Applicability of the Triad Concept for the Positional Specificity of Mammalian Lipoxygenases*

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Robert Vogel†, Christian Jansen‡, Jana Roffeis§, Pallu Reddanna‡, Pontus Forsvell¶, Hans-Eric Claesson¶, Hartmut Kuhn††, and Matthias Walther‡

From the †Institute of Biochemistry, University Medicine Berlin-Charité, Monbijoustrasse 2, D-10117 Berlin, Germany, the ‡School of Life Sciences, University of Hyderabad, Hyderabad-500046, Andhra Pradesh, India, ¶Orexo AB, S-10234 Stockholm, Sweden, and the ††Department of Medical Biophysics and Biochemistry, Karolinska Institutet, S-17177 Stockholm, Sweden

The nomenclature of lipoxygenases (LOXs) is partly based on the positional specificity of arachidonic acid oxygenation, but there is no unifying concept explaining the mechanistic basis of this enzyme property. According to the triad model, Phe-353, Ile-418, and Ile-593 of the rabbit 12/15-LOX form the bottom of the substrate-binding pocket, and introduction of less space-filling residues at either of these positions favors arachidonic acid 12-lipoxygenation. The present study was aimed at exploring the validity of the triad concept for two novel primate 12/15-LOX (Macaca mulatta and Pongo pygmaeus) and for five known members of the mammalian LOX family (human 12/15-LOX, mouse 12/15-LOX, human 15-LOX2, human platelet type 12-LOX, and mouse (12R)-LOX). The enzymes were expressed as N-terminal His tag fusion proteins in E. coli, the potential sequence determinants were mutated, and the specificity of arachidonic acid oxygenation was quantified. Taken together, our data indicate that the triad concept explains the positional specificity of all 12/15-LOXs tested (rabbit, human, M. mulatta, P. pygmaeus, and mouse). For the new enzymes of M. mulatta and P. pygmaeus, the concept had predictive value because the positional specificity predicted on the basis of the amino acid sequence was confirmed experimentally. The specificity of the platelet 12-LOX was partly explained by the triad hypothesis, but the concept was not applicable for 15-LOX2 and (12R)-LOX.

Lipoxygenases (LOXs) catalyze the stereo-specific peroxidation of free and/or esterified polyunsaturated fatty acids to corresponding hydroperoxy compounds and contribute to lipid signaling in mammalian cells (1, 2). According to the conventional nomenclature, LOX isoforms are classified with respect to their positional specificity of arachidonic acid oxygenation, but due to the increasing heterogeneity of the LOX family, this classification has become rather confusing in recent years. LOX genes are widely distributed in eukaryotes, and the corresponding enzymes have been characterized in plants (3), animals (4-5), human pathogenic microbes (6), and lower marine organisms (7, 8). LOX genes have also been detected in various bacteria (9, 10), but it remains unclear whether they have evolved from a joint ancient precursor or whether they were introduced by horizontal gene transfer (9, 11). The human genome contains six functional LOX genes that encode for six different LOX isoenzymes (12). Five are catalytically active as fatty acid dioxygenase (5-LOX, platelet type 12-LOX, 12/15-LOX, 15-LOX2, and (12R)-LOX), whereas eLOX3 exhibits a hydroperoxidase activity (13). The murine genome involves seven functional LOX genes (12).

Most LOXs exhibit a remarkable specificity of fatty acid oxygenation. They selectively oxygenate substrates to a single chiral product isomer (singular positional specificity). Other LOX isoforms (e.g. rabbit 12/15-LOX) exhibit a dual positional specificity because they are capable of oxygenating fatty acids to two chiral product isomers (14, 15). The 5-LOX from potato tubers oxygenates arachidonic acid to a complex product pattern, suggesting that the active site is flexible enough to accommodate substrates in various conformations (16). The structural basis for LOX specificity has been investigated in the past, and specificity determinants have been identified (17-21). The human 15-LOX2 can be converted to an 8-lipoxygenating enzyme when two critical amino acids are mutated (20). Vice versa, the mouse epidermis type 8-LOX can be converted to a 15-LOX when the corresponding residues are targeted (20). Multiple amino acid sequence alignments of a large number of (S)- and (R)-LOXs suggested that (R)-LOXs contain a critical Gly residue at the active site, which is an Ala in (S)-LOX, and mutagenesis studies on several LOX isoforms confirmed a role of this residue in reaction specificity (21, 22).

