce a 14R,15S-dihydroxy product,[57,71] which may be the result of 14,15-LTA_4 synthase activity in the mammalian 15-lipoxygenases.

The mechanism used in leukotriene synthesis may also aid in the synthesis of trioxygenated bioactive compounds lipoxin A4 (5S,6R,15S-trihydroxy-7E,9E,11Z,13E-eicosatetraenoic acid) and lipoxin B4 (5S,14R,15S-trihydroxy-6E,8Z,10E,12E-eicosatetraenoic acid). These compounds contain hydroxyls in the positions where the epoxide exists in 5,6-LTA_4 and 14,15-LTA_4 (lipoxin A4 contains hydroxyls at C5 and C6 and lipoxin B4 contains hydroxyls at C14 and C15). One pathway of lipoxin formation could be hydrolysis of the leukotriene expoxide followed by enzymatic oxygenation of C15 (in the case of lipoxin A4) or C5 (in the case of lipoxin B4) to produce the trioxygenated product.

SUMMARY AND CONCLUSIONS

The size difference (about 115 residues) and low homology between the amino-termini of plant and mammalian lipoxygenases indicates that the amino-

terminal β-barrel domain of the SB1 structure is not present in mammalian lipoxygenases. Analysis of conserved residues indicates that the catalytic domains of human lipoxygenases are structurally similar to the SB1 catalytic domain and that conserved residues duplicate orientations found in the SB1 structure.

Analysis of several interesting residues indicates their possible roles in lipoxygenases. Residue **D490** appears to form a salt bridge with R707 in plant lipoxygenases and with R487 in mammalian lipoxygenases. Residue H494, a candidate for ionic interaction with substrate, is uncharged and forms a hydrogen bond, which may be important for initiating the π-helical section of Helix 9. Residue N/H694 has an important function in lipoxygenases, perhaps involving oxygen binding, but could not be modeled as an iron ligand without shifting the main chains of nearby residues.

Analysis of unconserved residues in one pocket of the putative substrate binding site (residues 491, 552, 556, 557, 701, 747, 750, and 751), combined with results of mutagenesis studies, provides the underpinnings of a model which accounts for lipoxygenase positional specificity and stereospecificity. The model predicts that the methyl terminus of arachidonic acid binds in this pocket in 12- and 15-lipoxygenases and that the carboxy terminus of arachidonic acid binds in this pocket in 5- and 8-lipoxygenases. In 12- and 15-lipoxygenases, the bulkiness of hydrophobic residues in the pocket (especially residues 491, 556, and 557) affects how deeply substrate binds, thereby altering positional specificity. In 5- and 8-lipoxygenases, a similar mechanism would determine positional specificity—with the exception that some residues in the pocket would be hydrophilic to interact with the carboxy terminus of substrate. In 5-lipoxygenases, H747 and N557 may fulfill this role (hydrophilic residues are expected to be found in this area in 8-lipoxygenases when sequences become available).

In the catalytic mechanism of lipoxygenases, the ferric form of the enzyme accepts one electron from the substrate while a base abstracts a proton from the methylene carbon of the reactive 1,4-diene. The resulting intermediate could be described as having an Fe^{3+}-substrate organoiron bond or an Fe^{2+}-substrate free radical complex.[††] Dioxygen reacts with the intermediate, regenerating Fe^{3+} and producing a peroxidate anion, which then receives the proton from the base to give the hydroperoxide product. Rough placement of arachidonic acid in the three human lipoxygenase models positions the reactive pentadiene moiety in the vicinity of the empty iron coordination site (opposite **H690**) in the substrate binding cavity. The positioning is consistent with the organoiron intermediate and it is consistent with the free radical intermediate if the proton-abstracting base is near the unoccupied iron coordination site. There are three plausible candidates for the base in this area: the Nε of **H499,** the second oxygen of the terminal carboxylate (**I839**), or an iron-bound hydroxyl.

[††]Evidence in favor of an Fe^{3+}–carbon intermediate has been provided by kinetic studies with variations in substrate structure and by chemical analogy.[72,73] Evidence for the Fe^{2+}-free radical pathway is based on anaerobic reduction of Fe^{3+} by fatty acid substrate[74] and on detection of substrate free radicals.[75–77] The detection of substrate free radicals does not rule out the organoiron intermediate because an Fe^{3+}–carbon bond could undergo anaerobic dissociation to form Fe^{2+} and a free radical.

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