Mammalian lipoxygenases (LOxs) are categorized with respect to their positional specificity of arachidonic acid oxygenation. Site-directed mutagenesis identified sequence determinants for the positional specificity of these enzymes, and a critical amino acid for the stereoselectivity was recently discovered. To search for sequence determinants of murine (12R)-LOX, we carried out multiple amino acid sequence alignments and found that Phe\(^{390}\), Gly\(^{441}\), Ala\(^{455}\), and Val\(^{631}\) align with previously identified positional determinants of S-LOX isoforms. Multiple site-directed mutagenesis studies on Phe\(^{390}\) and Ala\(^{455}\) did not induce specific alterations in the reaction specificity, but yielded enzyme species with reduced specific activities and stereo random product patterns. Mutation of Gly\(^{441}\) to Ala, which caused drastic alterations in the reaction specificity of other LOX isoforms, failed to induce major alterations in the positional specificity of mouse (12R)-LOX, but markedly modified the enantioselectivity of the enzyme. When Val\(^{631}\), which aligns with the positional determinant Ile\(^{593}\) of rabbit 15-LOX, was mutated to a less space-filling residue (Ala or Gly), we obtained an enzyme species with augmented catalytic activity and specifically altered reaction characteristics (major formation of chiral (11R)-hydroxyeicosatetraenoic acid methyl ester). The importance of Val\(^{631}\) for the stereo control of murine (12R)-LOX was confirmed with other substrates such as methyl linolate and 20-hydroxyeicosatetraenoic acid methyl ester. These data identify Val\(^{631}\) as the major sequence determinant for the specificity of murine (12R)-LOX. Furthermore, we conclude that substrate fatty acids may adopt different catalytically productive arrangements at the active site of murine (12R)-LOX and that each of these arrangements may lead to the formation of chiral oxygenation products.

Lipoxygenases (LOXs)\(^8\) form a heterogeneous family of lipid peroxidizing enzymes that catalyze dioxygenation of free and/or esterified polyunsaturated fatty acids to their corresponding hydroperoxy derivatives (1). They are involved in the biosynthesis of eicosanoids (2) such as pro-inflammatory leukotrienes (3) and anti-inflammatory lipoxins (4), but have also been implicated in cell maturation (5), cancer (6), psoriasis (7), atherogenesis (8), and osteoporosis (9).

Mechanistically, the LOX reaction consists of four elementary reactions, the stereochemistry of which is tightly controlled (see Scheme 1): (i) stereoselective hydrogen abstraction from a bisallylic methylene, forming a carbon-centered fatty acid radical; (ii) \([+2]\) or \([-2]\) rearrangement of the fatty acid radical; (iii) stereospecific insertion of molecular dioxygen, forming an oxygen-centered hydroperoxy radical; and (iv) reduction of the hydroperoxy fatty acid radical to the corresponding anion. Our current understanding of how mammalian LOXs control the stereochemistry of the oxygenation reaction is derived from the x-ray structure of rabbit reticulocyte 15-LOX (10), from extensive mutagenesis studies on various LOX isoforms (11–15), and from experiments with chemically modified fatty acid substrates (16, 17). The substrate-binding cleft of the rabbit enzyme is a U-shaped pocket, the bottom of which is defined by a triad of amino acids (Phe\(^{393}\), Ile\(^{418}\), and Ile\(^{606}\)). A simple model for substrate alignment at the active site of this enzyme suggests that polyenoic fatty acids may slide into the substrate-binding pocket with their methyl end ahead (12). Molecular modeling (10, 18) and site-directed mutagenesis (12) suggest that the volume of the active site might be important for the positional specificity. This space-related hypothesis was initially opposed by the orientation-based model, which suggests the possibility of an inverse head-to-tail substrate alignment (19, 20). However, more recent experimental data suggest that both hypotheses appear to be valid (12, 17).

