Mammalian lipoxygenases constitute a heterogeneous family of lipid-peroxidizing enzymes, and the various isoforms are categorized with respect to their positional specificity of arachidonic acid oxygenation into 5-, 8-, 12-, and 15-lipoxygenases. Structural modeling suggested that the substrate binding pocket of the human 5-lipoxygenase is 20% bigger than that of the reticulocyte-type 15-lipoxygenase; thus, reduction of the active-site volume was suggested to convert a 5-lipoxygenase to a 15-lipoxygenating enzyme species. To test this “space-based” hypothesis of the positional specificity, the volume of the 5-lipoxygenase substrate binding pocket was reduced by introducing space-filling amino acids at critical positions, which have previously been identified as sequence determinants for the positional specificity of other lipoxygenase isoforms. We found that single point mutants of the recombinant human 5-lipoxygenase exhibited a similar specificity as the wild-type enzyme but double, triple, and quadruple mutations led to a gradual alteration of the positional specificity from 5S- via 8S-toward 15S-lipoxygenation. The quadruple mutant F359W/A424I/N425M/A603I exhibited a major 15S-lipoxygenase activity (85–95%), with (8S,5Z,9E,11Z,14Z)-8-hydroperoxyeicosa-5,9,11,14-tetraenoic acid being a minor side product. These data indicate the principle possibility of interconverting 5- and 15-lipoxygenases by site-directed mutagenesis and appear to support the space-based hypothesis of positional specificity.

Lipoxygenases (LOXs) constitute a heterogeneous family of lipid-peroxidizing enzymes that catalyze the dioxygenation of free and/or esterified polyunsaturated fatty acids to their corresponding hydroperoxy derivatives. In mammals LOXs are categorized with respect to their positional specificity of arachidonic acid oxygenation into 5-, 8-, 12-, and 15-LOXs (1, 2). In contrast, plant physiologists prefer a linoleic acid-related enzyme nomenclature since arachidonic acid is only a minor fatty acid in plants. Mammalian 5-LOXs are key enzymes in the biosynthesis of leukotrienes, which are important mediators of inflammatory and anaphylactic disorders (3, 4). During the past 10 years, 5-LOX inhibitors and leukotriene receptor antagonists have been developed as anti-asthmatic drugs, and some of them are now available for prescription (5, 6). Mammalian 15-LOXs have been implicated in peroxisome proliferation activating receptor-γ-mediated cell signaling (7), in cell development and maturation (8, 9), as well as in the pathogenesis of atherosclerosis (10, 11). The intracellular activity of LOXs is regulated on pre-translational, translational, and post-translational levels. Expression of the human 5-LOX is up-regulated by transforming growth factor (12), and melatonin represses the 5-LOX pathway in B-lymphocytes (13). The interleukins-4 (14) and -13 (15) induce 15-LOX expression in monocyte/macrophages, and this regulatory process involves activation of the transcription factor STAT6 (16) as well as JAK2 and Tyk2 kinases (17). Translation of the 15-LOX mRNA is prevented during early stages of red cell maturation when special non-histone nuclear proteins (heteronuclear ribonucleoproteins K and E1) bind to a repetitive sequence in the 3′-untranslated region (18). Although the positional specificity of arachidonic acid oxygenation is decisive for mammalian LOX classification, the structural reasons for the variation of this enzyme property remain unclear. The crystal structures of two plant (19–21) and one mammalian LOX (22) suggest a U-shaped hydrophobic substrate-binding pocket containing the catalytic non-heme iron. Site-directed mutagenesis studies identified Phe-353 (23), Tyr-603 and His-604 have been identified as critical amino acids for the positional specificity of the murine epidermis 8S- and the human epidermis-type 15S-LOX isoforms (28). Unfortunately, neither specificity-related mutagenesis data nor x-ray coordinates are currently available for the pharmacologically most relevant human leukocyte 5-LOX.

