Enantioselective Substrate Specificity of 15-Lipoxygenase 1[†]

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ABSTRACT: 15-Lipoxygenases are lipid-peroxidizing enzymes which have been implicated in the pathogenesis of various diseases, such as inflammation, atherosclerosis, and osteoporosis. Although the crystal structures for several lipoxygenase isoforms have been solved, there is little information on the substrate alignment at the active site and its impact on the catalytic mechanism. Investigating the oxygenation of specifically designed hydroxy fatty acids, we observed a pronounced enantioselectivity of 15-lipoxygenases for substrates carrying the oxygen moiety in close proximity to the site of hydrogen abstraction [16(*R*/*S*)-HETE, 17(*R*/*S*)-HETE]. To investigate the mechanistic basis for this unexpected behavior, we applied a strategy involving targeted substrate modification, site-directed mutagenesis, and structural modeling of the enzyme−substrate complex. Taken together, our data suggest that an (*S*)-hydroxy group in 16-HETE may form a hydrogen bridge between the substrate molecule and Gln548, which contributes to proper alignment of the fatty acid derivative at the active site of the enzyme. This interaction, which was not observed with 16(*R*)-HETE, 18(*R*)-HETE, or 18(*S*)-HETE, appears to be a major reason for the high degree of enantioselectivity during lipoxygenation of 16-HETE.

Lipoxygenases (LOXs)[1] are lipid-peroxidizing enzymes which convert free and esterified polyenoic fatty acids to the corresponding hydroperoxy derivatives (*1*, *2*). They have been implicated in the pathogenesis of chronic diseases such as inflammation (*3*), atherosclerosis (*4*), and osteoporosis (*5*) but also in targeted degradation of intracellular organelles during erythropoiesis (*6*). The catalytic cycle of the LOX reaction, which involves valency shuttling of the non-heme iron, consists of several elementary reactions, and their stereochemistry is tightly controlled (*7*, *8*). (i) Stereoselective hydrogen abstraction: Using arachidonic acid as substrate hydrogen can be abstracted from three different bisallylic methylenes, but most LOXs remove only a well-defined hydrogen atom. For instance, 15(*S*)-LOXs selectively abstract the *pro-S* hydrogen from C-13 whereas 5(*S*)-LOXs remove the *pro-S* hydrogen from C-7. (ii) Stereospecific oxygen insertion: If hydrogen is abstracted from a single bisallylic methylene, there are four stereochemical options for oxygen insertion, but most LOXs only select one of these positions. 15(*S*)-LOXs direct molecular dioxygen into the *S*-position at C-15, whereas 15(*R*)- or 11(*R*/*S*)-oxygenation is usually not catalyzed. The mechanistic basis for the stereochemical control of the LOX reaction is still elusive. Clearly, the stereochemistry of hydrogen abstraction is related to the alignment of the fatty acid substrate at the active site, and experiments with arachidonic acid isomers showing a positional isomerism of the double bonds suggested that fatty acid substrates slide into the substrate-binding pocket with their methyl ends ahead (*9*, *10*). For 15-lipoxygenation fatty acid substrates are aligned at the active site in such a way that the *pro-S* hydrogen at C-13 is located in close proximity to the catalytic non-heme iron (Scheme 1A). In contrast, for 12(*S*)-LOXs the *pro-S* hydrogen at C-10 is localized near the iron (Scheme 1B). In other words, for 12(*S*)-lipoxygenation fatty acids slide deeper into the substrate-binding pocket (*11*, *12*).

Many biocatalysts including lipid-metabolizing enzymes exhibit enantioselective substrate specificities (*13*−*16*). They only accept a certain substrate enantiomer but leave its mirror image molecule unmetabolized. Polyenoic fatty acids are nonchiral compounds, and thus, the question of whether LOXs do also exhibit enantioselective substrate specificity was initially not of major relevance. However, more recent findings indicated that LOXs also accept more complex substrates (*17*−*19*), and some of them contain chiral centers. Recent studies on 15-LOX-catalyzed oxygenation of hydroxylated fatty acids indicated that these substrates were less effectively metabolized when compared with their nonhydroxylated counterparts (*20*, *21*). However, in these studies the enantioselectivity of the oxygenation reaction was not explored. To investigate this topic in more detail, we prepared a series of hydroxyarachidonic acid derivatives

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[1] Abbreviations: RP-HPLC, reverse-phase high-performance liquid chromatography; SP-HPLC, straight-phase high-performance liquid chromatography; CP-HPLC, chiral-phase high-performance liquid chromatography; LOXs, lipoxygenases; GC/MS, gas chromatography/mass spectrometry; 15(*S*)-H(p)ETE, (15*S*,5*Z*,8*Z*,11*Z*,13*E*)-15-hydro(pero)xyeicosa-5,8,11,13-tetraenoic acid; 12(*S*)-H(p)ETE, (12*S*,5*Z*,8*Z*,-10*E*,14*Z*)-12-hydro(pero)xyeicosa-5,8,10,14-tetraenoic acid; 16(*R*/*S*)-HETE, (16(*R*/*S*),5*Z*,8*Z*,11*Z*,14*Z*)-16-hydroxyeicosa-5,8,11,14-tetraenoic acid; 16(*R*/*S*)-FETE, (16(*R*/*S*),5*Z*,8*Z*,11*Z*,14*Z*)-16-fluoroeicosa-5,8,11,-14-tetraenoic acid.

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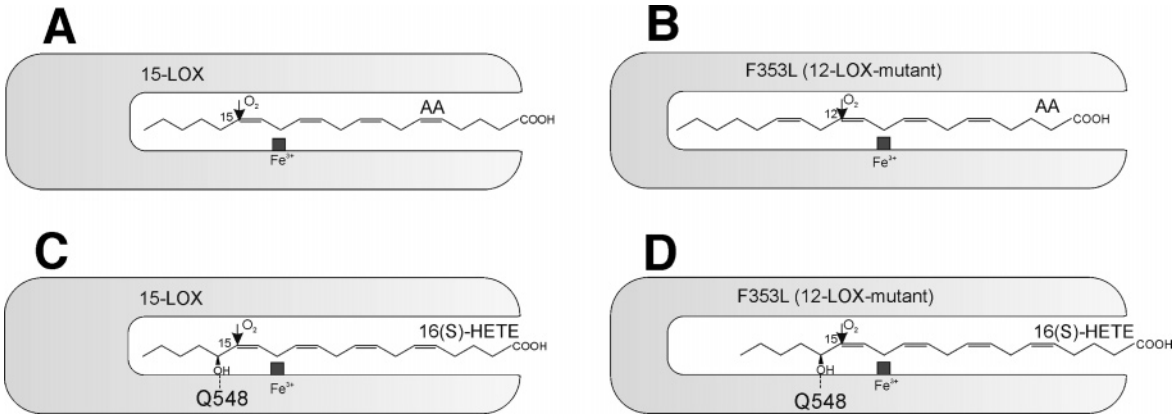
Scheme 1: Substrate Alignment of Arachidonic Acid Derivatives at the Active Site of 15-LOX Species[a]