Most of the mechanistic studies performed out in the past on the structural basis for the positional specificity of LOXs have been carried out on classical S-LOX isoforms (see Ref. 12 for review), but little is known about the corresponding mechanisms of the more recently discovered R-lipoxygenating enzyme species (21–23). However, during the preparation of this manuscript, a study that also included two R-LOXs was published (24). Multiple amino acid sequence alignments of R- and S-LOXs suggest that the R-lipoxygenating enzymes contain a conserved Gly in the central part of their primary structure. Mutation of Gly\(^{427}\) to Ala in (8R)-LOX from corals induces alterations in the positional specificity as well as changes in the enantioselectivity of the enzyme (pre-dominant formation of (12S)-HETE methyl ester). Similar modifications were observed when corresponding mutations were carried out with human (12R)-LOX, murine (8S)-LOX, and human (15S)-LOX2.
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To investigate the structural basis for the stereochemical control mechanisms of murine (12R)-LOX more comprehensively, we applied a strategy that involves targeted substrate modification and site-directed mutagenesis. For this purpose, we first identified putative sequence determinants for the reaction specificity by multiple amino acid sequence alignments and modified their side chains. The data obtained indicate that mutation of Ala455 and Phe359 led to the formation of a major sequence determinant for both the positional specificity and enantioslectivity of murine (12R)-LOX because the geometry of its side chain impacts the stereochemistry of both initial hydrogen abstraction and subsequent oxygen insertion.

EXPERIMENTAL PROCEDURES

Chemicals—The chemicals used were purchased from the following sources: arachidonic acid methyl ester (5Z,8Z,11Z,14Z-eicosatetraenoic acid methyl ester) and isopropyl β-d-thiogalactopyranoside from Sigma (Deisenhofen, Germany); HPLC standards of (12R)- and (12S)-HETEs, (15R)- and (15S)-HETEs, (11R)- and (11S)-HETEs, (8R)- and (8S)-HETEs, (9R,5Z,7E,11Z,14Z)- and (9S,5Z,7E,11Z,14Z)-9-hydroxy-5,7,11,14-eicosatetraenoic acids, and (5R)- and (5S)-HETEs from Cayman Chemical Co. Inc. (Ann Arbor, MI); sodium borohydride from Serva (Heidelberg, Germany); ampicillin from Invitrogen (Eggenstein, Germany); and HPLC solvents from Merck (Darmstadt, Germany). Restriction enzymes were purchased from New England Biolabs Inc. (Schwalbach, Germany). The QuikChange site-directed mutagenesis kit was from Stratagene (La Jolla, CA). The BaculoGold™ transfection kit and Sp9 insect cells were from BD Biosciences. Oligonucleotide synthesis was carried out by TIB MOLBIOL (Berlin, Germany). All other chemicals and solvents were of analytical grade.

Expression of (12R)-LOX—Murine (12R)-LOX was expressed in Escherichia coli as an N-terminally His-tagged fusion protein. For this purpose, the coding region of the cDNA was ligated into the pQE expression vector, and bacteria were transformed with the recombinant plasmid containing an ampicillin resistance gene as selection marker. Bacteria were cultured at 37 °C in 1 liter of LB medium containing 0.1 g/ml ampicillin to reach an absorbance at 600 nm of 0.5. The expression of the recombinant protein was induced by addition of isopropyl β-d-thiogalactopyranoside (1 mM final concentration). After 2 h of incubation, bacteria were spun down, washed twice, resuspended in 20 ml of phosphate-buffered saline (PBS), and lysed by sonication. Cell debris was removed by centrifugation, and the clear lysis supernatant was used as enzyme source. Activity assays were performed by addition of variable amounts of lysis supernatant to 0.5 ml of PBS containing arachidonic acid methyl ester (0.1 mM final concentration) as substrate. The mixture was incubated for 15 min at 37 °C, and the hydroperoxy lipids formed were reduced to the more stable hydroxy derivatives by addition of 0.1 ml of saturated solution of sodium borohydride in dry ethanol. After acidification to pH 3 (acetic acid), 0.5 ml of methanol was added, and the samples were kept on ice for 10 min. The protein precipitate was spun down, and aliquots of the clear supernatant were directly analyzed by reverse-phase (RP) HPLC for quantification of the LOX products.