For the time being, there are two hypotheses that rationalize the mechanistic differences between arachidonic acid 5- and 15-lipoxygenation (29). (i) The ‘orientation-based’ hypothesis suggests that, for 15-lipoxygenation, arachidonic acid may slide into the substrate binding pocket with its methyl terminus first...
and may adopt a sterically active conformation at the active site favoring oxygen insertion at C-15 of the arachidonic acid backbone. In contrast, for arachidonate 5-lipoxygenase, an inverse, head-to-tail substrate orientation was assumed (30). (ii) According to the “space-based” hypothesis, the substrate alignment at the active site is conserved among all LOX isoforms and the volume of the substrate binding pocket appears to be decisive for the positional specificity (31). Molecular modeling suggested that the substrate binding cavity of 5-LOXs is about 20% bigger than that of 15-LOXs (22, 31), and the additional space may allow an optimal substrate orientation for 5-lipoxygenation.

To test the “space-based” hypothesis, we attempted to alter the volume of the substrate binding pocket by site-directed mutagenesis and determined the positional specificity of the LOX mutants. Since previous experiments failed to convert mammalian 12/15-LOXs to 5-lipoxygenating enzyme species (24, 25, 27), an inverse mutagenesis strategy was selected. Crucial amino acids of the human leukocyte 5-LOX were replaced with the more space-filling counterparts present in the reticulocyte-type 15-LOXs. We found that reduction of the active site volume by multiple site-directed mutagenesis gradually altered the positional specificity from 5S- via 8S- toward 15S-lipoxygenation.

MATERIALS AND METHODS

Chemicals—The chemicals used were from the following sources: (5Z,8Z,11Z,14Z)-eicos-5,8,11,14-tetraenoic acid (arachidonic acid), (5S,8S,11Z,14Z)-5-hydroxy(peroxy)xyeicos-8,11,14-tetraenoic acid (5S-Hp(5E)ETE), CaCl₂, EDTA, ATP, and sodium borohydride from Serva (Heidelberg, Germany); ampicillin from Life Technologies, Inc. (Eggenstein, Germany); dipalmityloxy phosphatidylcholine, isopropyl-β-D-thio-galactopyranoside (IPTG), and ATP-Sepharose from Sigma-Aldrich (Deisenhofen, Germany); 12β,15β,17α,20α-tetrahydrolipoxygenase (15S-LOTE), (5S,9Z,12Z,14Z)-8-hydroxyeicos-5,9,11,14-tetraenoic acid (5S-HETE), (15S,5Z,8Z,11Z,13E)-5-hydroxy(peroxy)xy-eicosatetraenoic acid (5S-Hp(5E)ETE), (5S,15S,6S,8Z,11Z,13E)-5,15-dihydroxy-8,6,11,13-eicosatetraenoic acid (5S,15S-diHETE), and (8S,15S,5Z,8Z,11Z,13E)-5,15-dihydroxy-8,9,11,13-eicosatetraenoic acid (8S,15S-diHETE) from Cayman Chemical (distributed by Alexis GmbH, Grünberg, Germany). Restriction enzymes were purchased from New England Biolabs (Schwalbach, Germany). Phage T4 ligase, Pho polymerase, and sequencing kits were obtained from Roche Molecular Biochemicals (Mannheim, Germany), and the Escherichia coli strain HB 101 was purchased from Invitrogen (San Diego, CA). Oligonucleotide synthesis was carried out at TIB-Molbiol (Berlin, Germany). The human 5-LOX cDNA cloned into the Bluescript SK+ vector was a kind gift of Dr. A. Habenicht (Heidelberg, Germany).

Bacterial Expression and Site-directed Mutagenesis—In order to express the human 5-LOX as non-fusion protein, its cDNA was subcloned into the expression plasmid pPK 233-2. For this purpose we introduced a NcoI restriction site at the starting ATG and a HindIII site just behind the stop codon. The Ncol/HindIII restriction fragment was then ligated into the expression vector, and bacteria (HB 101) were transformed with the recombinant plasmid. Site-directed mutagenesis of the human 5-LOX was carried out by the polymerase chain reaction-overlap extension technique using mismatching synthetic oligonucleotides. The polymerase chain reaction products containing the mutations were digested with appropriate restriction enzymes and inserted into the wild-type expression plasmid. Transformed bacteria were replated. For each mutation 20–30 clones were screened for the expression of the mutant 5-LOX by restriction mapping and activity assay. Several 5-LOX-positive clones were sequenced. For the final activity assay, one sequenced clone was replated, five 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 to an optical density at 600 nm of about 0.5. The induction of expression was induced by addition of IPTG (1 mM final concentration). After 12 h at 30 °C, bacteria were spun down, washed with phosphate-buffered saline, resuspended in 0.5 ml of 0.1 M phosphate buffer, pH 7.4, containing 1 mM EDTA, and were kept on ice for 10 min. 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 activity assay or further LOX purification.

