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Probing the Substrate Alignment at the Active Site of 15-Lipoxygenases by Targeted Substrate Modification and Site-Directed Mutagenesis. Evidence for an Inverse Substrate Orientation†

Kristin Schwarz, Sabine Borngräber, Monika Anton, and Hartmut Kuhn*

Institute of Biochemistry, University Clinics (Charité), Humboldt University, Hessische Strasse 3-4, 10115 Berlin, Germany

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ABSTRACT: For oxygenation of polyenoic fatty acids by 12- and 15-lipoxygenases the methyl terminus of the substrate constitutes the signal for the initial hydrogen abstraction. In contrast, for 5-lipoxygenases an inverse head to tail substrate orientation has been proposed. However, recent structure-based sequence alignments suggested a conserved uniform substrate orientation for 5S- and 15S-lipoxygenation. Oxygenation of 15S-HETE derivatives by various wild-type and mutant lipoxygenases was investigated, and the evidence proved an inverse substrate orientation: (i) Substrate affinity and V_{max} of 15S-HETE oxygenation by arachidonic acid 15-lipoxygenases are >1 order of magnitude lower than the corresponding data for polyenoic fatty acids. 5S,15S- and 14R,15S-DiH(P)ETE were identified as major reaction products. (ii) Methylation of the carboxylate group of 15S-HETE augmented the reaction rate and shifted the reaction specificity strongly toward 5S-lipoxygenation. In contrast, methyl arachidonate was less effectively oxygenated than the free acid. Methylation of 15S-HETrE(8,11,14), which lacks the C5−C6 double bond, was without major impact on the oxygenation rate and on the product specificity. (iii) Introduction of a bulky glycerol moiety at the carboxylic group of 15S-HETE reversed the kinetic effects of methylation and led to a 14R-oxygenation of the substrate. (iv) When the product pattern of 15S-HETE oxygenation by the recombinant wild-type rabbit 15-lipoxygenase was compared with that formed by the Arg403Leu mutant, 5S- and 8S-lipoxygenations were augmented and 14R,15S-DiH(P)ETE formation was impaired. (v) Phe353Leu or Ile418Ala mutation of the same enzyme, which favored 12S-HETE formation from arachidonic acid, strongly augmented 8S-lipoxygenation of 15S-HETE methyl ester. These kinetic data and the alterations in the product specificity are consistent with the concept of an inverse head to tail substrate orientation during the oxygenation of 15S-HETE methyl ester and/or of free 15S-HETE by 15-LOXs. For 5S- and 8S-lipoxygenation, 15-HETE may slide into the substrate binding pocket with its carboxy terminus approaching the doubly allylic methylenes C-7 or C-10 to the non-heme iron.

Mammalian lipoxygenases (LOXs)[1] are classified with respect to their positional specificity of arachidonic acid oxygenation (*1*) into 5-, 8-, 12-, and 15-LOXs, but these isoforms may be further subdivided. For instance, arachidonate 12-LOXs can be classified into platelet-type and leukocyte-type enzymes (*2*), and in mouse skin an additional isoform was found (*3*). Similarly, arachidonate 15-LOX may be differentiated into reticulocyte-type enzymes (*4*) and epidermal-type 15-LOXs (*5*). Although the positional specificity of arachidonic acid oxygenation is the decisive parameter for LOX nomenclature, the mechanistic reasons for this enzyme property are not well understood. Earlier studies with a set of arachidonic acid isomers suggested that the positional specificity of LOX is not an invariant enzyme characteristic but depends on the alignment of the substrate at the active site (*6*). More recently, two independent primary determinants for the positional specificity of mammalian 12/15-LOX were identified (*7*−*9*). In addition, the crystal structures of three different LOX isoenzymes (*10*−*13*) and molecular modeling (*14*) led to a more comprehensive understanding of the enzyme−substrate interaction. For the human reticulocyte-type 15-LOX a three-point interaction between the enzyme and substrate polyenoic fatty acids has been suggested (*15*): (i) the physical contact of amino acids 418 and 419 with the methyl terminus of the substrate molecule; (ii) formation of a salt bridge between Arg403 and the negatively charged COO^- group of the fatty acid; (iii) a $\pi-\pi$ electron interaction of Phe414 with a double bond of arachidonic acid.