[a] Panels: (A) Alignment of arachidonic acid at the active site of wild-type 15-LOX. The substrate fatty acid penetrates the active site with its methyl end ahead, and the bisallylic methylene C-13 is located in close proximity of the non-heme iron. (B) Alignment of arachidonic acid at the active site of the F353L mutant. The substrate orientation is similar to that in (A), but due to the deeper substrate-binding pocket, the bisallylic methylene C-10 is located close to the non-heme iron. (C) Alignment of 16(*S*)-HETE at the active site of wild-type 15-LOX. The substrate orientation is similar to that in (A), but the 16(*S*)-OH group hydrogen bridges to Q548 and, thus, stabilizes the enzyme−substrate complex. (D) The substrate orientation is similar to that in (A), but the hydrogen bridge between the 16(*S*)-OH group and Q548 prevents deeper penetration of the substrate into the substrate-binding pocket. C-13 remains in proximity to the iron, and thus, 15-lipoxygenation is catalyzed.

[16(*S*/*R*)-HETE, 17(*S*/*R*)-HETE, 18(*S*/*R*)-HETE] and tested the enantiomers as substrates for the rabbit 15-LOX-1. We found that 16(*S*)-HETE (optical purity >98%) was strongly preferred as substrate over the corresponding 16-(*R*)-enantiomer. In contrast, the degree of enantioselectivity was less pronounced with 17- and 18-HETE (optical purity of both compounds >95%). The 16(*S*)-OH group in 16(*S*)-HETE appears to hydrogen-bridge with Gln548 (active site amino acid), and this interaction prevents the substrate from sliding deeper into the substrate-binding pocket.

MATERIALS AND METHODS

Chemicals. The chemicals used were from the following sources: sodium borohydride from Serva (Heidelberg, Germany), ampicillin from Gibco (Eggenstein, Germany), 13(*S*)-HpODE, isopropyl β-d-thiogalactopyranoside (IPTG), arachidonic acid, and soybean LOX-1 from Sigma-Aldrich (Deisenhofen, Germany), nitrosomethylurea and bis(trimethylsilyl)trifluoroacetamide (BSTFA) from Sigma (Deisenhofen, Germany), 10% $Pd/CaCO_3$ (catalyst for hydrogenation) from Aldrich (Taufkirchen, Germany), and HPLC solvents from Merck (Darmstadt, Germany). phage T4 ligase, Pwo-polymerase, and sequencing kits were obtained from Boehringer Mannheim (Mannheim, Germany), and the *Escherichia coli* strain HB 101 was purchased from Invitrogen (San Diego, CA). Oligonucleotide synthesis was carried out by TiB-Molbiol (Berlin, Germany). All solvents were of HPLC grade and were purchased from Baker (Deventer, The Netherlands). The hydroxy fatty acid enantiomers used in this study were synthesized as reported before (*21*, *22*).

Preparation of the Native Rabbit 15-LOX. The native reticulocyte-type 15-LOX was prepared from the stroma-free hemolysis supernatant of a reticulocyte-rich blood cell suspension (*23*) by ammonium sulfate precipitation, hydrophobic interaction chromatography, and anion-exchange chromatography on a preparative Mono-Q column (Pharmacia, Uppsala, Sweden). The final enzyme preparation was electrophoretically pure (<95%) and exhibited a linoleic acid turnover rate of about 30 s^{-1}.

Expression of the Recombinant Rabbit 15-LOX and Enzyme Purification. The recombinant wild-type 15-LOX and the various mutants were expressed in *E. coli* as His-tagged fusion proteins. For this purpose the 15-LOX cDNA was cloned into the pQE-9 expression plasmid (Qiagen, Hilden, Germany) between the *Sal*I and *Hin*dIII restriction sites. Bacteria were transformed with the recombinant plasmids, and routinely, 2 L of LB medium containing 100 mg/L ampicillin and 25 mg/L kanamycin were inoculated with a 15 mL overnight preculture. The bacteria were allowed to grow at 37 °C for 16 h, and then expression of the recombinant protein was induced by addition of 1 mM isopropyl 1-thio-β-D-galactopyranoside (final concentration). The cultures were kept for an additional 2 h at 30 °C, and then the bacteria were pelleted, washed (with phosphate-buffered saline), and resuspended in 30 mL of phosphate-buffered saline. Cells were disrupted with an Emulsiflex-C5 high-pressure cell homogenizer (Avestin, Ottawa, Canada), and the cell debris was spun down. The clear lysis supernatant was applied to a 0.4 mL nickel−agarose column (Qiagen, Hilden, Germany). The column was washed twice with washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0), and the adherent proteins were eluted by rinsing the column with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 200 mM imidazole, pH 8.0). Five 0.20 mL fractions were collected and the LOX activity was assayed in each of them. Routinely, more than 90% of the activity was recovered in fractions 2−4. These fractions were pooled, diluted 1:50 with 20 mM Tris-HCl buffer, pH 7.4, and loaded onto a Q-Sepharose column (gel bed volume 500 μL; Amersham Biosciences) for further purification by anion-exchange chromatography. After loading, the column was washed twice with 2 mL of 20 mM Tris-HCl buffer, pH 7.4, and then the enzyme was eluted with 120 mM KCl dissolved in the same buffer. Fractions of 0.25 mL were collected, activity was assayed, and the active fractions were pooled. For storage (−80 °C) the enzyme solution was supplemented with 10% glycerol.

Site-Directed Mutagenesis. Site-directed point mutations were performed using the QuickChange site-directed mu-

tagenesis kit (Stratagene, Amsterdam, The Netherlands), and the primary structures of mutated plasmids were confirmed by DNA sequencing. For the mutant 5−10 clones were screened by restriction mapping and activity assays to identify catalytically active LOX-positive clones. Expression and purification of the mutants were performed as described for the wild-type enzyme.

Assay Systems. Oxygenation kinetics of the different fatty acid derivatives were assayed spectrophotometrically, measuring the increase in absorbance at 235 nm in the substrate concentration range of 10−300 μM. The assay mixture was a 0.1 M phosphate buffer, pH 7.4, containing various concentrations of fatty acid substrate (sodium salt), and the reaction was started by addition of enzyme (15 μg). All measurements were carried out at room temperature.