Activity Assays and Enzyme Purification—For activity assays, those fermentation samples were selected that showed a comparable expression level of the 5-LOX species as indicated by immunoblotting. LOX activity was assayed in the lysate supernatants either spectrophoto metrically recording the increase in absorbance at 235 nm by HPLC quantification of the LOX products. For HPLC analysis, aliquots of the bacterial lysate supernatants were incubated for 15 min at room temperature with 0.1 mM arachidonic acid in the presence of 0.4 mM CaCl₂, 40 µg/ml dipalmityl phosphatidylcholine, and 0.1 mM ATP (final reaction volume of 0.5 ml). The hydroperoxy compounds formed were reduced with sodium borohydride to the corresponding hydroxy derivatives, 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. Activity assays of the purified enzyme preparations (see below) were carried out spectrophotometrically or by HPLC quantification of the LOX products. The spectrophotometric measurements, the assay mixture was a 0.1 M sodium/potassium phosphate buffer, pH 7.4, containing 0.4 mM CaCl₂, 0.1 mM EDTA, 0.1 mM ATP, 12 µg/ml dipalmityl phosphatidylcholine, and 0.1 mM arachidonic acid as substrate. To 1 ml of this mixture aliquots (50–200 µl) of the purified enzyme preparations were added, and the increase in absorbance at 235 nm was recorded at room temperature.

Enzyme preparations of the mutagenized 5-LOX were purified by FPLC on a semi-preparative MonoQ column (Amersham Pharmacia Biotech, Uppsala, Sweden) or by ATP affinity chromatography on an open bed ATP-Sepharose column (32). For this purpose, a LOX-active clone was picked with a sterilized toothpick and 10 ml of LB medium containing ampicillin (0.1 mg/liter) were inoculated. After overnight culture at 37 °C, 1 ml of this pre-culture was added to 200 ml of LB medium (0.1 mg/liter ampicillin) and the bacteria were grown at 37 °C to an optical density at 600 nm of about 0.5. Expression of the recombinant LOX species was then induced with 1 mM IPTG, and the culture was incubated for additional 12 h at 30 °C. Cells were spun down, washed with phosphate-buffered saline, and resuspended in 2 ml of 50 mM triethanolamine/HCl buffer, pH 7.5, containing 2 mM EDTA and 10 mM mercaptoethanol. Cells were lysed by sonication (three times for 20 s) with a Labsonic U-tip sonifier (Braun), and cell debris was removed by centrifugation.

For purification of the LOX species, aliquots of the supernatant were injected into FPLC and the chromatograms were developed with a linearly increasing sodium chloride gradient. Alternatively, aliquots of the supernatant were applied to an ATP-Sepharose column (gel volume of 3 ml). This column was washed with 10 ml of 50 mM triethanolamine/100 mM NaCl, 800 mM NaCl solution, and 1 M NaCl to remove unspecifically bound proteins, and the 5-LOX was then eluted with the same buffer containing 100 mM NaCl and 15 mM ATP. Fractions of 2 ml were collected, and the LOX activity was assayed. With these one-step purification procedures, the enzyme was purified about 500-fold, but we did not reach electrophoretic homogeneity (10–30% purity of the final enzyme preparation). Attempts to further purify the enzymes by classical column chromatography we experienced severe losses in enzyme activity. In separate experiments we compared the positional specificity of crude and purified enzyme preparations and did not observe significant differences.

