Stereocontrol of Arachidonic Acid Oxygenation by Vertebrate Lipoxygenases

NEWLY CLONED ZEBRAFISH LIP Ox YGENASE 1 DOES NOT FOLLOW THE ALA-VERSUS-GLY CONCEPT

Background: R-LOX carries a Gly at a critical position, but S-LOX contains an Ala there.

Results: Zebrafish LOX1 carries a Gly at this position but catalyzed 12S-lipoxygenation.

Conclusion: The Ala-versus-Gly concept is not applicable to zebrafish LOX1.

Significance: Prediction of LOX specificity on the basis of primary structure is not always accurate; functional studies are always required.

Animal lipoxygenases (LOXs) are classified according to their specificity of arachidonic acid oxygenation, and previous sequence alignments suggested that S-LOXs contain a conserved Ala at a critical position at the active site but R-LOXs carry a Gly instead. Here we cloned, expressed, and characterized a novel LOX isoform from the model vertebrate Danio rerio (zebrafish) that carries a Gly at this critical position, classifying this enzyme as putative arachidonic acid R-LOX.

Surprisingly, the almost exclusive arachidonic acid oxygenation product was 12S-H(p)ETE (hydro(pero)xyeicosatetraenoic acid), and extensive mutation around Gly-410 failed to induce R-lipoxygenation. This finding prompted us to explore the importance of the corresponding amino acids in other vertebrate S-LOXs. We found that Ala-to-Gly exchange in human 12-LOX and mouse 15-LOX induced major alterations in the reaction specificity with an increase of specific R-oxygenation products. For mouse 5-LOX and 12/15-LOX from rabbits, men, rhesus monkeys, orangutans, and mice, only minor alterations in the reaction specificity were observed. For these enzymes, S-HETE (hydroxyeicosatetraenoic acid) isomers remained the major oxygenation products, whereas chiral R-HETEs contributed only 10–30% to the total product mixture. Taken together these data indicate that the Ala-versus-Gly concept may not always predict the reaction specificity of vertebrate LOX isoforms.

Lipoxygenases (LOXs) are lipid-peroxidizing enzymes, which occur in pro- (1, 2) and eukaryotic (3, 4) cells but have not been detected in archaea. They catalyze the stereo-selective peroxidation of free and/or esterified polyunsaturated fatty acids to hydroperoxy derivatives (5, 6). The biological roles of LOXs have not yet clearly been defined, but plant LOXs have been implicated in the biosynthesis of phytohormones (7), flavor production (8), and mobilization of storage lipids during germination (9). Mammalian LOXs play a role in normal epidermal differentiation (10) and in the pathogenesis of inflammatory (11, 12) as well as hyperproliferative diseases (13, 14). The human genome contains six functional LOX genes, but in mice, seven LOX genes were identified; the mouse epidemis 12S-LOX gene is a functionless pseudogene in humans (15).

Most LOXs exhibit a remarkable specificity of fatty acid oxygenation. In contrast to non-enzymatic lipid peroxidation, which converts polyenoic fatty acid to a complex mixture of racemic hydroperoxide isomers, most LOXs selectively oxygenate their substrates to a single chiral product (circular reaction specificity). Other LOX isoforms, such as mouse, rabbit, and human 12/15-LOX, exhibit dual reaction specificities (16, 17, 18). They are capable of oxygenating arachidonic acid to chiral 12S- and 15S-H(p)ETE. The structural basis for the positional specificity of various LOX isoforms has been investigated in the past, and sequence determinants have been identified (19–25). Multiple amino acid sequence alignments (25, 26) of confirmed S- and R-LOXs as well as structural data for coral 8R-LOX (27) prompted the development of the Ala-versus-Gly hypothesis. This concept suggested that R-LOXs contain a small Gly at a critical position at the active site. In contrast, S-LOXs carry an Ala instead, and mutagenesis studies indicated the principle possibility of interconverting S- and R-LOX (25–29).