For analysis of the oxygenation products aliquots of purified enzyme preparations were incubated with various substrates (50 μM final substrate concentration) at room temperature for 35 min. For the 15-LOX and its mutants the assay mixture was 5 mL of sodium/potassium phosphate buffer, pH 7.4, containing 50 μM substrate fatty acid (as a sodium salt). The hydroperoxy fatty acids formed were reduced to the corresponding hydroxy derivatives by addition of 0.1 mL of a saturated methanolic solution of sodium borohydride. After acidification to pH 3, the lipophilic products were twice extracted with ethyl acetate. The organic extracts were combined. The solvent was evaporated. The residue was reconstituted in 0.5 mL of methanol, and aliquots were injected into the RP-HPLC.

Analytics. RP-HPLC was performed with a Shimadzu LC-6A liquid chromatograph connected to a Hewlett-Packard diode array detector 1040A. Separation of the fatty acid derivatives was performed on a Nucleosil C-18 column (Macherey-Nagel, Düren, Germany; 250 × 4 mm, 5 μm particle size) and a guard column (30 × 4 mm, 5 mm particle size; same vendor). The solvent system was a mixture of methanol/water/acetic acid (variable composition depending on the nature of the oxygenation products), and a flow rate of 1 mL/min was used. The absorbance at 235 nm was recorded. SP-HPLC was performed on a Nucleosil 100-7 column (Macherey-Nagel, Düren, Germany; 250 × 4 mm, 5 μm particle size) with the solvent system hexane/2-propanol/acetic acid (95:5:0.1 v/v) and a flow rate of 1 mL/min. Analysis of the chirality of LOX products was carried out either on a Chiralcel OB column with the solvent system hexane/2-propanol/acetic acid (97:3:0.1 v/v) or on a Chiralpack AD column with the solvent system hexane/methanol (98:2 v/v). In both cases a flow rate of 1 mL/min was used.

For GC/MS analysis the hydroxy fatty acids were prepared by RP- and/or SP-HPLC, methylated with diazomethane, and their hydroxy groups were silylated with bis(trimethylsilyl)-trifluoroacetamide (Sigma Chemicals, Deisenhofen, Germany) in dry pyridine. Gas chromatography/mass spectrometry was carried out on a Shimadzu GC-MS QP-2000 system equipped with a fused silica column, SPB 1 (10 m × 0.25 mm, coating thickness 0.25 μm). The injector temperature of 270 °C, ion source temperature of 180 °C, and electron energy of 70 eV were adjusted. The derivatized fatty acids were eluted with the following temperature program: isothermically at 180 °C for 2 min and then from 180 to 290 °C at a rate 5 °C/min.

Structural Modeling of the Enzyme/Substrate Complex. The model of the 15-LOX/16(*S*)-HETE complex was constructed on the basis of the crystal structure of rabbit reticulocyte 15-LOX (*24*) (PDB entry 1LOX). The missing residues 601−602, 210−211, and 177−187 as well as the 16(*S*)-HETE substrate were inserted using the molecular visualization program VMD (*25*), and energy was minimized using the molecular simulation program NAMD (*26*).

Preparation of 16(S)- and 16(R)-FETE. To a solution of either methyl 16(*R*)-HETE or 16(*S*)-HETE (5.0 mg, 0.015 mmol) in CH_2Cl_2 (1 mL) we added (diethylamino)sulfur trifluoride (4 μL, 0.036 mmol), and the mixture was stirred at −78 °C for 1 h. TLC analysis (50% ethyl acetate in hexane) indicated complete disappearance of the starting material. The reaction was stopped by addition of methanol (200 μL), and the entire reaction mixture was poured into a saturated solution of $NaHCO_3$. The lipophilic products were extracted twice with 2 mL of CH_2Cl_2, and the combined extracts were washed with H_2O and dried over Na_2SO_4. The solvent was removed under reduced pressure, and the reaction products were purified by RP-HPLC using methanol/water (85:15 v/v) as the solvent system. This procedure yielded about 4.4 mg (0.013 mmol) of 16-FETE methyl ester (RT = 15.6 min). The free acid [RT = 9.5 min, methanol/water/acetic acid (85:15:0.1 v/v)] was obtained in an overall yield of 82% by alkaline hydrolysis of the esters using NaOH (0.13 mmol) in a water/methanol solution.

Miscellaneous Methods. Protein concentrations were determined with 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 ethanol) were hydrogenated using 5 mg of 10% $Pd/CaCO_3$ from 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 as described above. Aliquots (2 μL) were analyzed by GC/MS. The kinetic constants (K_M and V_{max}) were obtained from Lineweaver−Burk plots. For these experiments the oxygenase activity with the different substrates was assayed at different fatty acid concentrations in the range between 10 and 500 μM. Initial oxygenation rates (increase in absorbance at 235 nm during the first 30 s of the progress curves) were used for calculations.

RESULTS

Enantioselective Oxygenation of Hydroxy Fatty Acid Isomers by the Rabbit 15-LOX. Hydroxylated polyenoic fatty acids are less effectively oxygenated by plant and mammalian LOXs when compared with their nonhydroxylated counterparts (*20*, *27*). However, the stereochemistry of this reaction was well controlled as indicated by the high degree of product specificity. To test whether hydroxy fatty acids containing a chiral center in the vicinity of the double bond system are oxygenated enantioselectively, we prepared racemic mixtures of 16(*S*/*R*)-HETE, 17(*S*/*R*)-HETE, and 18-(*S*/*R*)-HETE and determined the relative amounts of the substrate enantiomers that were not oxygenated by the rabbit

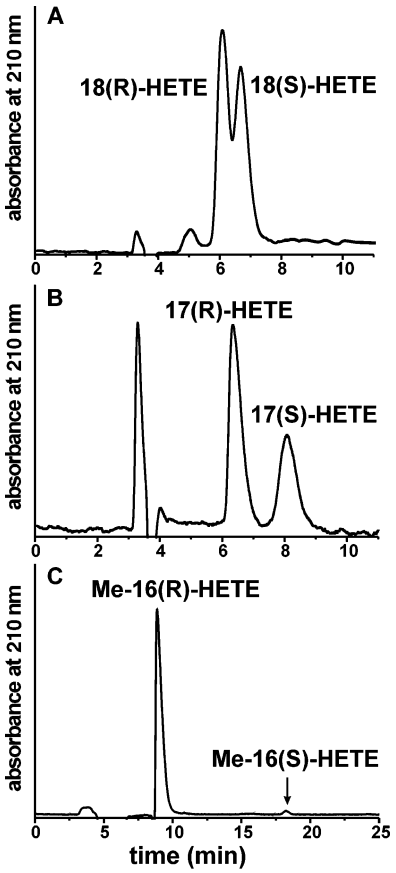


FIGURE 1: Enantioselective substrate conversion of 16(*S/R*)-HETE, 17(*S/R*)-HETE, and 18(*S/R*)-HETE by native rabbit 15-LOX. The pure native rabbit 15-LOX was incubated with racemic mixtures of 16-HETE, 17-HETE, and 18-HETE as described in Materials and Methods. After 10 min the sample was acidified, and the lipids were extracted. The solvent was evaporated, the products were reconstituted in 1 mL of methanol, the remaining substrate isomers were isolated by RP-HPLC and further analyzed by CP-HPLC [Chiralcel OB column, hexane/2-propanol/acetic acid (97:3:0.1 v/v), flow rate 1 mL/min for 18-HETE (panel A); Chiralcel OB column, hexane/2-propanol/acetic acid (97:3:0.1 v/v), flow rate 1 mL/min for 17-HETE (panel B); Chiralpack AD column, hexane/methanol (98:2 v/v), flow rate 1 mL/min for Me-16-HETE (panel C)].