Selected mutants of murine (12R)-LOX were expressed in Sp9 insect cells using the BaculoGold™ transfection system. For this purpose, the corresponding wild-type or mutant cDNA was ligated into the pVL1392 transfer vector containing a His tag at the N terminus of the enzyme. Highly purified recombinant transfer vectors were used for cotransfection of insect cells together with wild-type baculovirus. For this purpose, the recombinant transfer vector (5 μg) was mixed with 1 μg of BaculoGold™ DNA and added to a monolayer of Sp9 insect cells (2 × 10^6 cells/well) in TNM-FH medium. After 72 h, the culture supernatant containing recombinant baculovirus was used to re-infect freshly cultured Sp9 cells to produce high titer virus stocks. Finally, the high titer virus stock was diluted with culture medium to a multiplicity of infection of 5 and was used for infecting freshly cultured Sp9 cells (10^6 cells/ml). 3 days after infection, the cells were spun down, reconstituted in PBS, and sonicated with a tip sonifier (Braun, Melsungen, Germany). Cell debris was removed by centrifugation, and the clear lysis supernatant was used as enzyme source.

Mutagenesis—Site-directed mutagenesis was performed using the QuikChange mutagenesis kit following the manufacturer’s instructions. To identify mutant LOX clones, 10–20 clones were selected and screened for the mutations by restriction mapping and activity assays. Finally, mutations were confirmed by sequencing.

Analytes—HPLC was performed on a Shimadzu HPLC system connected to a Hewlett-Packard 1040A diode array detector. RP-HPLC was carried out on a Nucleosil C18 column (KS system, 250 × 4 mm, 5-μm particle size; Macherey Nagel, Duren, Germany) coupled with a guard column (30 × 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 (SP) HPLC was performed on a Zorbax silica column (250 × 4 mm, 5-μm particle size) with a solvent system of n-hexane/2-propanol/acetic acid (100:20:1 by volume) at a flow rate of 1 ml/min. For chiral-phase (CP) HPLC, we used a Chiralcel OD or a Chiralcel OB column (250 × 4 mm, 5-μm particle size) and a solvent system consisting of n-hexane/acetic acid (100:0.1 by volume) containing 2-propanol in various concentrations depending on the chemistry of the reaction products. The flow rate was 1 ml/min.

Gas Chromatography/Mass Spectrometry (GC/MS)—GC/MS was carried out on a Shimadzu GC/MS QP-2000 system equipped with a fused SPB1 silica column (10 m × 0.25 mm, 0.25-μm coating thickness) at an injector temperature of 270 °C. An ion source temperature of 180 °C and an electron energy of 70 eV were adjusted. The derivatized fatty acids were eluted with the following temperature program: isothermal run at 180 °C for 2 min and then from 180 to 290 °C at a rate of 5 °C/min.

Miscellaneous Methods—Protein concentrations were determined using the Roti-Quant kit (Carl Roth GmbH, Karlsruhe, Germany). Methylation of free carboxylic acids was achieved by bubbling ethereal diazomethane with argon (2 min) and transferring the diazomethane gas to the fatty acid solution. For more informative mass spectra, the methylated derivatives of the polyenoic fatty acids (10 μg dissolved in 1 ml of methanol) were hydrogenated using 5 mg of 10% palladium/CaCO₃ (Merck) as catalyst. Hydrogen gas was bubbled through this mixture for 2 min at room temperature. The solution was filtered to remove the catalyst; the solvent was evaporated; and the products were silylated. Aliquots (2 μl) were injected into the GC/MS system. Kₙ values for the formation of the different oxygenation products were determined by incubating the enzyme preparations with different concentrations of arachidonic acid methyl ester (7–100 μM) and quantifying the different HETE isomers separately by RP-HPLC. 8- and 9-HETEs were not well separated under our experimental conditions; and thus, the sum of both products was evaluated.
RESULTS