During the past decade, the zebrafish (Danio rerio) has emerged as a suitable model for early vertebrate development (30), and a number of targeted mutations in the zebrafish genome lead to phenotypic alterations that resemble human...
diseases (31). Zebrafish embryos develop externally, and the optical clarity of the embryos allows real time visualization of organogenesis (31, 32). Because of the model character of this organism for vertebrate development and because of the implication of LOX isoforms in skin differentiation (10), we searched a zebrafish peptide database (Ensembl) for potential LOX sequences and found 10 hits. When we compared these sequences with that of the human 15-LOX2, we found that Gly-410 of D. rerio LOX1 aligned with Ala-416 of human 15-LOX2. This residue has been implicated previously in stereoregulation of the LOX reaction (25, 27). However, when we tested the reaction specificity of the recombinant enzyme, we identified 12S-H(p)ETE as the major oxygenation product, which conflicts with the Ala-versus-Gly paradigm of stereoregulation of the LOX reaction. These data and the recent finding that A451G exchange in human eLOX3 hardly altered the product specificity of arachidonic acid oxygenation (33) prompted us to reevaluate the relative importance of this amino acid for the reaction specificity of several mammalian S-LOXs. We found that an Ala-to-Gly exchange induced partial alterations in the reaction specificity, and in most cases, the R-oxygenation products contributed between 10 and 30% to the overall product mixture. These partial alterations indicated the relevance of the critical Ala for the reaction specificity of the tested enzymes. However, they also suggest that for most mammalian LOX isoforms additional parameters are required to ensure a high degree of reaction specificity.

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, 5(±)-HETE, 8S-HETE, 8(±)-HETE, 9S-HETE, 9(±)-HETE, 11S-HETE, 11(±)-HETE, 12S-HETE, 12(±)-HETE, 15S-HETE, and 15(±)-HETE from Cayman Chemical (distributed by SPI-Bio, Montigny le Bretonneux, France); sodium borohydride and ampicillin from Invitrogen; isopropyl β-thiogalactopyranoside from Carl Roth GmbH (Karlsruhe, Germany); and HPLC solvents from Mallinckrodt Baker. Restriction enzymes were purchased from Fermentas (St. Leon-Rot, Germany). Oligonucleotide synthesis was performed at BioTec (Berlin, Germany), and nucleic acid sequencing was carried out at Eurofins MWG Operon (Ebersberg, Germany). The Escherichia coli strain XL1-Blue was purchased from Stratagene (La Jolla, CA).

Search for Lipooxygenases in Zebrafish Genome—First, we carried out a multiple sequence alignment of various animal LOX isoforms (ClustalW2) and searched for a conserved portion of the primary structure. We found a peptide (353WLLAKTWVRNAEFSSHEALTHLHSSHLL) that included two of the iron ligands and used the corresponding sequence of human 15-LOX2 as an in silico probe. Then, with this query sequence, we performed a BLASTP search in the Ensembl PEP_ALL zebrafish database. Hits were aligned with other animal LOXs, and sequences carrying an intact iron ligand sphere were considered potential LOXs.

Cloning and Bacterial Expression of D. rerio LOX1—The ATCC-10167880 zebrafish cDNA (IMAGE clone identification number 6795410) was purchased from LGC Standards (Wesel, Germany) and used as starting material for PCR cloning of D. rerio LOX1. The coding region of D. rerio LOX1 cDNA was amplified, and the 2 kbp PCR fragment was ligated into the pQE-9 bacterial expression vector. During the cloning strategy, the starting methionine of the LOX coding sequence was deleted, and the vector ATG, which was located in front of the multicloning site, was used as the start codon for bacterial expression.

Bacterial Expression and Site-directed Mutagenesis of Other LOX Isoforms—Wild-type and mutant LOX isoforms (human 12/15-LOX, rabbit 12/15-LOX, Macaca mulatta 12/15-LOX, Pongo pygmaeus 12/15-LOX, human 15-LOX2, and murine 5-LOX) were expressed as N-terminal His tag fusion proteins in E. coli as described before (20). For this purpose, the cDNAs were cloned into the pQE-9 prokaryotic expression plasmid in such a way that the starting methionine of the LOX coding sequence was deleted. Because of technical reasons, additional amino acids were incorporated at the N terminus of the tagged fusion protein. 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. Human 5-LOX was expressed in E. coli as a recombinant non-fusion protein, and activity assays were carried out as described before (34).