The substrate alignment for arachidonic acid 5-lipoxygenation has recently become a matter of discussion (*16*−

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* Author to whom correspondence should be addressed (telephone +49-30-2093 7539; fax +49-30-2093 7300).

[1] Abbreviations: RP-HPLC, reverse phase high-performance liquid chromatography; LOX(s), lipoxygenase(s); HPODE, hydroperoxyoctadecadienoic acid; 15S-HETE, 15(*S*)-hydroxy-5(*Z*),8(*Z*),11(*Z*),13(*E*)-eicosatetraenoic acid; 8S-HETE, 8(*S*)-hydroxy-5(*Z*),9(*E*),11(*Z*),14(*Z*)-eicosatetraenoic acid; 12S-HETE, 12(*S*)-hydroxy-5(*Z*),8(*Z*),10(*E*),14(*Z*)-eicosatetraenoic acid; 14R,15S-Di(P)ETE, 14(*R*),15(*S*)-dihydro(pero)xy-5(*Z*),8(*Z*),10(*E*),12(*E*)-eicosatetraenoic acid; 8S,15S-DiH(P)ETE, 8(*S*),15(*S*)-dihydro(pero)xy-5(*Z*),9(*EZ*),11(*Z*),13(*E*)-eicosatetraenoic acid; 5S,15S-DiH(P)ETE, 5(*S*),15(*S*)-dihydro(pero)xy-6(*E*),8(*Z*),11(*Z*),13(*E*)-eicosatetraenoic acid; LX B_4, lipoxin B_4 [5(*S*),14(*R*),15(*S*)-trihydro(pero)xy-6(*E*),8(*Z*),10(*E*),12(*E*)-eicosatetraenoic acid]; pfu, plaque-forming units.

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18). At present time there are two hypotheses that rationalize the positional specificity of 5-lipoxygenation: (i) the single orientation theory (*13*, *18*) and (ii) the inverse orientation hypothesis (*17*, *19*−*21*). According to the former theory all LOXs align the substrate fatty acids in such a way that the methyl end of the substrate molecule penetrates into the substrate binding pocket and the positional specificity depends solely on the volume of the substrate binding cavity. The inverse orientation hypothesis suggests that fatty acids may slide into the substrate binding cleft with their carboxylate group and, thus, the substrate may be inversely aligned at the active site. The possibility of such an inverse orientation was first concluded from mechanistic studies on the stereoselective hydrogen abstraction by the LOXs from corn and soybeans (*20*) as well as from the product pattern of 15S-HPETE oxygenation by the soybean LOX-1 (*22*). Later on, an inverse substrate orientation was suggested as a mechanistic reason for the oxygenation of linoleaidic acid by the soybean LOX-1 (*23*) and for the 9S-oxygenation of linoleic acid at neutral pH by the same enzyme (*24*). However, a strong argument for a conserved substrate orientation constitutes the large engergetic penalty (12−30 kJ/mol) associated with burying the charged carboxylate group in the hydrophobic environment of the substrate binding pocket (*18*).

It has been reported before that the arachidonic acid 15-LOXs from soybeans (isoenzyme 1) and rabbit reticulocytes are capable of catalyzing 5S-lipoxygenation of special substrates (*22*, *25*). The soybean enzyme converts 15S-HPETE to a mixture of 8S,15S- and 5S,15S-DiHPETE, and the stereochemical characteristics of this reaction are different from those of arachidonic acid oxygenation (*22*). Assuming an arachidonic acid-like alignment of 15-HETE at the active site, 5S-lipoxygenation, which involves hydrogen abstraction from C7 and a [−2] radical rearrangement (*19*), is difficult to understand. However, if one assumes an inverse head to tail substrate orientation, the stereochemistry of 15S-H(P)-ETE oxygenation can be explained without major problems. To investigate if 5S-lipoxygenation of 15S-HETE involves an inverse substrate orientation, we studied the reaction of wild-type and mutant LOXs with hydroxy fatty acids that were modified at the carboxylate terminus. From the data obtained one may conclude an inverse substrate orientation as a mechanistic reason for 5S-lipoxygenation of 15S-H(P)-ETE and/or its methyl ester by arachidonate 15-LOXs.