Mutagenesis of Positional Determinants Identified for Other LOX Isoforms—Previous mutagenesis studies on human and rabbit reticulocyte 15-LOXs identified Ile\textsuperscript{418} and Met\textsuperscript{419} and Phe\textsuperscript{353} and Ile\textsuperscript{593}, respectively, as sequence determinants for the positional specificity (11, 12), but no information is currently available on the importance of these residues for the specificity of murine (12\textsuperscript{R})-LOX. To test the relevance of these amino acids, we first performed multiple sequence alignments. Fig. 1A shows that Phe\textsuperscript{353} of rabbit 15-LOX aligns with Phe\textsuperscript{390} of murine (12\textsuperscript{R})-LOX. Similarly, Ile\textsuperscript{418} and Ile\textsuperscript{593} of the rabbit enzyme align with Ala\textsuperscript{455} and Val\textsuperscript{631} of the murine enzyme, respectively (Fig. 1, B and C). Because Ile is more space-filling than Ala and Val, one may conclude that murine (12\textsuperscript{R})-LOX may have a bigger substrate-binding pocket than rabbit 15-LOX. If the volume of the substrate-binding pocket is important for the positional specificity of murine (12\textsuperscript{R})-LOX, introduction of bulkier residues at these positions should alter the enzyme specificity in favor of C-13 hydrogen removal. In this case, 15- and/or 11-HETE methyl ester should be the major oxygenation product. To test this hypothesis, we first expressed murine (12\textsuperscript{R})-LOX in E. coli and then mutated Ala\textsuperscript{455} to a somewhat bulkier residue (Ile). TABLE ONE shows that this mutant exhibited a strongly reduced specific activity and a more random positional specificity compared with the wild-type enzyme. C-13 hydrogen abstraction was increased from 18\% (wild-type enzyme) to 43\% (A455I). Between the two positional isomers originating from C-13 hydrogen removal (15- and 11-HETEs), the formation of 11-HETE methyl ester, which involves [−2] rearrangement of the fatty acid radical, was dominant (33\% of the sum of HETE isomers). Interestingly, the 11-HETE methyl ester formed was chiral (R/S ratio of 98:2), and a similar R/S ratio was analyzed for 12-HETE. 15-HETE methyl ester contributed only ~10\% to the product mixture. To determine whether the alterations induced by the A455I mutation will become more pronounced when an even bulkier residue is introduced, we created the A455W mutant. This mutant was also less active than the wild-type enzyme, and C-10 hydrogen abstraction was dominant (50\%). (12\textsuperscript{R})-HETE methyl ester was the major oxygenation product (TABLE ONE), and an R/S ratio of 86:14 was determined. In addition, this mutant catalyzed significant C-7 hydrogen abstraction, resulting in the formation of similar amounts of 9- and 5-HETE methyl esters. Next, we thought about introducing a less space-filling Gly at position 455. However, sequence alignment with human (12\textsuperscript{R})-LOX indicated that this isoform has a Gly at this position; and thus, no major impact of the A455G mutation was expected. Summarizing these data, one may conclude that introduction of more space-filling residues at Ala\textsuperscript{455} impairs both the catalytic activity and positional specificity of the enzyme. The alterations in positional specificity can hardly be explained on the basis of the space-related hypothesis (10, 18) regardless of whether a straight (methyl end first) or an inverse (carboxylate first) substrate orientation might be involved.

To test the possible importance of Phe\textsuperscript{390} (12) for the positional specificity of murine (12\textsuperscript{R})-LOX, we first mutated Phe\textsuperscript{390} to a more space-filling residue (Trp). Unfortunately, this mutant had almost no activity (TABLE ONE); and thus, no product analysis was carried out. Next, the bulky Phe\textsuperscript{390} was mutated to a less space-filling residue (Ala). This mutant exhibited a reduced specific activity, and we observed a complex mixture of oxygenation products involving all possible positional iso-
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TABLE ONE

Impact of site-directed mutagenesis of putative specificity determinants on the specific activity and positional specificity of murine (12R)-LOX

| Enzyme | Impact on active-site volume | Specific activity | Product composition |
|--------|-------------------------------|-------------------|--------------------|
|        |                               |                   | C-13 hydrogen abstraction | C-10 hydrogen abstraction | C-7 hydrogen abstraction |
|        |                               |                   | 15-HETE | 11-HETE | 12-HETE | 8-HETE | 9-HETE | 5-HETE |
| Wild-type | aé                            | 100               | 0 | 18 | 63 (92:8) | 23 | 13 | 21 | 0 |
| A455I  | a                            | 10                | 10 | 33 (98:2) | 23 | 13 | 21 | 0 |
| A455W  | a                            | 45                | 8 | 12 | 33 (86:14) | 17 | 14 | 16 | 0 |
| F390W  | aé                           | 2                 | ND | ND | ND | ND | ND | ND | ND |
| F390A  | a                            | 50                | 8 | 14 | 29 (86:14) | 17 | 12 | 20 | 0 |
| V631F  | a                            | 4                 | ND | ND | ND | ND | ND | ND | ND |
| V631A  | a                            | 250               | 2 | 49 (99:1) | 14 | 16 | 19 | 0 |
| V631G  | a                            | 273               | 2 | 42 (98:2) | 13 | 15 | 29 | 0 |

As for the wild-type enzyme, 12-HETE (R/S ratio of 86:14) was identified as the major oxygenation product.