15-LOX. From Figure 1A it can be seen that oxygenation of 18(*R*/*S*)-HETE proceeded with little if any enantioselectivity. In contrast, using 16(*R*/*S*)- and 17(*R*/*S*)-HETE as substrate (Figure 1B,C), we observed a preponderance of the corresponding *R*-isomer in the mixture of unconverted hydroxy fatty acids. These data suggest that 16(*S*)- and 17(*S*)-HETE are preferentially oxygenated by the rabbit 15-LOX.

To quantify the degree of enantioselectivity of 16(*R*/*S*)- and 17(*R*/*S*)-HETE oxygenation more precisely, the substrate enantiomers were separated by chiral-phase HPLC and tested separately as LOX substrates. Here we found that 16(*S*)-(Figure 2A) and 17(*S*)-HETE (Figure 2B) were well oxygenated by the enzyme, whereas the corresponding *R* mirror images were less effective substrates. In fact, 16(*R*)-HETE was almost completely resistant toward 15-LOX-catalyzed oxygenation.

Next, we performed detailed kinetic studies on the oxygenation of 16- and 17-HETE enantiomers using the wild-type recombinant rabbit 15-LOX as catalyst. From Table 1 it can be seen that arachidonic acid (positive control) was well oxygenated by this enzyme species. K_M and V_{max}

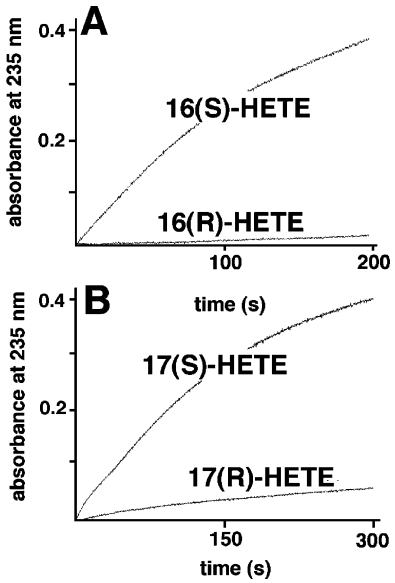


FIGURE 2: Substrate selectivity of native rabbit 15-LOX. The native rabbit 15-LOX (15 μg of protein/mL) was incubated with 16-HETE isomers (35 μM) and 17-HETE isomers (200 μM) as described in Materials and Methods, and the oxygenation reaction was followed spectrophotometrically, recording the absorbance at 235 nm.

determined here are in the range of the values reported previously for linoleic acid oxygenation by the native enzyme (*28*), and a catalytic efficiency coefficient (k_{cat}/K_M ratio) of 2.7 $(s\cdot\mu M)^{-1}$ was calculated.

16(*S*)-HETE was a less effective substrate than arachidonic acid as indicated by the lower k_{cat}/K_M ratio (Table 1). If one compares the k_{cat}/K_M ratios for the two 16-HETE enantiomers, a high degree of enantioselectivity becomes apparent (k_{cat}/K_M ratio for 16(*S*)-HETE of 21.6×10^{-3} $(s\cdot\mu M)^{-1}$ vs a k_{cat}/K_M ratio for 16(*R*)-HETE of $<0.7 \times 10^{-3}$ $(s\cdot\mu M)^{-1}$; enantioselectivity coefficient of >30.9). These data are consistent with the qualitative findings presented in Figure 2A. Similarly, a strong preference of the *S*-enantiomer was observed for 17-HETE although the extent of enantioselectivity was less pronounced (enantioselectivity coefficient of 7.4), and these results are consistent with the data shown in Figure 2B.

To test whether other 15-LOXs exhibit a similar enantioselectivity, we performed similar experiments with the soybean LOX-1. The kinetic studies on the oxygenation of 16(*S*)- and 16(*R*)-HETE revealed the following parameters: for 16(*S*)-HETE, $K_M = 53.8 \pm 14.0$ μM, $k_{cat} = 0.04 \pm 0.004$ s^{-1}, $k_{cat}/K_M = 0.7 \times 10^{-3}$ $(s\cdot\mu M)^{-1}$; for 16(*R*)-HETE, $K_M = 86.4 \pm 40.0$ μM, $k_{cat} = 0.01 \pm 0.002$ s^{-1}, $k_{cat}/K_M = 0.1 \times 10^{-3}$ $(s\cdot\mu M)^{-1}$, yielding a enantioselectivity coefficient of about 7. This value is more than 4-fold lower than that obtained for the rabbit enzyme, suggesting that rabbit 15-LOX exhibits a higher degree of enantioselectivity for 16-HETE oxygenation than the soybean enzyme.

Chirality of the Hydroxy Group Impacts the Positional Specificity of the LOX Reaction. With arachidonic acid as substrate the rabbit 15-LOX exhibits a dual positional specificity forming 15(*S*)-H(p)ETE and 12(*S*)-H(p)ETE in a ratio of about 10:1 (*29*). We confirmed these data under our experimental conditions for the recombinant enzyme species and observed a 15-/12-H(p)ETE ratio of 93:7 (Table 2). RP-HPLC analysis of the 16(*S*)-HETE oxygenation products

Table 1: Kinetic Parameters of Fatty Acid Oxygenation by Wild-Type Recombinant and Mutant (Phe353Leu) Rabbit 15-LOX[a]