Activity Assays—For the final activity assay, one sequenced clone was replated, five to eight 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 2.5 h at 30 °C, bacteria were spun down, washed, and resuspended in 0.5 ml of PBS. The cells were lysed by sonication with a Labsonic U-tip sonifier (Braun, Melsungen, Germany), cell debris was removed by centrifugation, and the lysis supernatant was used for the activity assay.

For a routine activity assay, arachidonic acid (100 μM final concentration) was added and incubated for 10 min at 25 °C. The hydroperoxy compounds formed were reduced with sodium borohydride, the mixture was acidified to pH 3, and 0.5 ml of ice-cold methanol was added. The protein precipitate was spun down, and aliquots of the clear supernatant were injected directly for quantification of the LOX products by RP-HPLC.

To check the expression level of recombinant LOX species, aliquots (50 μl) were taken for immunoblotting. For specific LOX staining an RGS-His antibody from Qiagen (Hilden, Germany) was used (1:5000) together with a peroxidase-conjugated anti-mouse IgG secondary antibody (Sigma; 1:5000).

Enzyme Purification—To test whether the reaction specificity of crude and purified LOX species was identical, some wild-type enzymes (12/15-LOX from rabbits, men, P. pygmaeus, and M. mulatta; human platelet 12-LOX; human 15-LOX2; human eLOX3; mouse 5-LOX; and D. rerio LOX1) and selected mutants were purified by affinity chromatography on a nickel-agarose (Qiagen) or a nickel-TED column (Machery-Nagel, Düren, Germany) column. In some cases, LOX was further
purified by Mono Q anion exchange chromatography. The standard purification procedure may be described as follows. LOX-active clones were picked with a sterilized toothpick, and 20–100 ml of LB medium (depending on the volume of the final culture) containing ampicillin (0.1 mg/liter) were inoculated. After overnight culture at 37 °C, 5-ml aliquots were taken off and processed for activity assay. The most active preculture was taken for large scale enzyme expression and purification. The precultures were added to 3–15 liters of LB medium (5 ml/liter) containing ampicillin (0.1 mg/liter), and bacteria were grown at 37 °C overnight. Expression of recombinant LOX was initiated by the addition of 1 mM isopropyl β-thiogalactopyranoside. After 2–6 h at 25–30 °C depending on the LOX isoform, the cells were spun down, resuspended in 40 ml of PBS, and lysed with an EmulsiFlex-C5 high pressure homogenizer (Avestin, Ottawa, Canada). Cell debris was spun down at 20,000 × g, and the supernatant was incubated with nickel-agarose beads or nickel-TED for 1 h at 4 °C. The nickel-agarose beads or nickel-TED, respectively, were 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). LOX activity of the elution fractions was tested using the activity assay, and enzyme-containing fractions were either supplemented with glycerol (10% final concentration), shock frozen in liquid nitrogen and stored at −80 °C, or further purified. For this purpose, enzyme fractions were desalted (Econo-Pac 10DG columns, Bio-Rad) and separated by FPLC using a Resource Q column (GE Healthcare).

**Analytcs**—HPLC analysis of the LOX products was performed on a Shimadzu instrument equipped with a 1040A Hewlett-Packard diode array detector by recording the absorbance at 235 nm. Reverse phase HPLC was carried out on a Nucleosil C₁₈ column (Machery-Nagel; KS system, 250 × 4 mm, 5-μm particle size) coupled with a guard column (30 × 4 mm, 5-μm particle size). A solvent system of methanol/water/acetic acid (80:20:0.1 by volume) was used at a flow rate of 1 ml/min. Straight phase HPLC (SP-HPLC) was performed on a Zorbax-SIL 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 (CP-HPLC). 15-HETE enantiomers were separated as free fatty acids on a Chiralcel OD column (Daicel Chemical Industries, Ltd.) using a solvent system consisting of hexane/2-propanol/acetic acid (100:5:0.1 by volume) and a flow rate of 1 ml/min. 9-HETE enantiomers were separated as methyl esters on a Chiralcel OB column (Daicel Chemical Industries, Ltd.) using a solvent system consisting of hexane/2-propanol/acetic acid (100:4:0.1 by volume) and a flow rate of 1 ml/min. 8-HETE enantiomers were also separated as methyl esters on a Chiralcel OD column using a solvent system consisting of hexane/2-propanol/acetic acid (100:3:0.1 by volume) and a flow rate of 1 ml/min. 12-HETE enantiomers were separated as methyl esters on a Chiralcel OD column using a solvent system consisting of hexane/2-propanol/acetic acid (100:5:0.1 by volume). 5-HETE enantiomers were separated as methyl esters on a Chiralcel OD column using a solvent system consisting of hexane/2-propanol/acetic acid (100:3:0.1 by volume) and a flow rate of 1 ml/min.