MATERIALS AND METHODS

Chemicals. Commercial sources of the chemicals were as follows: linoleic acid, arachidonic acid, and bis(trimethylsilyl)trifluoroacetamide (BSTFA) from Serva (Heidelberg, Germany); soybean lipoxygenase I (grade IV), 2-arachidonyl monoglyceride and α-tocopherol from Sigma Chemicals (Deisenhofen, Germany); HPLC standards of 15S-HETE, 5S,15S-DiHETE, 8S,15S-DiHETE, and lipoxin B_4 from Cayman Chemicals (distributed by Alexis Deutschland GmbH, Grünberg, Germany); 14R,15S-DiHETE and 14S, 15S-DiHETE from Biomol (Hamburg, Germany). All solvents were of HPLC grade and purchased from Baker (Deventer, The Netherlands).

Preparations. The rabbit reticulocyte LOX was purified from the hemolysate of a reticulocyte-rich blood cell suspension by ammonium sulfate precipitation and consecutive hydrophobic interaction and anion exchange chromatography (*26*). Preparations of the recombinant human 5-lipoxygenase (electrophoretically pure) and of the recombinant porcine leukocyte 12-lipoxygenase (partially purified by ammonium sulfate precipitation) were kindly provided by Dr. D. Riendeau (Merck Frosst, Canada) and Prof. S. Yamamoto (Tokushima, Japan), respectively. 2-[15S-HETE]-monoglyceride was prepared by the reaction of the soybean LOX-1 with 2-arachidonyl monoglyceride in 0.1 M sodium borate buffer, pH 9, containing 10 mM sodium deoxycholate. The reaction was followed by recording UV spectra of the incubation mixture, and when the absorbance at 240 nm showed no further increase, a molar excess of sodium borohydride was added, the mixture was acidified, the lipids were twice extracted with ethyl acetate, and the products were analyzed by RP-HPLC. One major product (90%) absorbing at 242 nm was detected. The chemical structure of this compound as 2-[15S-HETE]-monoglyceride was confirmed by SP-HPLC and GC/MS of the hydrolysis products.

Site-Directed Mutagenesis and Overexpression of the Mutant Enzyme. For site-directed mutagenesis of the rabbit 15-LOX the bacterial expression plasmid pKK 233−2 was used. Arg403Leu and Phe353Leu substitutions were carried out by PCR-mediated overlap extension technique using mismatching synthetic oligonucleotides. The cDNA constructs were sequenced to verify their primary structures. When bacteria were transformed with the recombinant plasmid, they expressed the mutant 15-LOX, but the level of expression was not sufficient for large scale enzyme preparation. Thus, we decided to overexpress the critical Arg403Leu mutant in the baculovirus/insect cell system. For this purpose the mutant 15-LOX cDNA was excised from the pKK 233-2 plasmid and was subcloned into the baculovirus transfer vector pVL1393. The resulting recombinant transfer plasmid pVL-LOX-L403 was amplified in *Escherichia coli*. One microgram of wild-type *Autographa californica* nuclear polyhedrosis virus (AcNPV) DNA was mixed with 2 μg of the pVL-LOX-L403 transfer plasmid and cotransfected into *Trichoplusia ni* insect cells (HighFive, Invitrogen Corp., San Diego, CA) using the lipofectin procedure. Recombinant baculovirus clones were isolated and purified by two to three rounds of viral plaque purification, and viral stocks with titers of $\sim 3 \times 10^8$ pfu/mL were produced. HighFive cells were cultured in spinner flasks at 27 °C. Cells were grown in Grace's medium supplemented with 10% heat-inactivated fetal calf serum and penicillin/streptomycin (final concentration = 100 μg/mL). For enzyme expression, HighFive cells (cell density = 8×10^5 cells/mL, viability = 98%) were infected with the recombinant baculovirus using a multiplicity of infection of 5. After 4 days, the cells were harvested, washed, and resuspended in 5 mL of 10 mM Bis-Tris buffer, pH 6.8, containing 2 mM EDTA and the proteinase inhibitors phenylmethylsulfonyl fluoride (40 mg/mL), leupeptin (1 mg/mL), and pepstatin A (1 mg/mL). This suspension was sonicated using a tip sonifier (Labsonic U, 50 W, six 15 s intervals with 30 s interruptions). After centrifugation at 10000*g* for 10 min at 4 °C, the supernatant was filtered (0.4 mm syringe filter, Millipore), and the filtrate was directly injected to fast liquid protein chromatography (FPLC) on a semipreparative Mono

Q HR 10/10 column. Proteins were eluted with a step gradient of sodium chloride. Fractions of 2 mL were collected, and the 15-lipoxygenase activity was assayed spectrophotometrically. The resulting enzyme preparation was ~70% pure and exhibited a linoleic acid oxygenase turnover rate of 1.6 s^{-1}.