Amino acid alignments and mutagenesis studies on the human 12/15-LOX (15-LOX1) suggested a role of Ile-418 and Met-419 in the positional specificity (17). Similar results have since been obtained for the rabbit ortholog (19), but corresponding mutations on the rat enzyme were not consistent with these results (23). These data and additional mutagenesis studies on various 12/15-LOX isoforms prompted the development of the “triad concept” explaining the reaction specificity of the rabbit 12/15-LOX (18, 19). This hypothesis emphasized that a triad of amino acids (Phe-353, Ile-418, and Ile-593) is important for the positional specificity. These residues form the bottom of a boot-shaped substrate-binding pocket (Scheme 1), and their side-chain geometry impacts the volume of the active site (19). Insertion of small residues at these positions increases the depth of the active site so that arachidonic acid can slide in...
 deeper into the pocket favoring hydrogen removal from C10 and, thus, 12-lipoxygenation (Scheme 1). This concept explained the reaction specificity of the rabbit 12/15-LOX, and scattered mutagenesis studies on other isoforms (24, 25) were consistent with this hypothesis. However, for other LOX isoforms, this concept was not applicable (26), and alternative hypotheses have been suggested (27). Recently, the 1.85 Å structure of a coral (BR)-LOX was solved (28). Based on the X-ray coordinates, the authors proposed an alternative model for substrate binding in a U-shaped pocket, which interconnects different surface areas of the protein (front door entrance and back door entrance). Although the functionality of the back door entrance has not been tested experimentally, the U-shaped concept challenges the triad hypothesis.

In light of this new information, we aimed at testing the applicability of the triad concept for various primate 12/15-LOX (human, *Macaca mulatta*), and *Pongo pygmaeus* as well as for mouse 12/15-LOX, human platelet type 12-LOX, human 15-LOX2, and mouse (12R)-LOX. Our data indicate that this concept explains the positional specificity of mammalian 12/15-LOX and has predictive value for the enzymes from *M. mulatta* and *P. pygmaeus*. However, it fails to explain the specificity of epidermal type 15-LOX2 and (12R)-LOX.

**MATERIALS AND METHODS**

**Chemicals**—The chemicals used were obtained from the following sources: arachidonic acid (5Z,8Z,11Z,14Z)-eicosatetraenoic acid) from Serva (Heidelberg, Germany); HPLC standards of (5S)-HETE, (12S)-HETE, 12(±)-HETE, (15S)-HETE, and 15(±)-HETE from Cayman Chemical (distributed by Spi Bio, Montigny le Bretonneux, France); sodium borohydride, ampicillin from Invitrogen, isopropyl-β-thiogalactopyranoside from Carl Roth GmbH (Karlsruhe, Germany). HPLC solvents were from New England Biosciences (Schwalbach, Germany). Oligonucleotide synthesis was performed at BioTez (Berlin, Germany), and nucleic acid sequencing was carried out at Eurofins MWG Operon (Ebersberg, Germany). The *Escherichia coli* strain XL-1 Blue was purchased from Stratagene (La Jolla, CA).

**Bacterial Expression and Site-directed Mutagenesis**—The various LOX isoforms (human 12/15-LOX, platelet 12-LOX, *M. mulatta* 12/15-LOX, *P. pygmaeus* 12/15-LOX, mouse 12/15-LOX, human 15-LOX2, murine (12R)-LOX) were expressed as N-terminal His tag fusion proteins in *E. coli* as described previously (29). For this purpose, the cDNAs were cloned into the pQE-9 procaroytic expression plasmid in such a way that the starting methionine of the LOX coding sequence was deleted. Because of technical reasons, the N terminus was elongated by additional amino acids including six consecutive His residues. Site-directed mutagenesis was performed using the QuikChange™ site-directed mutagenesis kit (Stratagene, Amsterdam, The Netherlands). For each mutant, 5–10 clones were selected and screened for LOX expression, and one clone was completely sequenced to confirm mutagenesis.