	WT 15-LOX				Phe353Leu			
substrate	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M [($s\cdot\mu M)^{-1}$]	enantio-selectivity coeff	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M [($s\cdot\mu M)^{-1}$]	enantio-selectivity coeff
arachidonic acid	8.16 ± 0.25	3.01 ± 0.22	2700×10^{-3}	na[b]	6.02 ± 0.91	8.7 ± 2.9	691×10^{-3}	na
16(*S*)-HETE	0.51 ± 0.07	23.6 ± 7.0	21.6×10^{-3}	>30.9	0.20 ± 0.01	22.7 ± 6.9	8.8×10^{-3}	14.7
16(*R*)-HETE	<0.02[c]	28.9 ± 9.0[c]	$<0.7 \times 10^{-3}$		0.08 ± 0.02	136 ± 59	0.6×10^{-3}	
17(*S*)-HETE	1.24 ± 0.23	236 ± 29	5.2×10^{-3}	7.4	0.24 ± 0.09	31.3 ± 12.3	7.6×10^{-3}	1.4
17(*R*)-HETE	0.18 ± 0.06	293 ± 40	0.7×10^{-3}		0.20 ± 0.08	36.8 ± 12.8	5.4×10^{-3}	

[a] Arachidonate derivatives were oxygenated by the pure rabbit reticulocyte 15-LOX and the F353A mutant (15 μg/mL) at pH 7.4 in the standard assay system as described in Materials and Methods. Apparent K_M and k_{cat} values were extracted from Lineweaver−Burk plots (measurements at five different substrate concentrations). k_{cat} values are given as turnover number under V_{max} conditions (substrate saturation). The enantioselectivity coefficient was calculated by dividing the k_{cat}/K_M of the *S*-isomer by the same ratio of the *R*-enantiomer. [b] na = not applicable. [c] Owing to the low oxygenation rate of 16(*R*)-HETE exact kinetic constants were difficult to determine; here we give rough estimates.

Table 2: Comparison of the Product Pattern Formed from 16- and 17-HETE Enantiomers[a]

	WT 15-LOX		Phe353Leu		Ile593Ala	
substrate	product	share (%)	product	share (%)	product	share (%)
arachidonic acid	15(*S*)	93	15(*S*)	28	15(*S*)	55
	12(*S*)	7	12(*S*)	72	12(*S*)	45
16(*S*)-HETE	15,16(*S*)	93	15,16(*S*)	89	15,16(*S*)	90
	12,16(*S*)	7	12,16(*S*)	11	12,16(*S*)	10
16(*R*)-HETE	—[b]		15,16(*R*)	6	—[b]	
			12,16(*R*)	94		
17(*S*)-HETE	15,17(*S*)	88	15,17(*S*)	3	15,17(*S*)	10
	12,17(*S*)	12	12,17(*S*)	97	12,17(*S*)	90
17(*R*)-HETE	15,17(*R*)	63	15,17(*R*)	1	15,17(*R*)	11
	12,17(*R*)	37	12,17(*R*)	99	12,17(*R*)	89
16(*S*)-FETE	15,16(*S*)	69	15,16(*S*)	25	15,16(*S*)	52
	12,16(*S*)	31	12,16(*S*)	75	12,16(*S*)	48
16(*R*)-FETE	15,16(*R*)	78	15,16(*R*)	24	15,16(*R*)	55
	12,16(*R*)	22	12,16(*R*)	76	12,16(*R*)	45

[a] Enzyme species (15 μg/mL) were incubated in a 5 mL assay volume (50 μM fatty acid substrate). Product preparation and RP-HPLC were carried out as described in Materials and Methods. Product structures were concluded from GC/MS data (hydrogenated derivatives). [b] Small amounts of unspecific products.

indicated one major conjugated diene (Figure 3A), which was homogeneous in SP- and CP-HPLC (data not shown). The electron impact mass spectrum of the hydrogenated TMS ether was characterized by the major α-cleavage fragments at *m*/*z* 159 and 343 as well as by an ion of minor intensity at *m*/*z* 487 (Table 3), indicating oxygen insertion at C-15 (Table 2). In contrast, using 16(*R*)-HETE as substrate only small amounts of unspecific reaction products were observed (Figure 3B), which is consistent with the spectrophotometric measurements (Figure 2A). With 17(*S*)-HETE as substrate we also observed an arachidonic acid like product pattern [ratio of 15-/12-oxygenation of 88:12 (Table 2)], and the structure of the major oxygenation products was confirmed by GC/MS (Table 3). For 17(*R*)-HETE 15-lipoxygenation was also dominant. However, the share of 12-lipoxygenation products was increased to about 30% (Table 2).

Impact of the Hydroxy Group on the Specificity of 12-Lipoxygenation. The positional specificity of arachidonic acid oxygenation is altered in favor of 12-lipoxygenation (*30*) when Phe353 is mutated to less space-filling amino acids (Phe353Leu exchange). To test whether a similar shift in positional specificity can be observed when 16- and 17-HETE enantiomers were used as substrate, we first determined the basic kinetic constants for the oxygenation of the four enantiomers using the Phe353Leu mutant. From Table 1 it can be seen that the k_{cat}/K_M ratio of arachidonic acid oxygenation is somewhat lower than that of the wild-type

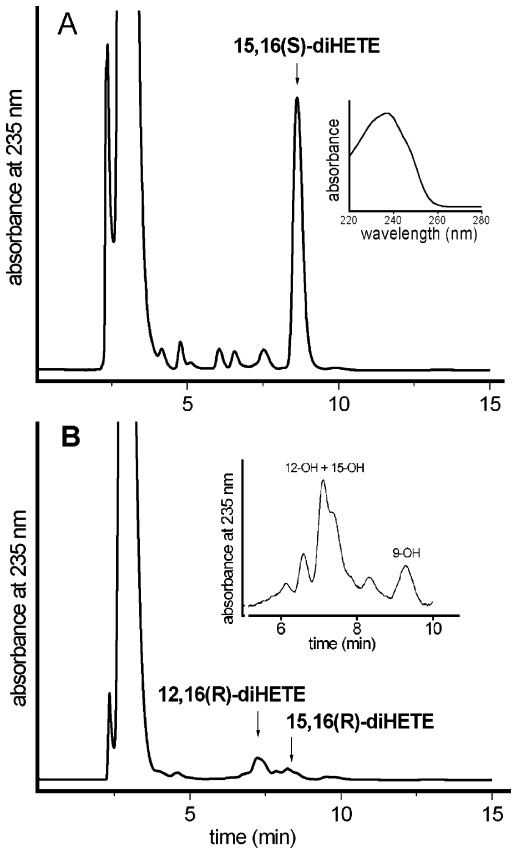
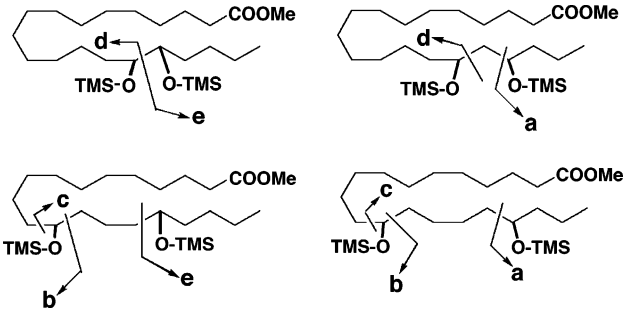