Assay Systems. The oxygenation of the hydroxy fatty acid derivatives was assayed either spectrophotometrically or oxygraphically with a homemade micro-Clark-type oxygen electrode. It has been reported before that 15-HETE is only effectively oxygenated when small amounts of hydroperoxy fatty acids acting as essential activators of the LOX were added to the assay system (*27*). During our studies we found that linoleic acid may also be used as activator. At the relatively high enzyme concentrations used for 15-HETE oxygenation, linoleic acid is rapidly converted to 13-HPODE, which in turn may act as enzyme activator. Because of this, we usually added small amounts of linoleic acid (5−10 μM) to our routine assay system.

The different LOX species were incubated with various substrates at room temperature. For the rabbit reticulocyte LOX and the porcine leukocyte 12-LOX a 0.1 M phosphate buffer, pH 7.4, was used; for the soybean LOX-1 a 0.1 M borate buffer, pH 9, was used. For the human 5-LOX the assay mixture was a HANKS buffer, pH 7.4, containing 12.5 mg/mL dipalmitoylphosphatidylcholine and 0.1 mM ATP. After 2 min of incubation, the hydroperoxy lipids formed by the LOXs were reduced with sodium borohydride, the mixtures were acidified to pH 4, and an equal volume of methanol was added. The protein precipitate was removed by centrifugation, and aliquots were injected to RP-HPLC analysis. For preparative purposes the lipophilic products were twice extracted with diethyl ether. The solvent was evaporated, and the remaining lipids were reconstituted in methanol and analyzed by HPLC and/or GC/MS. Methylation and propylation of 15S-HETE were carried out with diazomethane and diazopropane, respectively. For GC/MS the hydroxy fatty acids were converted to their corresponding trimethylsilyl ethers by the reaction with BSTFA in dry pyridine.

Analytics. If not stated otherwise, HPLC analysis and preparations were carried out on a Shimadzu LC-6A liquid chromatograph connected to a Hewlett-Packard 1040A diode array detector. 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). Solvent systems consisting of methanol/water/acetic acid (different compositions) and a flow rate of 1 mL/min were used. Products characterized by conjugated diene, conjugated triene, or conjugated tetraene chromophores were fractionated, the solvent was evaporated, and the residues were reconstituted in *n*-hexane and used for SP-HPLC or GC/MS analysis. 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 (100:10:0.1, v/v) and a flow rate of 1 mL/min. For quantification the following molar extinction coefficients were used: ϵ_{235} = 23 000 $(M\ cm)^{-1}$ for the conjugated dienes, ϵ_{270} = 40 000 $(M\ cm)^{-1}$ for the conjugated trienes, ϵ_{242} = 33 000 $(M\ cm)^{-1}$ for the double-conjugated dienes, and ϵ_{300} = 53 000 $(M\ cm)^{-1}$ for the conjugated tetraenes.

GC/MS was carried out on a Varian Vista 6000 gas chromatograph coupled to a Nermag 10-10 C mass spectrometer or 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). 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: from 130 °C with a rate of 50 °C/min to 200 °C, then with a rate of 5 °C/min to 260 °C followed by an isothermic postrun of 10 min.