**Enzyme Purification**—Wild-type enzymes and selected mutants were purified to apparent electrophoretic homogeneity by sequential affinity chromatography on a nickel-TED column and anion exchange chromatography. For purification, LOX-active clones were picked with a sterilized toothpick, and 100 ml of LB medium containing ampicillin (0.1 mg/liter) were inoculated. After overnight culture at 37 °C, a 5-ml aliquot was taken off of these precultures and processed for an activity assay. The most active clone was taken for large scale enzyme expression and purification. The 100 ml of preculture was added to 18 liters of LB medium containing ampicillin (0.1 mg/liter), and bacteria were grown at 37 °C to an optical density at 600 nm of about 0.5. Expression of recombinant LOX was initiated by the addition of 1 mm isopropyl-β-thiogalactopyranoside. The large scale culture was incubated at 30 °C for 2 h. Then cells were spun down, resuspended in PBS, and lysed with an EmulsiFlex-C5 high pressure homogenizer (Avestin, Ottawa, Canada). Cell debris was spun down, the supernatant was added to 1 ml of nickel-TED (Machery-Nagel, Düren, Germany), and the suspension was incubated for 1 h at 4 °C. The nickel-TED was transferred to an open bed column and washed with washing buffer (50 mM NaH2PO4, 300 mM NaCl, pH 8.0), and adhering proteins were eluted with elution buffer (50 mM NaH2PO4, 300 mM NaCl, 200 mM imidazole, pH 8.0). The LOX was purified to apparent electrophoretic homogeneity.
activity of the elution fractions was tested, employing the spectrophotometric assay. The pooled LOX containing nickel-TED fractions were desalted on an Econo-Pac 10DG column (Bio-Rad) and further purified by fast protein liquid chromatography on a Resource Q column (GE Healthcare).

Activity Assays—For the final activity assay, one sequenced clone was replated, at least four well separated colonies were picked, and the bacteria were cultured at 37 °C in 5 ml of LB medium containing 0.1 mg/ml ampicillin overnight. LOX expression was induced by the addition of isopropyl-β-thiogalactopyranoside (1 mM final concentration). After 2 h at 30 °C, bacteria were spun down, washed, and resuspended in 0.5 ml of PBS. Arachidonic acid was added on ice (100 μM final concentration), and cells were lysed by sonication with a Labsonic U-tip sonifier (Braun, Melsungen, Germany). The mixture was incubated for 15 min at 37 °C, the hydroperoxy compounds formed were reduced with sodium borohydride, and after acidification to pH 3 (acetic acid), 0.5 ml of methanol was added. The protein precipitate was spun down, and aliquots of the clear supernatant were injected directly for quantification of the LOX products to RP-HPLC. For purified enzyme preparations, LOX activity was assayed spectrophotometrically, recording the time-dependent increase in absorbance at 235 nm (Shimadzu UV-2100 photometer). The reaction mixture was treated accordingly (sodium borohydride, acetic acid, and methanol; see above), and after centrifugation, it was injected to RP-HPLC to examine the reaction specificity of the purified enzyme.

Analytics and Statistics—HPLC analysis of the LOX products was performed on a Shimadzu instrument equipped with Hewlett-Packard diode array detector 1040 A by recording the absorbance at 235 nm. Reverse phase HPLC was carried out on a Nucleodur C18 Gravity column (Marchery-Nagel, Düren, Germany; 250 × 4 mm, 5-μm particle size) coupled with a guard column (8 × 4 mm, 5-μm particle size). A solvent system of methanol/water/acetic acid (85:15:0.1, by volume) was used at a flow rate of 1 ml/min. Straight phase HPLC was performed on a Nucleosil 100-5 column (250 × 4 mm, 5-μm particle size) with the solvent system n-hexane/2-propanol/acetic acid (100:2:0.1 by volume) and a flow rate of 1 ml/min. Hydroxy fatty acid enantiomers were separated by chiral phase HPLC. The reaction specificity of wild-type primate 12/15-LOX. The 12/15-LOXs from humans, M. mulatta, and P. pygmaeus were expressed as recombinant His tag fusion proteins in E. coli and purified by affinity chromatography on nickel-TED, and their specificity of arachidonic acid oxygenation was analyzed by RP-HPLC (see “Materials and Methods”). The enantiomer composition of the major reaction products (insets) was quantified by chiral phase HPLC. The chemical structure of the reaction products was confirmed by cochromatography on RP-HPLC, straight phase HPLC, and chiral phase HPLC. The differences between the various mutants were highly significant (p < 0.001).