HPLC of Oxygenated Fatty Acids—HPLC was performed on a Shichmadzu system connected to a Hewlett Packard diode array detector 1040. Reverse phase-HPLC was carried out on a Nucleosil C-18 column (Macherey-Nagel, K5 system, 250 × 4 mm, 5-µm particle size) coupled with an appropriate guard column (30 × 4 mm, 5-µm particle size). For analysis of the mono-oxygenated fatty acids (HETE and HpETE isomers), a solvent system of methanol/water/acetic acid (80/20/0.1, by volume) was used at a flow rate of 1 ml/min. For the diHETE derivatives, the water content of the solvent system was somewhat increased (85/15/0.1, by volume) and the chromatographic scale was calibrated for conjugated dienes by injecting known amounts of 15HETE and for conjugated trienes using 8S,15S-diHETE as reference. Straight phase-HPLC (SP-HPLC) was performed on a Zorbax-SIL column (250 × 4.6 mm, 5-µm particle size) using a solvent system of n-hexane/2-propanol/acetic acid (100/20/0.1, by volume) at a flow rate of 1 ml/min. For enantiomer separation of the different hydroxy fatty acid isomers, chiral phase HPLC was employed using the following conditions: for 15S/HETE, analysis of the free acid on a Chiralcel OD column (250 × 4.5 mm, 5-µm particle size) with the solvent system n-hexane/2-propanol/acetic acid (80/20/0.1, by volume) and a flow rate of 1 ml/min; for 8S/HETE, analysis of the methyl ester on a Chiralcel OD column (250 × 4 mm, 5-µm particle size) with the solvent system n-hexane/2-propanol/acetic acid (100/40/0.1, by volume) and a
flow rate of 1 ml/min; for 5RS-HETE, analysis of the methyl ester on a Chiralcel OB column (250 × 4 mm, 5-µm particle size) with the solvent system n-hexane/2-propanol/acetic acid (100/5/0.1, by volume) and a flow rate of 1 ml/min.

Miscellaneous Methods—For basic kinetic characterization of the 5-LOX species, the oxygenation of arachidonic acid was quantified either spectrophotometrically or by RP-HPLC quantification of the LOX products. \( K_m \)-values were determined varying the substrate concentration in the range of 10–120 \( \mu \)M, and the linear part of the Lineweaver-Burk plot was evaluated. HPLC standards of racemic 5RS/HETE, 8RS/HETE, 12RS/HETE, and 15R/S/HETE were prepared by vitamin E-controlled autoxidation of arachidonic acid methyl ester and subsequent HPLC separation of the positional isomers. The free hydroxy fatty acids were obtained by alkaline hydrolysis of the methyl esters and subsequent reverse phase HPLC purification. For immunoblotting the bacteria were lysed as described for the activity assays. The cell debris was spun down, and aliquots of the lysis supernatants were applied to SDS-gel electrophoresis. The proteins were transferred to a nitrocellulose membrane by a semidry blotting procedure, and the blots were probed with a polyclonal antibody against the human 5-LOX (kind gift of A. Habenicht, Heidelberg, Germany). Anaerobiosis was achieved by repeated aeration of the assay mixture and subsequent flushing with argon. Hydroxy fatty acids were methylated with diazomethane in diethylether, and the resulting methyl esters were purified by RP-HPLC.

![FIG. 1. Product pattern formed by the purified recombinant human 5-LOX.](image)

A 200-ml fermentation of the human 5-LOX was performed, and the recombinant enzyme was purified by affinity chromatography on ATP-Sepharose. The active fractions were pooled and the product pattern of arachidonic acid oxygenation was analyzed by RP-HPLC as described under “Materials and Methods.” The chemical structure of the major product was confirmed by coinjection with authentic standards in RP- and SP-HPLC and by UV spectroscopy (inset). The \( S/R \) ratio (9:1) of the 5-HpETE formed was determined by chiral HPLC of the hydroxy fatty acid methyl ester obtained after suitable derivatization (reduction and methylation).

![FIG. 2. Amino acid alignment of mammalian 5- and 15-LOXs.](image)

The one-letter code for amino acids is used. The numbers indicate the position of the amino acid for the human 5-LOX.

RESULTS

Bacterial Expression of the Human 5-LOX and Characterization of the Recombinant Enzyme—Bacteria transformed with the recombinant expression plasmid containing the human 5-LOX cDNA express the functional enzyme as indicated by activity assays (Fig. 1) and immunoblot analysis (data not shown). An arachidonic acid oxygenase activity of 0.5 ± 0.1 \( \mu \)g of 5-HpETE formation/15-min incubation period (n = 27) was measured per milliliter of culture fluid. For more comprehensive characterization the recombinant enzyme was expressed in a 200-ml liquid culture and purified by affinity chromatography on ATP-Sepharose. The purified enzyme exhibited similar enzymatic characteristics (kinetic lag-phase, substrate specificity, suicidal inactivation) as crude enzyme preparations (bacterial lysis supernatant) and as the native enzyme prepared from human leukocytes.