**RESULTS**

**Lipoxygenase Sequences in Zebrafish Genome**—To search the zebrafish database for LOX sequences, we constructed in silico probes that mirror a highly conserved LOX oligonucleotide region. This region was identified by multiple sequence alignment and contained two of the four iron-liganding His residues of the rabbit enzyme (His-361 and His-366). Using this probe, we detected 10 different LOX cDNA sequences (supplemental Fig. 1), some of which originate from different genes. In addition, different alternative splice variant transcripts were found. Among these sequences, D. rerio LOX1 has been classified as 12-LOX, although its degree of sequence homology was not significantly higher when compared with other human LOX isoforms (Table 1). However, when we constructed a phylogenetic tree (supplemental Fig. 2), we found that this LOX isoform is most closely related to 12/15-LOX and platelet-type 12-LOX. Further inspection of this sequence (Fig. 1) revealed small amino acids (Val and Val) at positions 424 and 425 precluding 12-LOX activity according to the triad concept (20).
### Specifity of Vertebrate LOX

| Sequence   | Species                  | LOX Type |
|------------|--------------------------|----------|
| 400 KLLIPHTY RT NLNITLAREL LIVPGQVVD R  | human 15-LOX2 |          |
| 425 KLLIPHTRYN VQINSIGRAL LLNKGGLSAR   | mouse 12R-LOX  |          |
| 394 KLLTMLRYT LEINCRGRTQ LLSPEGIFKR    | zebrafish 12-LOX |          |
| 387 KLLIPHTY RT NLNIPARTG LVSDMGFDQ   | human 12/15-LOX |          |
| 388 KLLIPHTY RT NLNIPRANG LVSDFGTQD   | rabbit 12/15-LOX |          |
| 388 KLLIPHTY RT NLNIPRARS D LIsE1FGFDK  | mouse 12/15LOX  |          |
| 387 KLLIPHTY RT NLNIPARTG LVSDMGIDQ   | Macaca mul., 12/15-LOX |          |
| 387 KLLIPHTY RT NLNIPRARTQ LIsDGGFDQ   | Pongo pyg., 12/15-LOX |          |
| 387 KLLIPHTY RT NLNIPRARTQ LIsDGGFDQ   | human platelet 12-LOX |          |
| 387 KLLIPHTY RT NLNIPRARTQ LIsDGGFDQ   | human 5-LOX  |          |

Interestingly, Gly-410 of *D. rerio* LOX1 aligned with Ala-416 of human 15-LOX2 and Gly-441 of mouse 12R-LOX suggesting predominant R-oxygenation according to the Ala-versus-Gly concept. Thus, on the basis of the sequence data, one would expect that the *D. rerio* LOX1 might function as a 12R-oxygenase. In contrast, the other zebrafish LOX sequences carried an Ala or a Val at the position aligning with Gly-410 of *D. rerio* LOX1 so that arachidonic acid *S*-oxygenation was hypothesized.