1) to identify potential sequence determinants of other LOX isoforms. Human 12/15-LOX oxygenates arachidonic acid to a mixture of (12S)- and (15S)-H(p)ETE (30), and we confirmed this finding for the pure His tag fusion protein (Fig. 2). On the basis of the triad concept, a similar reaction specificity was predicted for the P. pygmaeus LOX because the positions of the three sequence determinants were occupied by amino acids with rather bulky side chains. HPLC analysis of arachidonic acid oxygenation products confirmed this suggestion (Fig. 2). In contrast, the LOX from M. mulatta contained a small amino acid at the position that aligns with Ile-418 of the rabbit enzyme, and thus, according to the triad concept, it should catalyze arachidonic acid 12-lipoxygenation. In fact, the purified recombinant His tag fusion protein converted arachidonic acid mainly to (12S)-H(p)ETE, with (15S)-H(p)ETE being a minor side product (Fig. 2).

Mutagenesis of Specificity Determinants of Human 12/15-LOX

Mutation of Ile-418/Met-419 to residues that were present at these positions in 12-lipoxygenating enzyme species converted human 12/15-LOX to a 12-lipoxygenating enzyme species (17). However, the impact of the other two members of the determinant triad has not been investigated. For this study, we first
Mutations of Phe-353 (Borngräber I Determinant)—Introduction of a small amino acid at 353 (F353L) converted human 12/15-LOX to a 12-lipoxygenating enzyme species (88% 12-H(p)ETE). When we further reduced the size of the side chain at this position (F353A) to force complete conversion of the reaction specificity, the enzyme lost its catalytic activity (<1% residual activity), and the reaction specificity was strongly impaired. For this mutant, we observed large shares of 11-HETE (26%) and 8-HETE (28%) in addition to 12-HETE (40%) and 15-HETE (6%).

Mutations of Ile-593 and Met-419 (Sloane Determinant)—When Ile-418 of the human 12/15-LOX was mutated to V, which is present at this position in 12-lipoxygenating enzymes, the positional specificity was partially altered in favor of 12-H(p)ETE formation (12-H(p)ETE/15-H(p)ETE ratio of 60:40) (17). When we introduced an even smaller Ala (I418A), almost complete conversion of the positional specificity was observed (Table 1). Next, we mutated the amino acid located adjacent to Ile-418 to a smaller residue (M419V) and found partial conversion of the positional specificity toward 12-lipoxygenation (Table 1).

Mutations of Ile-593 (Borngräber II Determinant)—Finally, we introduced a less space-filling residue at Ile-593 (I593A) and also observed an increase in the share of 12-H(p)ETE (Table 1). It should be stressed at this point that all major oxygenation products formed by the mutants were chiral, showing a strong preponderance of the S enantiomer (>90%).