Single Point Mutations of Sequence Determinants—Structure-based sequence alignment of mammalian 5- and 15-LOXs (Fig. 2) suggested potential sequence determinants for the positional specificity of the human 5-LOX, and these residues were targeted by site-directed mutagenesis. In order to decrease the volume of the substrate-binding pocket, we mutated these amino acids to the more space-filling counterparts present in reticulocyte-type 15-LOXs. Single point mutations at positions 424 (A424I) or 425 (N425M) led to enzyme species forming significant amounts of 8S-HpETE in addition to 5S-HpETE, as indicated by HPLC analysis of the corresponding hydroxy derivatives obtained after borohydride reduction of the primary oxygenation products (Fig. 3). In contrast, A603I and C561F exchange did not influence the positional specificity (Table I). Phe-353, which constitutes a sequence determinant of mammalian 12/15-LOXs, aligns with Phe-359 of the human 5-LOX. In order to reduce the volume of the active site, we mutated Phe-359 to more bulky Trp. From Table I it can be seen that the F359W mutant exhibited an altered positional specificity compared with the wild-type enzyme. Although 5S-HpETE was still the major oxygenation product, 8S-HpETE contributed about 30% to the product mixture. Here again the corresponding hydroxy fatty acids obtained after borohydride reduction of the peroxo fatty acids formed were analyzed. The F359H mutant formed little more 8S-HpETE than the wild-type control, but significantly less than the F359W mutant (Table II). These minor alterations may be explained by the fact that a Phe-His exchange may not significantly alter the volume of the substrate binding pocket since both amino acids are of similar size (33). When smaller site chains were introduced at this position (F359L, F359V), inactive enzyme species resulted (Table II). It may be speculated that enlargement of the active site leads to a sloppy substrate.
Positional specificity of multiple point mutants of the human 5-LOX

The LOX activity was expressed as micrograms of HETE formation/ml of fermentation culture during a 15-min incubation period. Product formation was quantified by RP-HPLC analyzing the HETEs obtained after borohydride reduction of the HpETEs formed. The activity data represent the means of five independent measurements with different LOX-positive clones.

| Mutant LOX activity | Product pattern | μg HETE/ml culture fluid | share in % |
|---------------------|-----------------|--------------------------|-----------|
|                     | 5-HETE          | 8-HETE                   | 15-HETE   |
| Wild-type           | 0.58            | >95<sup>a</sup>          | <5        | 0         |
| N425M               | 0.47            | 76<sup>a</sup>           | 24<sup>a</sup> | 0         |
| N425M/F359W         | 0.58            | 59<sup>a</sup>           | 41<sup>a</sup> | 0         |
| N425M/A424I         | 0.40            | 22<sup>a</sup>           | 78<sup>a</sup> | 0         |
| F359WA424I          | 0.94            | <5                       | 90<sup>a</sup> | 6<sup>a</sup> |
| F359WN425M/A424I    | 0.09            | 5                        | 55<sup>a</sup> | 42<sup>a</sup> |

<sup>a</sup> S/R ratio > 9:1.

Mutations of the Sequence Determinants—To further reduce the volume of the substrate-binding pocket, we combined the effective single mutations listed in Table I. When N425M was combined with F359W and A424I (F359W/N425M, A424I/N425M double mutants), the share of 8S-HpETE formation was strongly augmented (Table III). Here again, we did not observe significant amounts of chiral 15-HETE when analyzing the product mixture after borohydride reduction. After combining the F359W exchange with an A424I mutation, we no longer observed a major 5-HpETE formation. Instead, 8S-HpETE was identified as main oxygenation product (Table III). Interestingly, this double mutant converted arachidonic acid consistently to a small (5%) but significant share of chiral 15S-HpETE, as indicated by HPLC analysis of the hydroxy fatty acids obtained after borohydride reduction. This effect was even more pronounced when the F359WA424I/N425M triple mutant was constructed. As indicated in Fig. 4, this mutant enzyme converted arachidonic acid to an almost 1:1 mixture of 8S- and 15S-HpETE. Although the rate of arachidonic acid oxygenation was lower than that of the wild-type controls (0.1 μg of HpETE/ml of culture fluid culture versus 0.58 μg/ml culture fluid of the wild-type enzyme), product formation was completely enzyme-controlled as indicated by the high degree of chirality of the hydroperoxy fatty acids formed (Fig. 4, insets).