FIGURE 3: Formation of conjugated dienes during the oxygenation of 16(*S*)- and 16(*R*)-HETE by the recombinant rabbit 15-LOX. The purified wild-type recombinant rabbit 15-LOX (15 μg/mL) was incubated in the standard assay (1 mL assay volume) in the presence of either 40 μM 16(*S*)-HETE (panel A) or 40 μM 16(*R*)-HETE (panel B). The hydroperoxy products formed were reduced with sodium borohydride (see Materials and Methods), the sample was acidified, the proteins were precipitated with 1 mL of ice-cold methanol, and after centrifugation aliquots of the clear supernatant were injected to RP-HPLC using methanol/water/acetic acid (75:25:0.1 v/v) as a solvent system (flow rate 1 mL/min). The chemical structures of the products were deduced from GC/MS data obtained for the hydrogenated derivatives. (A) Oxygenation products of 16(*S*)-HETE. Inset: UV spectrum of the major product indicating the presence of a conjugated diene chromophore. (B) Oxygenation products of 16(*R*)-HETE. Inset: SP-HPLC analysis (see Materials and Methods) of the diHETE isomers prepared by RP-HPLC, indicating the formation of at least two products.

Table 3: Mass Spectral Data of Lipoxygenase Products



substrate	product	key ions (*m*/*z*) and relative abundance (%)
17-HETE	12,17-diHETE	145 (45.5)[a], 301 (51.0)[b], 303 (33.5)[c], 487 (0.8) [M^+]
	15,17-diHETE	145 (100)[a], 343 (40.0)[d], 487 (0.5) [M^+]
16-HETE	12,16-diHETE	159 (47.0)[e], 301 (55.1)[b], 303 (34.5)[c], 487 (0.4) [M^+]
	15,16-diHETE	159 (66.9)[e], 343 (51.0)[d], 487 (0.5) [M^+]

enzyme. Comparison of this ratio for 16(*S*)- and 16(*R*)-HETE suggested an enantioselective oxygenation of the substrate by this arachidonic acid 12-lipoxygenating enzyme species. As for the wild-type enzyme the *S*-enantiomer was more efficiently oxygenated, and an enantioselectivity coefficient of 14.7 was calculated. However, when 17(*S*)- and 17(*R*)-HETE were used as substrates, the degree of enantioselective preference of the 17(*S*)-enantiomer was rather limited for the Phe353Leu mutant (enantioselectivity coefficient of 1.4).

Comparing the composition of the oxygenation products formed by the wild-type 15-LOX and its Phe353Leu mutant, we found that 16(*S*)-HETE was predominantly oxygenated at C-15 (Table 2). This result was quite remarkable since it contrasts the dominant 12-lipoxygenation of arachidonic acid by the Phe353Leu mutant. Even more surprisingly, we observed a major 12-oxygenation when 16(*R*)-HETE was used as the substrate for this enzyme mutant. Thus, there was a pronounced difference in the positional specificity between 16(*S*)- and 16(*R*)-HETE oxygenation by the Phe353Leu mutant. In contrast, the two 17-HETE enantiomers were predominantly oxygenated at C-12 by this enzyme mutant. Taken together, these data indicate that Phe353Leu exchange strongly alters the positional specificity of the oxygenation of most substrates [arachidonic acid, 16(*R*)-HETE, 17(*R*)-HETE, 17(*S*)-HETE] in favor of 12-lipoxygenation except from 16(*S*)-HETE. For this hydroxy fatty acid the wild-type enzyme and the Phe353Leu mutant exhibited a similar positional specificity; both enzyme species introduce molecular dioxygen at C-15. In other words, if the hydroxy group at C-16 is present in the *S*-configuration, alterations in the positional specificity induced by site-directed mutagenesis appear to be prevented. This was, however, not the case if a 16(*R*)-OH group is present.

To test whether this effect is also apparent with other 12-lipoxygenating mutants, we performed an experiment with the Ile593Ala mutant of the rabbit 15-LOX (*12*). This mutant oxygenated 16(*S*)-HETE mainly at C-15 (Table 2). The relative share of 12,16(*S*)-HETE formation was only 10%. In contrast, the degree of 12-lipoxygenation was much higher when arachidonic acid, 17(*S*)-HETE, or 17(*R*)-HETE was used as substrate. Thus, Ile593Ala exchange induces alterations in the stereochemical characteristics of hydroxy fatty acid oxygenation similar to what we observed for the Phe353Leu mutant of the enzyme.

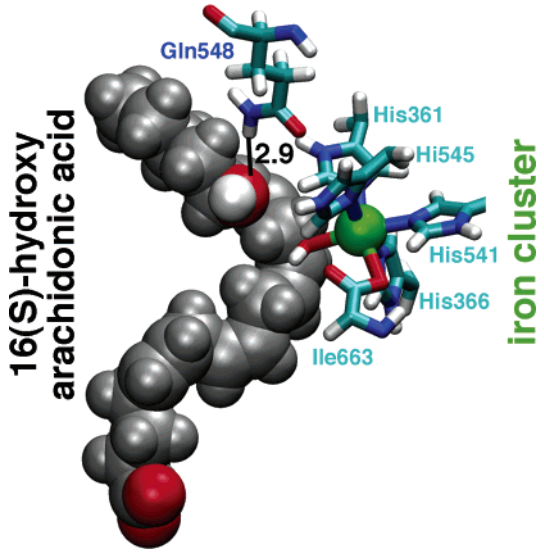


FIGURE 4: Model of the 15-LOX/16(*S*)-HETE complex. The model is based on the X-ray coordinates of a rabbit 15-LOX/inhibitor complex. The (*S*)-hydroxy group in 16(*S*)-HETE is in hydrogen-bonding distance (2.9 Å) to the amide nitrogen of Gln548. The modeling procedure is described in Materials and Methods.

Mechanistic Investigations. Mutation of the space-filling Phe353 to the less bulky Leu in the rabbit 15-LOX provides additional space at the active site (*12*, *28*), which allows arachidonic acid to slide further into the substrate-binding pocket (Scheme 1A,B). If one interprets our data on the positional specificity of 16(*S*)-HETE oxygenation in light of this hypothesis, it may be concluded that the 16(*S*)-hydroxy group prevents the substrate from sliding deeper into the active site. In contrast, a 16(*R*)-hydroxy group may not be capable of accomplishing such hindrance. To explain this phenomenon, we hypothesized that the 16(*S*)-OH group might hydrogen-bridge to a neighboring amino acid, which arrests the substrate in a position optimal for oxygen insertion at C-15 (Scheme 1C,D). In contrast, 16(*R*)-HETE may slide deeper into the substrate-binding pocket forming an enzyme/substrate complex, the structure of which is optimal for 12-lipoxygenation. To test this hypothesis, we first modeled the 15-LOX/16(*S*)-HETE complex and found that the hydroxy group is localized in close proximity to Gln548 and, thus, may hydrogen-bridge with this amino acid (Figure 4).