To test whether the effect introduced by mutation of a single sequence determinant can be reversed by mutation of another constituent of the determinant triad, we employed a multiple mutagenesis strategy. The wild-type enzyme (Table 1) converted arachidonic acid mainly to (15S)-H(p)ETE (share of 15-H(p)ETE was 86.0%). In contrast, the F353L mutant was a 12-lipoxygenating enzyme species (share of 15-H(p)ETE was 12%). Next, we introduced a bulky Phe at Ile-418 into the 12-lipoxygenating F353L mutant. According to the triad concept, this mutation should reverse the spatial effect induced by F353L, and thus, the share of 15-H(p)ETE formation was expected to be increased again. In fact, the F353L/I418F double mutant oxygenated arachidonic acid to a mixture of 15-H(p)ETE and 12-H(p)ETE (share of 15-H(p)ETE was 36%) (Table 1), and the increase in 15-HETE formation was highly significant (p < 0.001) when compared with the F353L mutant. Finally, we introduced a small Ala at Ile-593 to force again a decrease in 15-H(p)ETE formation. The F353L/I418F/I593A triple mutant converted arachidonic acid to a mixture of 12- and 15-H(p)ETE, and the share of 15-H(p)ETE was significantly lower (Table 1) than that produced by the F353L/I418F double mutant (30% versus 36%, p < 0.05). It should be stressed at this point that the triple mutant only exhibited some 30% residual catalytic activity when compared with the wild-type enzyme. These data suggest that the triad model of the reaction specificity, which was developed for the rabbit 12/15-LOX (19), also explains the specificity of the human enzyme.

Mutagenesis of Specificity Determinants in 15-Lipoxygenating P. pygmaeus 12/15-LOX

When we applied a similar mutagenesis strategy to 12/15-LOX of P. pygmaeus, we found that separate mutation of the specificity determinants altered the reaction specificity as predicted by the triad hypothesis.

Borngräber I Determinant—Introduction of a small residue at Phe-353 (F353L) converted the enzyme to an arachidonic acid 12-lipoxygenating enzyme, and similar results were obtained for the F353V mutant (Table 2). When we introduced an even smaller A (F353A), an almost inactive mutant resulted (<2% residual activity).

Sloane Determinants—As expected, I418A exchange induced dominant arachidonic acid 12-lipoxygenation (Table 2). Interestingly, the specific activity of this mutant was more than 9-fold higher than that of the wild-type enzyme, suggesting a more optimal substrate alignment at the active site. Next, we mutated Met-419 (adjacent to Ile-418) to a less space-filling residue (M419V) and observed partial conversion of the positional specificity toward 12-lipoxygenation (Table 2).

Borngräber II Determinant—As for the human enzyme, I593A exchange only induced partial alterations in the positional specificity (Table 2), suggesting that Phe-353 and Ile-418 have higher priority for the reaction specificity than Ile-593.

We next created double and triple mutants to test spatial interactions between the sequence determinants. As indicated in Table 2, introduction of a less bulky residue at Phe-353 (F353L) induced an almost complete switch in positional specificity in favor of 12-lipoxygenation, and this finding was con-
firmed in this series of experiments (Fig. 3). When we next mutated Ile-418 in the F353L mutant to a more space-filling amino acid (F353L/I418F), we observed an increased share of 15-H(p)ETE (13% for F353L versus 35% for F353L/I418F). Thus, the effect on the reaction specificity induced by F353L was partially reversed in the F353L/I418F double mutant. This reversal was significantly (p < 0.01) more pronounced when a bulkier Trp was inserted at Ile-418; the share of 15-H(p)ETE formation for the F353L/I418F double mutant was increased to 48.4 ± 1.4%. Finally, we introduced a small Ala at Ile-593 of the F353L/I418F double mutant in order to reduce 15-H(p)ETE formation again. Indeed, we found that the share of 15-H(p)ETE formation was lowered to about 24% for the F353L/I418F mutant. This finding of positional specificity of the two enzymes. Introduction of more space-filling residues at Ile-593 should alter the positional specificity of LOX isomers in favor of 15-lipoxygenation. However, when we assayed the positional specificity of the I593F mutant, we did not find significant differences from the wild-type enzyme. When introducing an even bulkier Trp, the share of 15-HETE was slightly up (2.8 ± 1.4%, p < 0.01). These mutagenesis data suggest that the triad concept can be applied for the 12-lipoxygenating 12/15-LOX of M. mulatta. However, for this LOX isoform, the Borngräber determinants (Phe-353 and Ile-593) are not of major importance.