It has been reported before that I593A exchange altered the positional specificity of the rabbit 15-LOX (25), but the inverse strategy with the human 5-LOX (A603I) was not successful (Table I). It may be speculated that reduction of the active site volume achieved by this mutation was not sufficient to alter the enzyme specificity. However, when A603I exchange was carried out with the F359WA424I/N425M triple mutant, a LOX species was created, which exhibited a major 15LOX activity (Fig. 5). We found that the F359WA424I/N425M/A603I quadruple mutant exhibited an impaired oxygenase activity (mutant: 0.16 ± 0.08 μg of HETE/ml of culture fluid, n = 20; wild-type control: 0.58 μg of HETE/ml of culture fluid, n = 27) and that the share of 15S-HpETE formation varied between 75% and 90%. Here again, the high degree of chirality (Fig. 5, insets) of the two major reaction products indicated that the dioxygenase reaction was completely enzyme-controlled.

Multiple point mutations are always dangerous since the

binding and, thus, to a strongly reduced enzymatic activity.

Taken together these results indicate that Phe-359, Ala-424, and Asn-425 may be considered as sequence determinants for

the positional specificity of the human 5-LOX and that introduction of more space-filling residues at these positions leads to an increased share of 8S-HpETE formation. Unfortunately, these alterations were only partial, and we did not observe any chiral products of 15-lipoxygenation.
three-dimensional structure may be altered at different sites, and, thus, the enzyme may lose activity. In fact, our triple and quadruple mutants exhibited reduced substrate affinity when compared with the native 5-hydroxylase, we analyzed the primary oxygenation products prepared under non-reducing conditions. Since hydroperoxy fatty acids are not well separated in RP-HPLC from the corresponding hydroxy compounds, the oxygenated fatty acids were prepared by RP-HPLC and further analyzed by SP-HPLC. From Fig. 6 (panel A), it can be seen that the wild-type 5-LOX converts arachidonic acid mainly to 5-HpETE. Using the F359W/A424I/N425M/A603I mutant as catalyst, the major oxygenation product co-eluted with an authentic standard of 15-HpETE (panel B). When product preparation was carried out under reducing conditions, we detected the corresponding hydroxyl derivatives (Fig. 6), and these data confirm that fatty acid hydroperoxides constitute the primary reaction products. Under anaerobic conditions, arachidonic acid oxygenation was prevented, and experiments with H_{2}^{18}O did not show any incorporation of heavy oxygen isotopes. These data indicate that the quadruple 5-LOX mutant remains a true LOX and that multiple mutations do not convert the LOX to a fatty acid hydroxylase.

Arachidonic acid methyl ester was not a suitable substrate for the wild-type 5-LOX and its F359W/A424I/N425M/A603I mutant. This finding was not surprising for the wild-type enzyme since similar data have been reported before for the native enzyme (34). However, the 15-lipoxygenating quadruple mutant was expected to oxygenate methyl arachidonate since other 15-lipoxygenating enzyme species (reticulocyte-type 15-LOX) accept fatty acid methyl esters as substrate. Our finding that this 5-LOX mutant is not capable of oxygenating arachidonic acid methyl ester suggests that the enzyme species exhibits a substrate specificity, which is more closely related to the wild-type 5-LOX and/or to the epidermis-type 15-LOX (28).

It has been reported before that the positional specificity of certain LOX isoforms strongly depends on the pH of the reaction mixture (35). To exclude that the differences in the positional specificity of the 5-LOX mutants may be due to a different pH sensitivity, we investigated the pH dependence of both the wild-type 5-LOX and its 15-lipoxygenating quadruple mutant. As shown in Fig. 7, the activity of both enzyme species showed somewhat different pH profiles. For the wild-type 5-LOX, a pH optimum of 8.0 was determined under our experimental conditions and 5-HpETE turned out to be the exclusive oxygenation product over the entire pH range. The F359W/A424I/N425M/A603I quadruple mutant oxygenated arachidonic acid most effectively at pH 8.5, and 15-HpETE was identified as major reaction product at all pH values tested.