The crystal structure of rabbit 15-LOX predicted the importance of Ile693 for the positional specificity (10), and site-directed mutagenesis studies confirmed this prediction (12). To reduce the volume of the active site of murine (12R)-LOX, we next introduced bulkier residues at this position. When Val631 of murine (12R)-LOX was mutated to an amino acid with a larger side chain (Ile or Phe), a graded reduction of the specific activity was observed (residual activities of 24 and 4% for the V631I and V631F mutants, respectively). Moreover, these mutants exhibited a strongly impaired positional specificity (data not shown). Finally, we mutated Val631 to an amino acid carrying a smaller side chain (Ala or Gly) to increase the volume of the substrate-binding pocket. Here, we observed a strong increase in the catalytic activity and more specific alterations in the reaction specificity. For both mutants, we identified (11R)-HETE as the major oxygenation product. In contrast, 12-HETE methyl ester was strongly reduced (TABLE ONE).

In E. coli, murine (12R)-LOX was expressed at only low levels, and purification of the recombinant enzyme was not possible. For more detailed investigations, the wild-type enzyme and the most interesting mutants (V631A and V631G) were overexpressed as His-tagged fusion proteins in the baculovirus/insect cell system and purified on a nickel-agarose column. The enzyme expressed in insect cells exhibited a 4-fold higher specific activity with methyl linoleate as substrate (10 μg of HETE/μg of enzyme in E. coli versus 38 μg of HETE/μg of enzyme in insect cells), and similar alterations were observed in the methyl arachidonate system. Compared with the wild-type enzyme, the V631A mutation induced a 6.6-fold increase in methyl linoleate oxygenase activity. For the V631G mutant, the increase was 5.6-fold. The alterations in the product pattern observed for the "bacterial enzyme" were confirmed in the insect cell system. Here again, 11-HETE methyl ester was identified as the major oxygenation product for the V631A mutant (Fig. 2A), and analysis of the enantiomer composition indicated a strong preponderance of the R-isomer (Fig. 2C). The 8-HETE methyl ester formed by the mutant enzyme was also chiral, but the S-enantiomer was dominant (Fig. 2C). This finding contrasts with the stereochemistry of the 8-HETE formed by the wild-type enzyme (Fig. 2B). These data suggest that, for the formation of 8-HETE, the substrate fatty acids might be aligned differently at the active site of the two enzyme species.

Summarizing these data, one concludes that the V631A and V631G mutations lead to a strong increase in the catalytic activity and to major
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Reactivity with the Mechanistic Probe 20-HETE Methyl Ester—When rabbit 15-LOX reacts with methyl arachidonate, formation of (15S)-HETE methyl ester is dominant; in contrast, when 20-HETE methyl ester is used as substrate, 5-lipoxygenation prevails, and an inverse head-to-tail substrate orientation was suggested (25). To test whether or not 20-HETE methyl ester is oxygenated by murine (12R)-LOX with a different positional specificity compared with methyl arachidonate, we incubated the wild-type enzyme and its V631A mutant with 20-HETE methyl ester and compared the specific activities and product patterns. TABLE TWO shows that for both the wild-type enzyme and its V631A mutant, 20-HETE methyl ester was oxygenated at a higher rate than methyl arachidonate. As with other substrates (methyl arachidonate and methyl linoleate), the wild-type enzyme was a less effective catalyst than the V631A mutant when reacting with 20-HETE methyl ester (54 μg diHETE formed per μg of LOX protein versus 304 μg of diHETE formed per μg of LOX protein during a 15-min incubation period).