*D. rerio* LOX1 Oxygenates Arachidonic Acid Mainly to 12S-H(p)ETE—To test the reaction specificity of *D. rerio* LOX1, we expressed the enzyme as an N-terminal His tag fusion protein in *E. coli*, purified the enzyme by affinity chromatography on nickel-agarose, and quantified the pattern of arachidonic acid oxygenation products. RP-HPLC (Fig. 2) suggested that as predicted 12-H(p)ETE was the dominant conjugated diene synthesized during a 15-min incubation period; other HETE isomers were only formed in minor quantities. SP-HPLC of the purified 12-H(p)ETE peak (not shown) confirmed that the major reaction product was 12-H(p)ETE but also revealed that small amounts of 9-H(p)ETE were produced, which is not well separated from 12-H(p)ETE in RP-HPLC. To analyze the enantioomer composition of 12-HETE, chiral phase HPLC was carried out. As indicated in Fig. 2B, 12S-HETE was the major oxygenation product. This result was rather surprising because the sequence data predicted major 12R-oxygenation. To explore whether alterations in the bulkiness of the side chains of Gly-410 might force changes in the stereochemistry, we mutated this Gly to Ala, Leu, and Phe. The G410A mutant exhibited 83.0% and converted arachidonic acid almost exclusively to 12S-H(p)ETE. Unfortunately, the G410L and the G410F mutants were catalytically inactive. To test the possible contribution of neighboring amino acids, we created two double mutants (C408S/I409 and T412A/Q413V) in which we gently reduced the volume of the side chains to favor 12R-lipoxygenation. Unfortunately, these mutants were catalytically inactive so possible alterations in the product composition could not be evaluated.

Impact of Ala-to-Gly Exchange on Reaction Specificity of Various Mammalian LOX—It has been reported before (25) that A416G exchange strongly altered the reaction specificity of arachidonic acid oxygenation of human 15-LOX2 in favor of 11R-HETE formation. In our hands, wild-type 15-LOX2 oxygenated arachidonic acid almost exclusively to 15S-H(p)ETE. In contrast, the A416G mutant formed 11R-H(p)ETE as the major reaction product (71%), and 15S-HETE was synthesized in smaller amounts (Fig. 3), which is consistent with previously reported results (25).

Mammalian 12/15-LOXs exhibit dual positional specificities, and dominant 15-lipoxygenating LOXs (rabbits, men, and orangutans) can be separated from mainly 12-lipoxygenating enzymes (mice, rats, pigs, and rhesus monkeys). This classification may be of evolutionary interest (35). We first performed Ala-to-Gly exchanges on 15-lipoxygenating 12/15-LOX. For human and rabbit 12/15-LOX, we observed partial alterations in the reaction specificity. The major reaction product for both wild-type enzyme and the corresponding Ala-to-Gly mutant was 15S-H(p)ETE. However, the share of 11R-H(p)ETE, which was virtually absent for the two wild-type enzymes, amounted to 23% for the corresponding Ala-to-Gly mutants (Table 2), and the differences were statistically highly significant (*p* < 0.001, *t* test). Similar results were obtained with the 12/15-LOX from orangutan (*P. pygmaeus*). To confirm similar alterations for 12-lipoxygenating 12/15-LOX, we first performed corresponding mutations on the I418A mutant of the rabbit 12/15-LOX. It has been reported before that this artificial enzyme species converts arachidonic acid almost exclusively to 12S-H(p)ETE (36). According to the Ala-versus-Gly concept, 8R-HETE was expected to be a major product of arachidonic acid oxygenation by the I418A/A404G double mutant, and we observed that this compound contributed about 12% to the overall product mixture. Similarly, the A404G (mouse) and A403G (rhesus monkey) mutants of 12-lipoxygenating 12/15-LOX synthesized significant amounts of 8R-H(p)ETE (15% for mouse LOX and 14% for rhesus monkey LOX).