Basic kinetic characterization of the wild-type 5-LOX, its 8-lipoxygenating double mutant, and its 15-lipoxygenating quadruple mutant revealed that the mutant isoforms exhibit a somewhat reduced substrate affinity when compared with the

FIG. 4. Arachidonic acid 15-lipoxygenation by the F359W/A424I/N425M 5-LOX triple mutant. The wild type and mutant 5-LOX species were expressed in E. coli (1 liter of culture) and purified by FPLC on a Mono Q-Sepharose column. LOX-containing fractions were pooled, activity assays were carried out, and the oxygenation products extracted under reducing conditions were analyzed by RP-HPLC. Insets, analysis of the enantiomer composition of 15- and 8-HETE obtained after suitable derivatization (reduction and methyl-
The enzyme species were prepared as described in the legend to Fig. 6. Aliquots of the pooled LOX-active fraction of the ATP-Sepharose affinity chromatography were incubated in the standard assay system using different hydroxy fatty acids as substrate (20 μM final concentration, 0.5 ml of assay sample). After borohydride reduction the incubation mixture was acidified, acidic methanol (1% acetic acid) was added to reach a final concentration of 50%, and the entire mixture was injected for RP-HPLC analysis of the dihydroxy fatty acids (see “Materials and Methods”). The chemical structure of the various diHETE isomers was confirmed by UV spectroscopy and coinjection with authentic standards.

### TABLE V

| Substrate        | Wild-type 5-LOX | F359W/A424I/N425M/A603I |
|------------------|-----------------|--------------------------|
| 5S-HETE          | No              | 5,15-DiHETE 0.14          |
| 12S-HETE         | 5,12-DiHETE 0.13| No 0                     |
| 15S-HETE         | 5,15-DiHETE 0.43| 8,15-DiHETE 0.03         |

DISCUSSION

For mammalian 12- and 15-LOXs the amino acids, which align with the Phe-353 (23), Ile-418, Met-419 (24), and Ile-593 (25) of the rabbit reticulocyte 15-LOX, have been identified as sequence determinants for the positional specificity. However, the question of whether these amino acids may also be important for the specificity of the pharmacologically most relevant mammalian 5-LOXs has not been investigated so far. Since previous site-directed mutagenesis studies and chimera formation failed to convert mammalian 12- and 15-LOXs to 5-lipoxygenating enzyme species (24, 25, 27), we followed an inverse strategy and mutated critical amino acids of the human 5-LOX to the more space-filling counterparts present in mammalian 15-LOXs. Our data suggest that Phe-359, Ala-424, Asn-425, and Ala-603 of the human 5-LOX are involved in positioning substrate fatty acids at the active site and, thus, may be considered as sequence determinants for the positional specificity. When these amino acids alone or in combination with each other were mutated to more space-filling residues, we observed a gradual conversion of the human 5-LOX to 8- and further to 15S-lipoxygenating enzyme species. These results, which indicate the principle possibility of interconverting 5- and 15-lipoxygenating enzyme species, are rather surprising since the stereochirality of 5S- and 15S-lipoxygenation is quite different and both isoenzyme classes do not share a high degree of amino acid identity (64% between human 5-LOX and reticulocyte-type 15-LOX).

wild-type enzyme ($K_m$ of 63 μM for the F359W/A424I double mutant, 57 μM for the F359W/A424I/N425M/A603I quadruple mutant, and 35 μM for the wild-type 5-LOX). However, the differences observed were not very dramatic.

Using different hydroxy fatty acids as substrate, we investigated the question of whether the various 5-LOX species are capable of catalyzing hydrogen abstraction from different bisallylic methylenes. We found that the wild type 5-LOX strongly favors C-7 hydrogen abstraction. 5S-HETE, which lacks a C-7 bisallylic methylene, was not oxygenated by this enzyme. In contrast, 12S-HETE and 15S-HETE containing C-7 bisallylic methylenes were effectively oxygenated at C-5 (Table V). The 15-lipoxygenating quadruple mutant was less restrictive with respect to the site of hydrogen abstraction. 5S-HETE, which contains two bisallylic methylenes (C-13 and C-10), was oxygenated at C-15 and at C-12, indicating that the mutant enzyme can catalyze hydrogen abstraction from both bisallylic methylenes to an almost similar extent (Table V). 15S-HETE, which lacks the C-13 bisallylic methylene, was oxygenated at C-8, and this reaction involves a C-10 hydrogen removal. Interestingly, the reaction rate of 15S-HETE oxygenation was more than 1 order of magnitude lower than that of 5S-HETE conversion. 12S-HETE, which only contains a C-7 bisallylic methylene, was not oxygenated by the F359W/A424I/N425M/A603I mutant. This result is quite plausible since the quadruple mutant was not capable of catalyzing C-7 hydrogen abstraction from arachidonic acid (Fig. 5).