To compare the product patterns of 20-HETE methyl ester oxygenation, the diHETE methyl ester isomers were prepared by RP-HPLC, hydrolyzed, and further analyzed by SP-HPLC. Fig. 4 shows that three major reaction products (I–III) were formed by the wild-type enzyme. Product I was identified as 12,20-diHETE methyl ester by co-injection with an authentic standard in SP-HPLC. GC/MS data of the hydroxynated derivative of product II indicated its chemical structure as 8,20-diHETE. Thus, the wild-type enzyme catalyzed major 8-oxygenation of 20-HETE methyl ester, and this reaction involved C-10 hydrogen abstraction and [−2] radical rearrangement. In contrast, with arachidonic acid methyl ester, 12-lipoxygenation (C-10 hydrogen abstraction but [+2] radical rearrangement) was dominant (TABLE THREE). When the V631A mutant was used as catalyst, 9,20-diHETE methyl ester was identified as major oxygenation product (Fig. 4). 8,20-diHETE methyl ester, the major product of wild-type enzyme catalysis, contributed only 10% to the product mixture. Formation of 9,20-diHETE methyl ester involved C-7 hydrogen abstraction and [−2] radical rearrangement. Thus, the V631A mutation altered both the site of hydrogen abstraction and the direction of radical rearrangement.

Mutagenesis of the Putative Determinant for Enantioselectivity—Multiple sequence alignments indicate that R-LOXs contain a conserved glycine in the central part of their primary structure, which is substituted with alanine or serine in S-LOXs. Site-directed mutagenesis of this residue impacts the reaction specificity of four different LOX isoforms (24). For this study, we performed similar mutagenesis experiments and mutated Gly243 to Ala in murine (12R)-LOX. If the concept developed previously (24) were applicable for murine (12R)-LOX, one would expect a major formation of (8S)-HETE. Although we observed a moderate increase in 8-lipoxygenation, (12R)-HETE remained the major oxygenation product (Fig. 5). When we analyzed the enantiomer composition of the 8-HETE methyl ester formed by the wild-type enzyme, we detected a preponderance of the R-enantiomer. In contrast, (8S)-HETE methyl ester prevailed using the G441A mutant as catalyst. Thus, the G441A mutation altered the enantiomer composition of 8-HETE methyl ester in favor of S-lipoxygenation. To increase the extent of alterations induced by the G441A mutation, we prepared the G441V mutant, which carries a bulkier residue at this position. Unfortunately, this mutant was catalytically silent.

When linoleic acid methyl ester was used as substrate for murine (12R)-LOX, (9R)-HODE methyl ester was the major reaction product, and the minor site product (13-HODE methyl ester) was predominantly in the S-configuration (Fig. 6). However, we observed strong alterations

TABLE TWO

Specific activities of wild-type (12R)-LOX and mutant V631A expressed in Sf9 insect cells with 20-HETE methyl ester as substrate

| Substrate | Specific activity | Major oxygenation products |
|-----------|------------------|----------------------------|
| Wild-type enzyme |                      |                             |
| Methyl-AA | 1                | 12-HETE (63%), 11-HETE (15%) |
| Methyl-20-HETE | 1.4             | 8,20-diHETE (60%), 12,20-diHETE (30%), 9,20-diHETE (10%) |
| V631A mutant |                  |                             |
| Methyl-AA | 1.4              | 11-HETE (49%), 9-HETE (17%), 8-HETE (16%) |
| Methyl-20-HETE | 8.0             | 9,20-diHETE (60%), 12,20-diHETE (30%), 8,20-diHETE (10%) |
in the positional specificity upon reacting the G441A mutant with methyl linoleate. Here, (13\(_S\))-HODE methyl ester was the major reaction product, and (9\(_R\))-HODE was formed only in small amounts (TABLE THREE). These results are also consistent with the recently introduced concept of the importance of the Gly441 for the positional specificity (24). Here again, the G441V mutant was catalytically silent.

**DISCUSSION**

The overall reaction specificity of LOXs is a result of the stereochemistry of the three catalytic elementary reactions: (i) stereoselectivity of hydrogen removal, (ii) direction of radical rearrangement, and (iii) stereospecific oxygen insertion. Unfortunately, no direct experimental data that prove the structure of LOX-substrate complexes are currently available. However, there are models for enzyme-substrate interactions that are based on extensive mutagenesis experiments (11–15, 24) and on targeted substrate modification (16, 17). Taken together, these previous studies suggested that the depth of substrate entry into the binding cleft, the orientation (straight or inverse) of the substrate alignment at the active site, and the presence of blocking amino acid side chains appear to be important for the stereochemical control mechanisms.