Table 1. Oxygenation of Various Substrates by Lipoxygenases of Different Positional Specificity[a]

enzyme	relative reaction rate (%)			
	arachidonic acid	methyl arachidonate	15S-HETE	methyl 15S-HETE
5-LOX (human)	100 (2.0)	<2	100	12
12-LOX (leukocyte)	100 (2.1)	13	139	132
15-LOX (rabbit)	100 (3.2)	6	6	65
15-LOX (soybean)	100 (3.0)	<2	3	30

[a] The lipoxygenases were incubated with various substrates (200 μM arachidonic acid and methyl arachidonate, 30 μM 15S-HETE and its methyl ester) for 2 min at room temperature as described under Materials and Methods. As activator of the enzyme, 5 mM 13(*S*)-hydroperoxylinoleic acid was added. The reaction was stopped by addition of sodium borohydride. After acidification, an equal volume of methanol was added, protein precipitate was removed by centrifugation, and aliquots were injected directly onto the RP-HPLC column. The oxygenation rate of arachidonic acid for each enzyme was set at 100%. In parentheses the formation of HETEs (μg/mL·min) is given. For arachidonate derivatives, the formation of 5-, 12-, and 15-H(P)ETE was quantified. The reaction of 15S-HETE was quantified by measuring the disappearance of the substrate (decrease in absorbance at 235 nm).

RESULTS

Oxygenation of Arachidonate Derivatives by Various Lipoxygenases. LOXs of various positional specificities are capable of oxygenating 15S-HETE and its methyl ester in the presence of small amounts of hydroperoxy fatty acids acting as activator of the enzyme (Table 1). All LOXs tested oxygenate free arachidonic acid more efficiently than the corresponding methyl ester. However, when 15S-HETE and its methyl ester were used as substrate, the LOXs tested behaved differently. The recombinant human 5-LOX oxygenated 15S-HETE at a rate comparable to arachidonic acid, and 5S,15S-DiH(P)ETE was identified as the major oxygenation product (not shown). These data were not surprising because both arachidonic acid and 15S-HETE contain a doubly allylic methylene at C-7 that may be recognized by the enzyme for initial hydrogen removal. As methyl arachidonate, 15S-HETE methyl ester was a less effective substrate than the corresponding free acid.

The porcine leukocyte 12-LOX oxygenated 15S-HETE at a somewhat higher rate than arachidonic acid, and 14R, 15S-DiH(P)ETE was identified as the major oxygenation product. Previous studies suggested that 15S-HETE was a poor substrate for this enzyme, whereas 15S-HPETE was rapidly oxygenated (*2*). In fact, we did not observe an oxygenation of 15S-HETE when hydroperoxy linoleic acid (13-HPODE) acting as activator of the enzyme was absent.

Table 2. Kinetic Constants of the Lipoxygenase Reaction with Different Substrates

	enzyme			
	rabbit 15-LOX		soybean 15-LOX	
substrate	K_M (μM)	V_{max} (s^{-1})	K_M (μM)	V_{max} (s^{-1})
arachidonic acid	5.0	8.3	8.5	225
linoleic acid	12.8	12.6	20.0	320
15-H(P)ETE	94.9[a]	0.4[a]	440[b]	25[b]

[a] 15S-HETE as substrate. [b] 15S-HPETE as substrate. The kinetic constants for the soybean enzyme were taken from refs *29* and *22*.

Table 3. Relative Oxygenation Rates of 15S-HETE Derivatives[a]

substrate	relative oxygenation rate (%)
15S-HETE	100
15S-HETE methyl ester	1250
15S-HETE propyl ester	350

[a] The reticulocyte 15-lipoxygenase was incubated with different substrates (130 μM) at 4 °C in 0.1 M phosphate buffer, pH 7.4, in the presence of 20 μM hydroperoxylinoleic acid. Oxygen uptake was measured with a micro-Clark-type oxygen electrode. The oxygenation rate of 15S-HETE (5.2 nmol/mL·min) was set at 100%.