A403G exchange in the human platelet 12-LOX induced more drastic alterations. The wild-type enzyme oxygenated arachidonic acid almost exclusively to 12S-H(p)ETE. In contrast, for the A403G mutant, 8R-H(p)ETE (57%) was the major arachidonic acid oxygenation product (Fig. 4), whereas 12S-H(p)ETE contributed about 43% to the product mixture (Table 2).
To test the applicability of the Ala-versus-Gly concept for human 5-LOX, we created the A411G mutant but did not detect any catalytic activity. However, similar amounts of enzyme protein were detected in the bacterial lysate supernatant indicating comparable expression levels of wild-type and mutant enzyme species. Thus, A411G exchange induced a loss of catalytic activity. This negative outcome prompted us to test the Ala-to-Gly exchange for mouse 5-LOX. From Fig. 5A, it can be seen that wild-type mouse 5-LOX converts arachidonic acid to 5-H(p)ETE, which was predominantly in the S configuration as indicated by chiral phase HPLC (Fig. 5B). Although the major oxygenation product formed by the corresponding A411G mutant (Table 2) was also 5-H(p)ETE (42.9 ± 4.2%), significant amounts of 12-H(p)ETE (27.6 ± 7.5%) and 9-H(p)ETE (29.4 ± 3.6%) were also analyzed (Fig. 5A). Chiral phase HPLC (Table 2) indicated that 5-H(p)ETE was predominantly (>95%) the S-enantiomer, whereas 9-H(p)ETE (S:R ratio, 19:81) and 12-H(p)ETE (S:R ratio, 17:83) were mainly R (Fig. 5C and Table 2). These data indicate that for mouse 5-LOX the A411G exchange partially altered the positional specificity of the enzyme. If one considers the fact that 5S- and 8S-H(p)ETE are the major reaction products of wild-type mouse 5-LOX, the formation of 9R- and 12R-H(p)ETE by the A411G mutant could be predicted by the Ala-versus-Gly concept (27).

**DISCUSSION**

Previous multiple sequence alignments indicated that S-LOXs contain Ala or Ser residues at the position that aligns with Ala-416 of human 15-LOX2 (25, 26). In contrast, R-LOXs carry a smaller Gly instead. When this Gly in various R-LOXs was mutated to a more space-filling Ala the share of S-oxygenation products was increased (25, 27, 37). When the inverse strategy (Ala-to-Gly exchange) was applied for various S-LOXs (25, 38, 39), R-H(p)ETE isomers were identified among the major oxygenation products. These data prompted the Ala-versus-Gly concept (6), which may be of predictive value. More recently, a linoleic acid 9R-LOX was detected in the filamentous cyanobacterium *Nostoc* sp. 7120. Surprisingly, this enzyme carried an Ala at the critical position, which would have qualified it as an S-LOX (28). When this Ala was mutated to a less bulky Gly, there was hardly any alteration in the reaction specificity. However, when more space-filling residues (Val and Ile) were introduced, 13S-H(p)ODE was the major linoleic acid oxygenation product. The relative share of 13S-H(p)ODE formation increased with the bulkiness of the amino acid side chain at this critical position. Similar results were obtained for the 48-kDa mini-LOX from *Anabaena* sp. PCC 7120 (29).
When we investigated the LOX pathway of *D. rerio* (zebrafish), a model organism that is frequently used to study vertebrate embryogenesis (31, 32), we detected 10 different LOX transcripts in the zebrafish database (Ensembl). Among them, only *D. rerio* LOX1 contained a Gly at the critical position (Gly-410). On the basis of this observation and applying the triad concept of the positional specificity of LOX, arachidonic acid 12R-lipoxygenation was predicted for this enzyme. However, surprisingly, 12S-H(p)ETE was identified as the major oxygenation product. According to the Ala- versus-Gly concept, introduction of a smaller residue at this position should force arachidonic acid R-oxygenation, but because Gly is the smallest proteinogenic amino acid, we were unable to follow this strategy. When we introduced less bulky residues in the immediate surrounding of Gly-410, because Gly is the smallest proteinogenic amino acid, we were unable to follow this strategy. When we introduced less bulky residues in the immediate surrounding of Gly-410, inactive enzyme species resulted. Thus, there is little room for additional mutagenesis studies to explore the mechanistic basis for the lack of applicability of the Ala-versus-Gly concept for *D. rerio* LOX1. To explore whether or not *D. rerio* LOX1 might be the only exception from the Ala-versus-Gly concept, we searched publicly available fish expressed sequence tag databases for similar LOX sequences and found a partial LOX sequence (440 N-terminal amino acids) in *Phoxinus phoxinus* (a small fish from the family of Cyprinidae) that shares an amino acid identity of 80% with the *D. rerio* LOX1. Gly-410 of the *D. rerio* LOX1 was aligned with a Gly in this LOX. Although no functional data are currently available for this *P. phoxinus* LOX, the high degree of sequence identity with the zebrafish enzyme suggests the existence of another S-lipoxygenating isozyme that carries a Gly at this critical position.