In this study, we investigated the structural basis for the reaction specificity of murine (12\(_R\))-LOX, testing the relevance of those amino acids that have previously been identified as sequence determinants for other LOX isoforms (12, 24). For this purpose, four different regions in the primary structure of murine (12\(_R\))-LOX were targeted (Fig. 1). First, we mutated the determinants of Sloane et al. (11) and Borngräber and co-workers (12) and found that the resulting mutants exhibited a reduced catalytic activity and a stereo random positional specificity regardless of whether space-filling or less bulky amino acids were introduced (TABLE ONE). This unspecific product pattern suggested that the mutant enzyme species might have lost stereochemical control. Alternatively, one may assume that substrate fatty acids may adopt different orientations in the binding cavity.

**TABLE THREE**

Composition of the reaction products formed from fatty acid methyl esters by wild-type and mutant (G441A) murine (12\(_R\))-LOXs

| Enzyme          | Methyl arachidonate | Methyl linoleate |
|-----------------|---------------------|------------------|
|                 | Relative specific activity | % | % | Major products | R/S ratio | % | % | Major products | R/S ratio |
| Wild-type       | 100 | 12-HETE(69%), 8-HETE(15%) | 92:8, 80:20 | 100 | 9-HODE(78%), 13-HODE(22%) | 92:8, 18:82 |
| G441A           | 49  | 12-HETE(48%), 8-HETE(38%) | 65:35, 30:70 | 103 | 9-HODE(20%), 13-HODE(80%) | 74:26, 15:85 |

FIGURE 4. SP-HPLC of reaction products formed from 20-HETE methyl ester by (12\(_R\))-LOX species. The recombinant (12\(_R\))-LOX species expressed in the baculovirus/insect cell system was incubated for 15 min with 20-HETE methyl ester (100 \(\mu\)M final concentration) in PBS. After addition of sodium borohydride, the lipophilic products were extracted, and the diHETE isomers were separated by RP-HPLC. The methyl esters were hydrolyzed under alkaline conditions, and the free fatty acid derivatives were analyzed by SP-HPLC using a solvent system of \(n\)-hexane/2-propanol/acetic acid (100:0.1). Peaks were identified as 12,20-dihETEs by co-injection with authentic standards. Peaks II and III were identified as 8,20- and 9,20-dihETEs, respectively, by GC/MS analysis of the hydrogenated compounds. OTMS, trimethylsilyl ether.
different catalytically productive arrangements at the active site and that each of these arrangements may lead to the formation of a different oxygenation product. In fact, the high degree of optical purity of some minor reaction products (Fig. 2) and the different $K_M$ values obtained for the formation of (11$R$)- and (12$R$)-HETEs by the V631A mutant (Fig. 3) support this hypothesis. A similar steric multiplicity of the enzyme-substrate complex has been suggested before for prostaglandin-endoperoxide H synthase-1 (26).

When Val$^{631}$, which aligns with Ile$^{593}$ of rabbit 15-LOX (determinants of Borngräber and co-workers (12) and Jisaka et al. (15)), was mutated to a more space-filling residue (Phe), an inactive enzyme was created. In contrast, mutation of Val$^{631}$ to a less bulky residue (Ala or Gly) induced a strong increase in the catalytic activity and specific alterations in the product pattern. For these mutants, optically pure (11$R$)-HETE methyl ester was analyzed as the major product (Fig. 2 and TABLE ONE), and the results indicated a tight stereochemical control of oxygen insertion. Biosynthesis of (12$R$)-HETE methyl ester involves C-10 hydrogen abstraction and $\cdot$$H_2$ radical rearrangement. In contrast, synthesis of (11$R$)-HETE methyl ester required C-13 hydrogen abstraction and $\cdot$$H_2$ radical rearrangement. As indicated in Scheme 2A, molecular oxygen was inserted from opposite sides of the plane determined by the double bonds. The structural reasons for these differences are rather complex because all major elementary reactions of the LOX cycle, hydrogen abstraction, radical rearrangement, and oxygen insertion (Scheme 1), are impacted. According to the antarafacial character, 12$R$-lipoxygenation is initiated by abstraction of the $D$-hydrogen from C-13. For this reaction, the $D$-hydrogen should be localized in close proximity to the non-heme iron; and according to the antarafacial character, two principal reaction products ((12$R$)- and (8$S$)-HETEs) were expected...
(Scheme 2A). However, if 8S-oxygenation is sterically hindered (presence of a bulky side chain), (12R)-oxygen insertion may be preferred. Such steric hindrance has recently been suggested as the mechanistic difference between R- and S-LOXs (24). Arachidonic acid 11R-oxygenation, which was observed for the V631A and V631G mutants, is initiated by abstraction of the l-hydrogen from C-13. Thus, for this reaction, the l-hydrogen should be located close to the iron. Such an arrangement would be possible only if one assumes minor alterations in substrate conformation, e.g., rotation around one of the single bonds (Scheme 2B). Such a rotation displaces the d-hydrogen from the enzyme-bound iron, but positions the l-counterpart near the metal. Abstraction of the l-counterpart from C-13 enables either 11R- or 15S-lipoxygenation. Because we did not see major formation of 15-HETE (TABLE ONE), C-15 may be blocked for oxygen insertion.