**Fig. 7.** pH profiles of the LOX activity of the wild type 5-LOX and its F359W/A424I/N425M/A603I mutant. Enzyme preparation was carried out as described in the legend to Fig. 6. The different pH values were adjusted in a phosphate/borate buffer mixture by the addition of 1 N HCl or 1 N NaOH. The relative LOX activity was determined by quantifying the formation of hydroxy fatty acids (after borohydride reduction) by RP-HPLC after a 15-min incubation period. No major alterations in the positional specificity were observed during the entire pH range.
In order to find out whether the effects observed are peculiar for the human leukocyte 5-LOX, we carried out mutagenesis studies on a potato tuber 5-LOX (36). The recombinant wild-type enzyme oxygenated arachidonic acid to a mixture of 5- and 8-HpETE, as indicated by RP-HPLC of the corresponding hydroxy compounds obtained after borohydride reduction (5-HpETE/8-HpETE ratio of 65:35). No 15-HpETE formation was observed. Val-580 of this enzyme, which aligns with Asn-425 of the human 5-LOX, was mutated to a more space-filling His, and the resulting V580H mutant converted arachidonic acid to a 60:20:20 mixture of 5-, 8-, and 15-HpETE (data not shown). With this enzyme, a single point mutation altered the positional specificity in favor of 15S-lipoxygenation. However, the alterations were only partial and we did not carry out multiple mutations on this plant LOX.

The x-ray data of the rabbit 15-LOX (22) and more recent mutagenesis studies on this enzyme (25) suggested that the bottom of the substrate-binding pocket is defined by the side chains of Phe-353, Ile-418, and Ile-593. The walls of the cavity are lined by hydrophobic residues, but in the proximity of the iron center there are several polar amino acids. The opening of the substrate-binding cleft may be capped by Arg-403, which for the human leukocyte 5-LOX (36). The recombinant wild-type enzyme species created in this study may be used in molecular test systems searching for isoform-specific LOX inhibitors. Thus, unspecific LOX inhibitors, which have no preference for a particular LOX isoform, may not be developed as potential drugs, because of probable unwanted side effects. However, the observation that the volume of the active site of mammalian 5-LOXs is bigger than that of other isoforms may be useful for the development of specific 5-LOX inhibitors. The mutant enzyme species created in this study may be used in molecular test systems searching for isoform-specific LOX inhibitors.

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15. The substrate-binding pocket is defined by the side chains of Phe-353, Ile-418, and Ile-593. The walls of the cavity are lined by hydrophobic residues, but in the proximity of the iron center there are several polar amino acids. The opening of the substrate-binding cleft may be capped by Arg-403, which was modeled to interact with the substrates’ carboxylate (22, 37). Arg-403 of the rabbit enzyme aligns with Lys-409 of the human 5-LOX (Fig. 2), and we mutated this lysine to a positively charged arginine (K409R) and to an apolar leucine (K409L). Surprisingly, we did not observe major differences when the positional specificity and the specific activities of the wild type and mutant enzyme species were compared. These data suggest that Lys-409 of the human 5-LOX may not be of major importance for enzyme/substrate interaction.

In mammals 5- and 15-LOXs may have different biological functions. Thus, unspecific LOX inhibitors, which have no preference for a particular LOX isoform, may not be developed as potential drugs, because of probable unwanted side effects. However, the observation that the volume of the active site of mammalian 5-LOXs is bigger than that of other isoforms may be useful for the development of specific 5-LOX inhibitors. The mutant enzyme species created in this study may be used in molecular test systems searching for isoform-specific LOX inhibitors.