For most human LOX isoforms, the Ala- versus-Gly concept is partly applicable. Corresponding mutations on 15-LOX2 (25) and 12R-LOX (27) strongly altered the enantioselectivity, and similar effects were observed here for the platelet 12-LOX (Fig. 4). However, for human 12/15-LOX, more subtle alterations (25%) in the reaction specificity were observed, and similar data were obtained for the orangutan ortholog (Table 2). Even less pronounced alterations (15%) were found when corresponding mutations were introduced in 12-lipoxygenating 12/15-LOXs (Table 2). Human 5-LOX was inactivated when A411G exchange was performed suggesting catalytic relevance of Ala-411. However, even in the presence of direct structural data for human 5-LOX (40), the catalytic function of this residue during 5-LOX reaction remains to be defined. Mouse 5-LOX remained catalytically active after A411G exchange and exhibited an altered reaction specificity (Fig. 5 and Table 2). These data indicate that the Ala- versus-Gly concept may be partly applicable for mammalian 5-LOXs. Structures of both human 5-LOX and rabbit 12/15-LOX (Protein Data Bank codes 3O8Y and 2P0M, respectively) are very similar, and *Ala*-411 (5-LOX) and Ala-404 (12/15-LOX) are located at almost the same position (supplemental Fig. 3) in proximity to the catalytically active iron, leaving just enough space for fatty acid binding. Our finding that mouse 5-LOX follows at least in part the Ala-versus-Gly concept further corroborates a similar substrate alignment at the active site of 5- and 12/15-LOX, a question that has been a matter of discussion for many years.

Human eLOX3 carries an Ala at this position (Ala-451), and 9-H(p)ETE (31%) and 5-H(p)ETE (20%) were identified previously as major conjugated diene-containing arachidonic acid oxygenation products (33). A451G exchange only induced minor alterations in the specificity of arachidonic acid oxygenation because the percentage of 9-H(p)ETE increased to 38% at the expense of 5-H(p)ETE. Chiral phase HPLC revealed that 9-H(p)ETE formed by the A451G mutant was 67% 9R compared with a racemic mixture for the wild-type enzyme (33).

The molecular mechanism by which an Ala-to-Gly exchange impacts the stereochemistry of the LOX reaction is still a matter of discussion. Originally, a mechanistic concept was developed (25) that was based on the assumption that molecular dioxygen is selectively targeted by the enzyme to a defined carbon atom of the enzyme-bound fatty acid radical (targeting hypothesis). The more space-filling Ala side chain (compared with Gly) was suggested to block oxygen penetration to C11 of the carbon-centered pentadienyl radical, preventing arachidonic acid 11R-oxygenation. In contrast, the less space-filling Gly residue may allow oxygen targeting to the 11R-position. This concept was later refined on the basis of direct structural data for the coral 12-LOX (27). For 12/15-LOXs, the antarafacial character of the LOX reaction prohibits oxygen insertion at the 15R- and 11S-positions. Although the mechanistic basis for the antarafacial character has never been explored in detail, it might be possible that oxygen is just not available at this position. This might be related to the more hydrophilic iron cluster, which may hinder intraenzyme oxygen diffusion to these carbon atoms. Consequently, oxygen may only be inserted at the 15S- or 11R-position, which is located at the
TABLE 2
Reaction specificity of various mammalian LOX isoforms and corresponding Ala-to-Gly mutants

Lipoxygenase isoforms were expressed in E. coli, and the product specificity of arachidonic acid oxygenation was determined as described under “Materials and Methods.” The major reaction product is indicated in bold italic numbers. The S/R ratio is given in parentheses; this ratio was not determined for products that contributed less than 12% to the total product mixture.