The mouse 12/15-LOX carries three small residues at the triad positions (Leu-353, Val-418, and Val-593), and the native enzyme exhibits major arachidonic acid 12-lipoxygenation (31). The recombinant protein converted arachidonic acid to 12-H(p)ETE (76.4 ± 3.1%), with 15-H(p)ETE being a minor product (23.5 ± 3.1%). When we introduced more bulkier residues, the share of 15-H(p)ETE increased (Borngräber I: 50.9 ± 1.6% for L353F; Borngräber II: 64.5 ± 6.2% for V593F; Sloane: 66.6 ± 5.1% for V418F). Although these changes were not complete they were highly significant when compared with wild type (p < 0.001 for all mutants).

Mutagenesis Studies on Human Platelet 12-LOX

Although the human platelet 12-LOX shares a high degree (65%) of amino acid conservation with the human 12/15-LOX, it oxygenates arachidonic acid exclusively to 12-H(p)ETE (32). A similar reaction specificity was previously reported for the recombinant enzyme (24), and we confirmed this result in our study (Fig. 5). Previous mutations of the Sloane determinants (A417I and K416Q/A417I/V418M) only induced minor
Specificity of Primate LOX

Mutagenesis of Human 15-LOX2 and Murine 12R-LOX Does Not Support the Triad Concept

For the human 15-LOX2, Asp-602 and Val-603 have been identified as sequence determinants for the positional specificity (20, 33). To test applicability of the triad concept for this LOX isomerase, we mutated the corresponding amino acids and obtained the following results. (i) The pure wild-type enzyme converted arachidonic acid to (15S)-H(p)ETE, with 12-H(p)ETE being almost undetectable (<1%). (ii) When Phe-365, which aligns with Phe-353 of the rabbit enzyme (Borngräber I), was mutated to Leu (F365L), we found a small but significant increase in 12-H(p)ETE formation (5.0 ± 1.0%, p < 0.01), but the catalytic activity dropped down to about 8%. (iii) Ile-418 of the rabbit enzyme (Sloane determinant) aligned with Ser-430 of human 15-LOX2. This amino acid was mutated to a less bulky residue (S430A) to force 12-lipoxygenation. However, analyzing the reaction products formed, we only observed very small amounts of 12-H(p)ETE (2.0 ± 0.1%). Finally, we exchanged Ala-606 (Borngräber II determinant) to a smaller Gly (A606G) but did not observe major alterations in positional specificity (2.0 ± 0.2% 12-H(p)ETE). These data suggest that Phe-365, Ser-430, and Ala-606 of human 15-LOX2 do not function as major sequence determinants and that the triad concept may not be applicable for this enzyme species.

Previously, we attempted to convert the murine (12R)-LOX to a 15-lipoxygenating enzyme species by mutating the corresponding amino acids. The F390W (Borngräber 1) and the V631F (Borngräber 2) mutants were catalytically inactive. When mutating the Sloane determinants (A455I and A455W), we created enzyme species with strongly reduced specific activity, which converted arachidonic acid to a mixture of all positional H(p)ETE isomers (except 5-H(p)ETE)) (26). These data suggest that the triad concept also may not be applicable for (12R)-LOX.