When we mutated Gly441 to Ala in murine (12R)-LOX (determinant of Coffa (24)) (Fig. 1), we observed only minor alterations in the positional specificity (Fig. 5), and this result appeared to contradict the role of this amino acid in the reaction specificity of LOXs. However, analysis of the enantiomer composition of the 8-HETE methyl ester formed by both the wild-type enzyme and its G441A mutant revealed obvious differences in the enantiomer ratio. (8R)-HETE was dominant for the wild-type enzyme, but (8S)-HETE methyl ester prevailed for the mutant. The altered enantiomer composition of 8-HETE methyl ester is consistent with the concept of the importance of this amino acid. However, our data do not support a strict linkage between stereo- and regioselectivity of oxygen insertion (24).

Murine (12R)-LOX strongly prefers methylated polyenoic fatty acids (22, 27). Here, we confirmed these data with three different substrates: arachidonic acid, linoleic acid, and 20-HETE. This is a unique enzyme property because all other LOX isoforms known so far, including the human ortholog, prefer free fatty acids. These data suggest that the charged free carboxylate may hinder catalysis. One way to explain this mechanistic peculiarity is to assume an “inverse” substrate alignment at the active site. In this case, the substrate fatty acid may slide into the substrate-binding pocket with its carboxylate ahead (Scheme 2A). Bur-
yng a charge inside the hydrophobic environment of the substrate-binding pocket may be thermodynamically hindered; and thus, free fatty acids might not be accepted as substrates for this LOX isoform. However, methylation eliminates the charge and may thus overcome thermodynamic hindrance. A similar situation was reported previously for the oxygenation of 15-HETE by rabbit reticulocyte and soybean S-LOXs (28, 29). Both enzymes catalyze major 5S- and 8S-lipoxygenation of 15-HETE, and this reaction involves an inverse substrate alignment. The thermodynamic hindrance of such an enzyme-substrate complex is mirrored by a low substrate affinity and a strongly impaired reaction rate. However, methylation of the free carboxylate strongly increases both the substrate affinity and the reaction rate (28).

When 20-HETE methyl ester is used as substrate for murine (12R)-LOX, a different substrate-binding scenario may be discussed. With this substrate, the wild-type enzyme catalyzed mainly 8-lipoxygenation, which involved C-10 hydrogen abstraction and [-2] radical rearrangement. In contrast, with methyl arachidonate, C-10 hydrogen abstraction and [±2] radical rearrangement was catalyzed (12-lipoxygenation). The different direction of radical rearrangement can be explained if the two substrates are inversely aligned to each other at the active site. If methyl arachidonate slides into the substrate-binding pocket with its carboxylate head, 20-HETE methyl ester may penetrate the active site with its methyl end first. Increasing the volume of the substrate-binding pocket by the V631A mutation may allow the substrate to move in deeper so that hydrogen abstraction from C-7 (formation of 9,20-diiHETE) can be catalyzed. Unfortunately, this hypothesis does not explain the opposite directions of radical rearrangement, but this might be related to blocking effects of large amino acid side chains (24).

In summary, our results on the structural basis of the reaction specificity of murine (12R)-LOX indicate that Val<sup>631</sup> is a major sequence determinant for the positional specificity of this enzyme. Mutation of other potential candidate amino acids that are of major importance for other LOX isoforms leads mainly to partially inactive enzyme species exhibiting random positional specificity. The mechanisms involved in these alterations appear to be rather complex and cannot be explained solely on the basis of the space- and/or orientation-related hypothesis. However, a combination of these two theories with the recently described shielding mechanism (24) may provide a plausible explanation for the observed alterations.

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