| Specificity of wild-type enzyme | LOX isoforms   | Mutant     | Product | Share of total products |
|--------------------------------|---------------|------------|---------|-------------------------|
| Dominant 15-lipoxygenating     | Human 15-LOX  | A416G      | 15-HETE | 26 (86:14)              |
|                                | Human 12/15-LOX | A403G   | 11-HETE | 71 (15:85)              |
|                                | Rabbit 12/15-LOX | A404G  | 15-HETE | 23 (9:91)               |
|                                | P. pygmaeus 12/15-LOX | A403G | 15-HETE | 23 (9:91)               |
| Dominant 12-lipoxygenating     | Rabbit 12/15-LOX (I418A) | A418A/A404G | 12-HETE | 73 (97:3)               |
|                                | Mouse 12/15-LOX | A404G    | 12-HETE | 65 (90:10)              |
|                                | M. mulatta 12/15-LOX | A403G   | 8-HETE  | 15 (5:95)               |
|                                | Human platelet 12-LOX | A403G   | 8-HETE  | 86 (96:4)               |
|                                | Mouse 5-LOX    | A411G     | 5-HETE  | 43 (99:1)               |
| Dominant 5-lipoxygenating      | Human 5-LOX    | A411G     | 5-HETE  | Inactive                |
|                                | Mouse 5-LOX    | A411G     | 8-HETE  | 42 (97:3)               |
|                                | Mouse 5-LOX    | A411G     | 8-HETE  | <2                      |

* RP-HPLC was used to quantify the reaction products.
* SP-HPLC was used for product quantification.

FIGURE 4. SP-HPLC analysis of arachidonic acid oxygenation products formed by wild-type human platelet-type 12-LOX and its A403G mutant. Wild-type and A403G mutant of the human platelet 12-LOX were expressed in E. coli as described under “Materials and Methods.” Bacteria were spun down and lysed, and aliquots of the stroma-free lysis supernatant were used for activity assays (see "Material and Methods"). The conjugated dienes were collected and further analyzed by SP- and CP-HPLC. A, wild-type enzyme; B, A403G mutant; insets, enantiomer analysis by CP-HPLC.

same side of the fatty acid backbone. In 15S-LOXs, the Ala side chain was suggested to block directly or indirectly (25, 27) 11R-oxygen insertion, favoring 15S-lipoxygenation. When we calculated the energy landscape for oxygen distribution at the active site of the rabbit 12/15-LOX-arachidonic acid complex, we found that the probability of oxygen occupancy around C15 of the fatty acid is about 7-fold higher than around C11 (41). This result is consistent with the reaction specificity of this enzyme, but it also suggests that oxygen targeting may not be the only mechanism controlling the stereochemistry of both radical rearrangement (+2 or −2 direction) and oxygen insertion. If these two processes were only controlled by oxygen availability one would expect to find the formation of significant amounts (up to 15%) of 11R-H(p)ETE by the wild-type enzyme. However, the share of 11-H(p)ETE formation never exceed 2%, and unpublished chiral analyses indicated a racemic mixture. Moreover, looking at the oxygen distribution map, one gets the impression that oxygen availability in a small volume unit (large enough to cover C1–C5 of a pentadienyl system) might not be sufficient different to explain the high degree of reaction specificity of most LOXs. This might be different for other LOX isoforms, but unfortunately, oxygen distribution maps for other LOX are currently not available.

If oxygen targeting (targeting hypothesis) is not the only way for controlling the stereochemistry of the LOX reaction, what are potential alternative mechanisms? The carbon-centered fatty acid radical that is formed via initial hydrogen abstraction for controlling the stereochemistry of the LOX reaction, what are potential alternative mechanisms? The carbon-centered fatty acid radical that is formed via initial hydrogen abstraction is usually regarded as a planar pentadienyl radical in which the electron density is equally distributed over the entire pentadienyl moiety. However, if the π-electron density is focused at a certain position the oxygen-accepting carbon atom may be predetermined. There are at least two principle ways to achieve this goal (Scheme 1). (i) The dislocation hypothesis states that if the planar symmetric pentadienyl radical

3 H. Kuhn, unpublished data.
formed during hydrogen abstraction is structurally distorted at the active site of the enzyme asymmetric allylic radicals (42) may be formed in which the electron density is focused at a certain (for instance 15S-position) position. In addition to these three theories, the reduction hypothesis may explain the enantioselectivity of LOXs. If oxygenation of the fatty acid radical is a reversible process (43), which may proceed randomly, the product pattern may become stereospecific if reduction of the peroxy radical is stereoselective. Which of these four potential mechanisms is dominating remains unclear at the moment. It might even be the case that a combination of several of these mechanistic elements, such as oxygen targeting and radical distortion, is responsible for the high degree of reaction specificity of most LOXs.

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