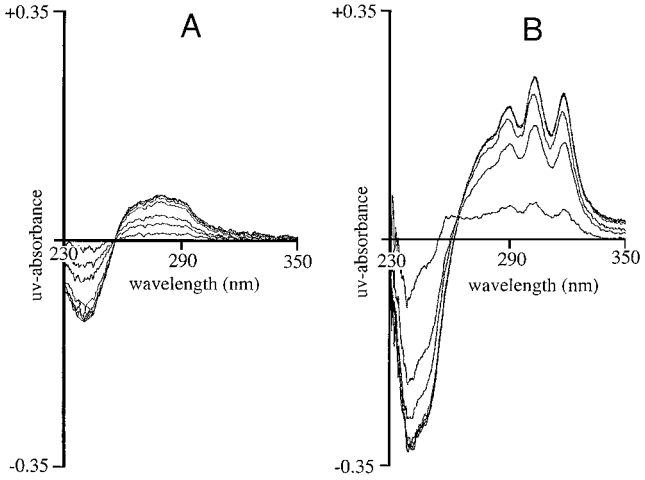


FIGURE 1: Spectral changes of the reaction mixture during the oxygenation of 15S-HETE (A) and 15S-HETE methyl ester (B). The rabbit reticulocyte 15-lipoxygenase was incubated with 30 μM substrate in the presence of 5 μM linoleic acid in 0.1 M phosphate buffer, pH 7.4, at 15 °C for 16 min. The UV spectra of the incubation mixtures were recorded every 2 min, and the spectrum at the very beginning of the reaction was set as background.

This phenomenon was observed not only for the porcine leukocyte 12-LOX but also for the 15-LOXs of rabbit reticulocytes and for the soybean enzyme. In contrast to the 5-LOX, which prefers free 15S-HETE over its methyl ester, the porcine leukocyte 12-LOX oxygenated 15-HETE methyl ester at a similar rate as its free acid.

For the 15-LOXs from rabbit reticulocytes and soybeans, 15S-HETE was a very poor substrate. When compared with arachidonic acid, the oxygenation rate of 15S-H(P)ETE (V_{max}) and the substrate affinity were >1 order of magnitude lower (Table 2). Because both enzymes require an $n-8$ doubly allylic methylene for hydrogen abstraction, which is lacking in 15S-HETE, the low reaction rate becomes plausible. The low substrate affinity may be due to the thermodynamic energy barrier associated with burrying either the charged carboxylic group (soybean LOX) or the polar C-15 hydroxy group (rabbit enzyme) in the hydrophobic environment of the substrate binding cleft. Most interestingly, the methyl ester of 15S-HETE turned out to be a much better substrate than the free acid (Table 1). This result was quite unexpected, because free polyenoic fatty acids are preferred over their methyl esters by both enzymes. To investigate the mechanistic reasons for this unusual kinetic behavior, we studied the oxygenation of 15S-HETE by 15-LOXs in more detail.

Mechanistic Studies on 15S-HETE Oxygenation by Wild-Type 15-LOXs. Oxygraphic measurements of 15-HETE oxygenation by the rabbit reticulocyte 15-LOX (Table 3) confirmed the HPLC finding that 15S-HETE methyl ester is a much better substrate than the free acid (Table 1). Analysis of the products formed from 15S-HETE and its methyl ester, revealed remarkable differences. The spectral changes of the incubation mixture (Figure 1A) suggested the formation of conjugated trienes during 15S-HETE oxygenation, and RP-HPLC (Figure 2A) indicated 14R,15S-DiH-(P)ETE as the major oxygenation product. In addition, variable amounts of 5S,15S-DiH(P)ETE and 8S,15S-DiH-(P)ETE isomers were also detected (Table 4). It should be mentioned that the 14R,15S-DiH(P)ETE/5S,15S-DiH(P)ETE ratio was rather variable when different enzyme preparations were used. With some preparations of the native enzyme an almost exclusive 14R/15S-DiH(P)ETE formation was observed, whereas with other batches this ratio was 2:1. Moreover, we observed a difference in the 14R,15S-DiH-(P)ETE/5S,15S-DiH(P)ETE ratio when the native 15-LOX (Table 4) was compared with the recombinant wild-type enzyme (Table 6). However, in all cases 14R,15S-DiH(P)-ETE was the major oxygenation product. With 15S-HETE methyl ester as substrate, conjugated tetraenes were formed as indicated by the spectral changes of the incubation mixture (Figure 1B), and RP-HPLC (Figure 2B) identified lipoxin B_4 as the major conjugated tetraene. However, this compound did only contribute ~20% to the mixture of oxygenation products (Table 4). As the major product of 15-HETE methyl ester oxygenation, 5S,15S-DiH(P)ETE was identified (70%). This compound could not be detected in the spectrophotometric assay because its UV spectral properties are similar to those of the substrate. Most interestingly, we detected only very small amounts 14R,15S-DiH(P)ETE, which constituted the major product of free 15S-HETE oxygenation. Summarizing these results, one may conclude that methylation of 15S-HETE was of dual consequence for its oxygenation by 15-LOXs: (i) it augmented the oxygenation rate (Table 3) and (ii) altered the positional specificity, favoring the formation of products that involve C-7 hydrogen abstraction (Figure 2). These alterations suggest that 15S-HETE and its methyl ester may be aligned inversely at the active site of 15-LOX. The formation of 14R,15S-DiH(P)-ETE involves a hydrogen removal from C10 and a [+4] radical rearrangement (oxygen insertion takes place four carbon atoms away from the hydrogen removal in the direction of the methyl end of the fatty acid substrate), suggesting an arachidonic acid-like alignment. In contrast, 5S,15S-DiH(P)ETE formation involves C-7 hydrogen removal and [−2] radical rearrangement (oxygen insertion takes place two carbon atoms away from the hydrogen removal in the direction of the carboxylic end of the fatty