To obtain experimental evidence for such enzyme/substrate interaction, we exchanged the OH group in 16(*R*/*S*)-HETE with fluorine. This halogen occupies similar space as an OH group but is not capable of forming a hydrogen bridge. When 16(*S*)- and 16(*R*)-fluoro-5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid [16(*S*)-FETE and 16(*R*)-FETE, respectively] were oxygenated by the native rabbit 15-LOX (Figure 5A,B), we observed major oxygen insertion at C-15 independent of the stereochemistry of the chiral center. In addition, smaller amounts of the corresponding 12-lipoxygenation products were detected. In contrast, when the arachidonic acid 12-lipoxygenating mutant Phe353Leu was used as catalyst, 12-lipoxygenation was the dominant process for both 16(*S*)-FETE and 16(*R*)-FETE (Figure 5C,D). Thus, in contrast to 16(*S*)-HETE the fluorine-substituted derivative [16(*S*)-FETE] appears to

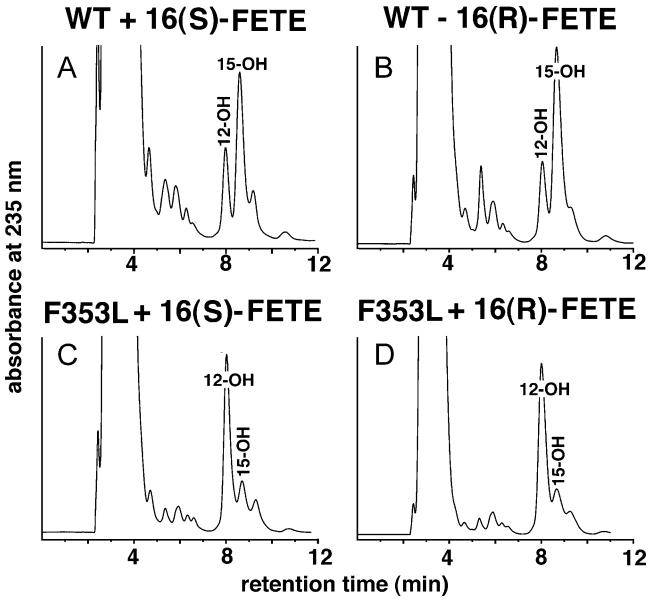


FIGURE 5: Formation of conjugated dienes during the oxygenation of 16(*S*)- and 16(*R*)-FETE by the recombinant rabbit 15-LOX and its Phe353Leu mutant. The purified wild-type recombinant rabbit 15-LOX (15 μg/mL, panels A and B) or its Phe353Leu mutant (panels C and D) was incubated in the standard assay (1 mL assay volume) in the presence of either 50 μM 16(*S*)-FETE or 50 μM 16(*R*)-FETE. The hydroperoxy products formed were reduced with sodium borohydride, the sample was acidified, the proteins were precipitated with 1 mL of ice-cold methanol, and after centrifugation aliquots of the clear supernatant were injected to RP-HPLC using methanol/water/acetic acid (80:20:0.1 v/v) as a solvent system (flow rate 1 mL/min). The chemical structures of the products were concluded from GC/MS data obtained for the derivatized hydrogenated derivatives.

slide deeper into the substrate-binding pocket and adopts a configuration at the active site that enables major 12-lipoxygenation. These data are consistent with our hypothesis that the 16(*S*)-OH group prevents deeper penetration of the substrate molecule via the formation of a hydrogen bridge with a neighboring amino acid.

To further confirm the modeling-derived hypothesis that Glu548 might constitute the putative binding partner for the 16(*S*)-OH group of the substrate (Figure 4), we mutated this amino acid to a Leu, which is not capable of forming a hydrogen bridge. Unfortunately, Gln548Leu exchange resulted in an enzymatically inactive enzyme species, and thus, no major conclusions could be drawn from this experiment. It should, however, been stressed that this result was not surprising. Glu548 constitutes a second-order iron ligand and has been implicated in stabilizing the overall structure of the iron ligand sphere (*24*). Gln548Leu exchange might alter the coordination geometry of the iron cluster leading to an inactive enzyme species.

DISCUSSION

The stereochemistry of LOX-catalyzed oxygenation of polyenoic fatty acids is tightly controlled on two levels: (i) Experiments with stereospecifically labeled polyenoic fatty acids indicated that most LOX isoforms abstract selectively one of the two hydrogen atoms attached to a prochiral bisallylic methylene (*31*, *32*). (ii) Insertion of molecular dioxygen into the fatty acid substrate also proceeds stereoselectively since either an (*S*)-hydroperoxide (S-LOX) or the corresponding *R* mirror image (R-LOX) is formed (*7*). In this study we investigated a third element in stereochemical control of the LOX reaction, enantioselective substrate specificity. Polyenoic fatty acids (PUFAs) constitute the most effective substrates for LOXs, and most naturally occurring PUFAs are nonchiral compounds. Thus, there was no need to ask the question of whether LOXs may exhibit enantioselective substrate specificity. More recent results, however, indicated that complex polyenoic fatty acid derivatives may also serve as LOX substrates, and some of them contain asymmetric carbon atoms (*20*−*22*). These data prompted us to explore whether mammalian arachidonic acid 15-LOXs exhibit an enantioselective substrate specificity and whether this property may be related to the positional specificity of the enzyme. For this purpose we prepared a set of hydroxy arachidonic acid isomers, which carry the hydroxy group at C-16 [16(*R*/*S*)-HETE], C-17 [17(*R*/*S*)-HETE] or C-18 [18-(*R*/*S*)-HETE], and tested the substrate behavior of the enantiomers for the 15-LOXs from rabbit reticulocytes and soybeans. We found that both enzymes exhibit enantioselective substrate specificity, but its degree depended on the fatty acid structure. 16(*S*)-HETE was strongly preferred as substrate over the corresponding *R*-enantiomer (Figure 2A). With 17-HETE the degree of enantioselectivity was lower (Table 1) but still existent. In contrast, with 18-HETE we did not observe preferential oxygenation of either enantiomer (Figure 1A). These data indicate that the degree of enantioselective substrate specificity of hydroxy fatty acid oxygenation depends on the distance of the chiral center (C-16, C-17, C-18) from the site of hydrogen abstraction (C-13). If this distance is short (16-HETE), a high degree of enantioselectivity was observed. With increasing distances enantioselectivity is gradually lost. In summary, one may conclude that with certain substrates LOXs constitute enantioselective enzymes capable of removing a defined substrate enantiomer from a racemic mixture but leave the mirror image untouched.