DISCUSSION

According to their phylogenetic relatedness, 12/15-LOX form a separate subfamily of mammalian LOX (5), and different orthologs exhibit variable positional specificities. Most mammalian species (mouse, rats, pigs, cattle, and M. mulatta) express 12-lipoxygenating 12/15-LOX, but in humans and P. pygmaeus, the enzyme constitutes a 15-lipoxygenating isoform. Rabbits are the only animal species for which separate genes encoding for a 12- and a 15-lipoxygenating 12/15-LOX have been described (34). Previous mutagenesis studies on the human 12/15-LOX suggested Ile-418 and Met-419 as sequence determinants for the positional specificity (17), and similar experiments on the rabbit ortholog indicated that Phe-353 (18) and Ile-593 (19) may also play a similar role. On the basis of these data, the triad hypothesis was developed, which explained the molecular basis of the positional specificity of the rabbit enzyme (35). This concept suggested that for 12/15-LOX, the amino acids aligning with Phe-353, Ile-418/Met-419, and Ile-593 of the rabbit enzyme may function as sequence determinants for the positional specificity and that their side chain geometry is of particular importance. In the crystal structure, these residues are clustered and form the bottom of the substrate-binding pocket and thus determine the depth of the pocket. If these positions are occupied by small residues, fatty acid substrates are capable of penetrating deeper into the substrate-binding pocket so that hydrogen abstraction from C10 (12-lipoxygenation) is favored (Scheme 1). In contrast, if more...
space-filling residues are located at these positions, hydrogen is mainly abstracted from C13 (15-H(p)ETE formation) (19, 35). Although this concept explains the positional specificity of the rabbit enzyme, its applicability to other LOX isoforms has not been tested systematically. Here we attempted to fill this gap and found that the 15-lipoxygenating 12/15-LOXs of humans and 
P. pygmaeus as well as the 12-lipoxygenating enzymes from 
M. mulatta and mice follow this concept. The relative importance of the triad constituents is variable for the different 12/15-LOX isoforms. For human and 
P. pygmaeus 12/15-LOXs, Phe-353 (Borngräber I) and Ile-418 (Sloane) play a major role because single mutations of these amino acids to less space-filling residues convert the enzyme almost completely to a 12-lipoxygenating enzyme species. These alterations could be reversed partially when we introduced a more bulky residue at either position (Table I and Fig. 3). As for the rabbit enzyme, single mutation of Ile-593 induced partial alterations in the positional specificity of both enzymes (Tables 1 and 2). Thus, for human, rabbit, and 
P. pygmaeus 12/15-LOX Phe-353 and Ile-418 may be considered first order determinants of the positional specificity, whereas Ile-593 functions as a second order determinant.

The 12-lipoxygenating 12/15-LOX from 
M. mulatta carries a bulky Phe at 353, and further increase of the space requirement at this position (F353W) hardly impacted the positional specificity. Similar observations were made for mutagenesis at Ile-593. Thus, for this LOX isoform, Phe-353 and Ile-593 only play a minor role as sequence determinants, whereas mutations at Val-418 induced drastic changes (Fig. 4). These data suggest that for this 12-lipoxygenating enzyme, the Sloane determinants are most important for reaction specificity. For the 12-lipoxygenating 12/15-LOX from mice, only partial alterations of the positional specificity were obtained when single mutations of the triad constituents were introduced.

Previous studies on the human platelet 12-LOX indicated that combined mutagenesis of the Sloane determinants (Lys-416, Ala-417, and Val-418) lead to an enzyme species that produced about 10–20% 15-H(p)ETE in addition to the major oxygenation product 12-H(p)ETE (24). The share of 15-H(p)ETE was further increased to about 60% when all non-conserved amino acids located between positions 398–429 (7 amino acids) were mutated to the corresponding residues present in the 15-lipoxygenating human 12/15-LOX. Unfortunately, this enzyme mutant only exhibited a residual activity of about 1%, and the enantiomer composition of 15-H(p)ETE as indicator for its enzymatic origin was not determined. In the present study, we found that combined mutagenesis of the Sloane determinants leads to partial alterations in the positional specificity of this enzyme and that both 12- and 15-H(p)ETE originated from enzymatic catalysis (strong preponderance of the S enantiomer). In contrast, mutagenesis studies at the other triad constituents (Phe-352 and Ile-593) did not induce major specificity alterations. Taken together, one may conclude that the triad concept may be applicable for platelet type 12-LOXs but that the Borngräber determinants may not play a major role.

For the time being, it remains unclear whether the reaction specificity of 5-LOXs can also be explained on the basis of the triad hypothesis. Mutagenesis of the triad constituents of rabbit 12/15-LOX to amino acids present at this position in human 5-LOX led to inactive enzyme mutants (36). However, introduction of the rabbit 12/15-LOX determinants into the human 5-LOX led to a gradual increase in (15S)-H(p)ETE formation. In fact, the quadruplet mutant F359W/A424I/N425M/A603I exhibited a major (15S)-lipoxygenase activity (85–95%) with (8S)-H(p)ETE as a minor side product (36). These data suggest that the triad concept might also be applicable at least in part for 5-LOXs, but additional mutagenesis studies on other 5-lipoxygenating isoforms are required to confirm this hypothesis.

The mutagenesis data obtained for the mouse 12R-LOX and the human 15-LOX2 indicated that the triad hypothesis is apparently not applicable for the epidermis type LOX subfamily. Thus, the concept does not constitute a comprehensive model explaining the reaction specificity of all LOX isoforms.

Recently, attempts were made to develop a general scheme explaining the reaction specificity of all LOX (22, 27). This scheme was further developed on the basis of the crystal structure of the coral 8R-LOX (28). The key feature of the new concept is a single active site amino acid, which is conserved as an Ala in most (5)-LOXs (21, 22, 27). In contrast, R-LOXs carry a smaller Gly at this position. This critical amino acid, either alone or in connection with other active site residues (28), appears to control the stereochemistry of the oxygenation reaction by switching the position of oxygen insertion into the pentadienyl radical initially formed by hydrogen abstraction.

Mutagenesis data on several mammalian LOX isoforms (human 15-LOX2, (12R)-LOX, and human pl12-LOX) strongly support this concept since a large share (30–60%) of (R)-lipoxygenation products were formed by the mutant enzymes. However, for other LOX isoforms (mouse 12/15-LOX, 
P. pygmaeus, and 
M. mulatta), the Ala-to-Gly or Gly-to-Ala exchange at this position only induced minor alterations (5–15%) in the reaction specificity, and other enzymes (Danio rerio) lost their catalytic activity. Thus, we concluded that both the Coffa model and the triad concept may not be applicable to all LOX isoforms.

In addition, a novel concept for the substrate-binding pocket of LOX (U-shaped versus boot-shaped binding pocket) was introduced that challenges the triad concept (28). The x-ray coordinates of the coral (8R)-LOX suggest a U-shaped substrate-binding site that reaches the protein surface on both ends (front door and back door entrance). However, for the coral (8R)-LOX, the back door entrance is closed by a salt bridge, and for the soybean and rabbit enzymes, substantial rearrangement of other structural elements is required to open the “back door entrance.” It remains to be shown whether the back door entrance is really of functional relevance. Furthermore, it remains unclear how the mutagenesis data on the triad constituents reported here can be explained by this model. These residues, which have been suggested to form the bottom of the boot-shaped substrate pocket, would be rather distant from the fatty acid substrate in the U-shape model, and thus, it is hard to explain the impact of their side chain geometry on the reaction specificity of arachidonic acid oxygenation.

The principle weakness of all concepts developed to explain the positional specificity of LOX is the lack of direct experimental proof. There is no structural information on catalytically
productive LOX-fatty acid complexes so that the substrate alignment at the active site remains speculative. Moreover, oxygen penetration to the active site and its movement within the substrate binding pocket is not really understood. However, the structural models developed on the basis of amino acid alignments, targeted substrate modification, and site-directed mutagenesis might help to work out experimental strategies to comprehensively clarify the structural basis of LOX specificity. On the other hand, it might even be possible that different LOX isoforms employ different mechanisms to determine their reaction specificity, and thus, there may not be a unifying concept for the reaction specificity of all LOX isoforms.

REFERENCES

1. O’Donnell, V. B., Maskrey, B., and Taylor, G. W. (2009) Methods Mol. Biol. 462, 5–23
2. Wymann, M. P., and Schneiter, R. (2008) Nat. Rev. Mol. Cell Biol. 9, 162–176
3. Liavonchanka, A., and Feussner, I. (2006) J. Biol. Chem. 281, 303–312
4. Yu, Z., Schneider, C., Boeglin, W. E., Marnett, L. J., and Brash, A. R. (2003) J. Biol. Chem. 278, 1145–1153
5. Kühn, H., and Thiele, B. J. (1999) J. Biol. Chem. 274, 23679–23682
6. Neau, D. B., Gilbert, N. C., Bartlett, S. G., Boeglin, W., Brash, A. R., and Newcomer, M. E. (2009) Biochemistry 48, 7906–7915
7. Borngräber, S., Roufis, J., Reimann, I., Huth, A., Borngräber, S., Kühn, H., and Thiele, B. J. (1998) J. Biol. Chem. 273, 149–152
8. Berghäfer, S., Bögel, W. E., and Brash, A. R. (1996) J. Biol. Chem. 271, 20949–20956