Terminal (20-HETE) and subterminal (19-HETE, 18-HETE, 17-HETE, 16-HETE) hydroxylated fatty acids are produced in various mammalian cells and tissues, and interesting biological activities have been reported. When rabbit kidneys are perfused in vitro in the presence or absence of various hormones (angiotensin II, bradykinin, vasopressin), variable amounts of different HETE isomers have been detected, with 19- and 17-HETE being the major products (*33*). These metabolites have been implicated in regulation of renal perfusion and in tubular transport mechanisms. Polymorphonuclear leukocytes form large amounts of 16-(*R*)-HETE when incubated with arachidonic acid in the absence of calcium ionophore (*34*). This metabolite inhibits neutrophil aggregation and prevents adhesion of the cells to the vascular wall. In a rabbit model of thromboembolic stroke 16(*R*)-HETE suppresses the increase in intracranial pressure (*35*). Hence, stimulation of 16(*R*)-HETE synthesis may be considered a novel therapeutic strategy to treat and/or prevent stroke. 16-HETE has also been identified as a major arachidonic metabolite in the phospholipid bilayer of platelet membranes (*36*), but functionality of this minor membrane component remains elusive.

In mammalian cells terminal and subterminal HETE isomers are usually formed via the cytochrome P-450 pathway. In the murine intestinal tract a special isozyme

(CYP2C40) has been identified, which converts arachidonic acid in a regio- and stereospecific manner to 16(*R*)-HETE (*37*). Our finding that there is a stereoselective conversion of 16(*S*)-HETE from a racemic 16-HETE mixture suggests an alternative biosynthetic pathway for 16(*R*)-HETE formation, which may involve 15-LOX. If a racemic mixture of 16(*R,S*)-HETE is formed by fatty acid hydroxylases, stereoselective secondary conversion of 16(*S*)-HETE will lead to enrichment of 16(*R*)-HETE. Generalizing these experimental data, one may conclude that such a biosynthetic scheme will lead to enrichment of one enantiomer from a racemic product mixture. These data indicate that a stereospecific pattern of hydroxylated polyenoic fatty acids does not prove the LOX origin. Selective removal of a certain optical isomer from a racemic mixture by hydroxy fatty acid metabolizing enzymes may lead to a similar pattern of oxygenation products, but the biosynthetic mechanism is quiet different.

Mutagenesis of the space-filling Phe353 to a less bulky Leu converts the rabbit 15-LOX to an arachidonic acid 12-lipoxygenating enzyme species (*30*). A similar change in positional specificity was also observed with 16(*R*)-, 17(*R*)-, and 17(*S*)-HETE as substrate. Surprisingly, we found that the positional specificity of 16(*S*)-HETE lipoxygenation remained unchanged. With this substrate an arachidonic acid like oxygenation pattern was observed. This result was quite surprising since the 16(*S*)-OH group appeared to prevent further penetration of the substrate into the substrate-binding pocket as indicated in Scheme 1. As a mechanistic explanation for this unexpected observation, we hypothesized that the 16(*S*)-OH group may hydrogen-bridge to an amino acid residue in a region closely adjacent to the iron cluster. In fact, structural modeling of the 15-LOX/16(*S*)-HETE complex suggested such a hydrogen bridge between the 16(*S*)-OH group and Gln548 (Figure 4). In contrast, a 16(*R*)-OH group was not capable of hydrogen bridging to any surrounding amino acid, and thus, the substrate may not be hindered from penetrating deeper into the active site of the 12-lipoxygenating Phe353Leu mutant. Our results obtained with the fluorine-substituted 16-HETE derivatives [16(*S*)- and 16(*R*)-FETE] are consistent with this hypothesis. In the immediate surrounding of the iron cluster there are two candidate amino acids, the side chains of which are capable of hydrogen bridging (Glu357 and Gln548). However, structural modeling suggested that Glu357 is far distant from the 16(*S*)-OH group, and thus, formation of a hydrogen bridge appears impossible. To obtain experimental evidence for this modeling-based assumption, we carried out additional site-directed mutagenesis studies. When Glu357 was mutated to a Leu in the Phe353Leu mutant, we obtained a catalytically active double mutant, which oxygenated 16(*S*)-HETE predominantly at C-15. These data indicate that in this double mutant 16(*S*)-HETE is still prevented from sliding deeper into the substrate-binding pocket. Thus, Glu357 appears not to be a suitable binding partner for the 16(*S*)-OH group.

Next, a similar mutagenesis strategy was carried out for Gln548. Unfortunately, mutation of this residue in the wild-type rabbit 15-LOX to a Leu (Gln548Leu exchange) resulted in an enzymatically inactive enzyme species. These data were not surprising since Glu548 constitutes a second-order iron ligand and has been implicated in stabilizing the overall structure of the iron ligand sphere. Gln548Leu exchange might alter the coordination geometry of the iron cluster leading to an inactive enzyme species. An alternative explanation of our data was the possibility that the 16(*S*)-OH group may hydrogen-bridge directly to one of the first-order iron ligands. Although such a scenario was unlikely in light of our modeling studies, we mutated each of the first-order iron ligands (His 361, His366, His541, His545, Ile663) to test whether we can induce 12-lipoxygenation of 16(*S*)-HETE. Unfortunately, we obtained inactive enzyme protein for each of the single mutants, and thus, no major conclusions could be drawn. When we quantified the enzyme-bound iron for the purified preparations of these mutants, we found a strongly reduced iron content. A similar behavior has been reported before for the human 5-LOX (*38*). These data indicate that these mutants lack the essential metal ion, and these data explain their catalytic silence. In light of these findings we cannot completely rule out the possibility that the primary iron ligands may contribute to substrate alignment at the active site although our modeling studies appear to argue against such a scenario.

In summary, our experimental data indicate that plant and mammalian 15-LOX-1 exhibits enantioselective substrate specificity with hydroxy fatty acids, which carry the hydroxy group in close proximity to the site of hydrogen abstraction (16-HETE, 17-HETE). The (*S*)-hydroxy group in 16-HETE appears to hydrogen-bridge to the active site amino acid Gln548, and this enzyme−substrate interaction prevents the substrate from sliding deeper into the substrate-binding pocket of 12-lipoxygenating enzyme mutants (Phe353Leu and Ile593Ala) so that the hydroxy fatty acid is properly aligned for oxygen insertion at C-15. In contrast, because of steric constraints no hydrogen bridge is formed with 16-(*R*)-HETE as substrate, and thus, this hydroxy fatty acid is optimally oriented at the active site for 12-lipoxygenation.

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