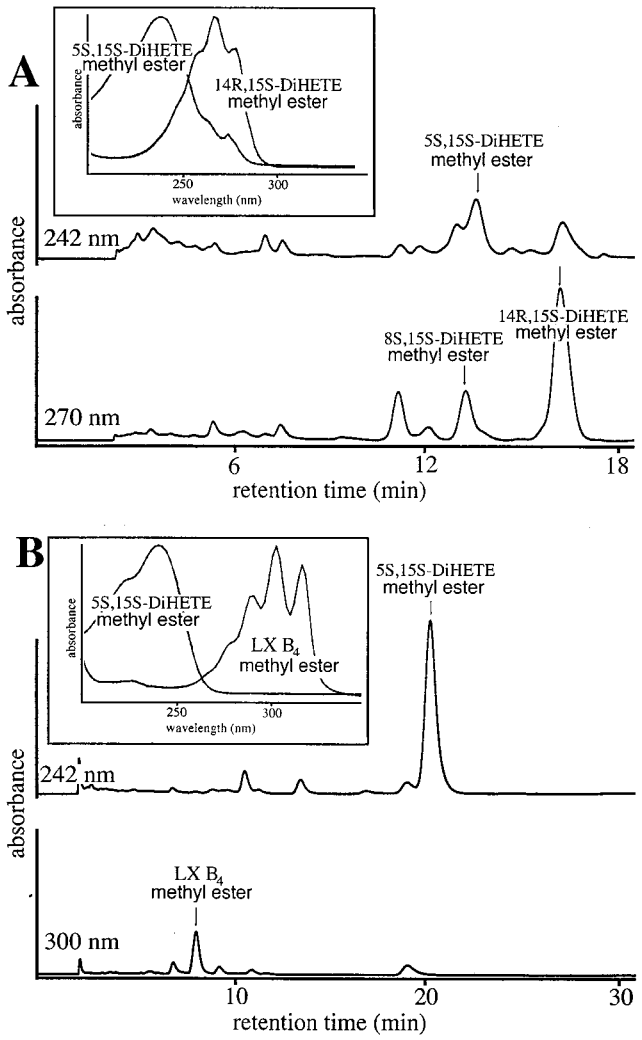


FIGURE 2: Reaction products formed from 15S-HETE derivatives by the reticulocyte 15-LOX. The incubation was carried out as described in the legend to Figure 1. The reaction was stopped by addition of sodium borohydride. After acidification to pH 3, the products were twice extracted with ethyl acetate, methylated with diazomethane, and analyzed by RP-HPLC on a Zorbax ODS colunm (250 × 4.6 mm, 5 mm particle size). (A) Reaction products of 15S-HETE oxygenation: Solvent system of methanol/water/acetic acid (75:25:0.1), flow = 1 mL/min. The retention times of some authentic standards are indicated by the arrows above the traces. (Insets) UV spectra of the major products. (B) Reaction products of 15S-HETE methyl ester oxygenation: Solvent system of methanol/water/acetic acid (70:20:0.1), flow = 1 mL/min. The retention times of authentic standards are indicated by the arrows above the traces. The chromatograms at both wavelengths are scaled for the major products. (Insets) UV spectra of the major products.

acid substrate). These differences may be explained by an inverse head to tail substrate orientation.

An increase in the oxygenation rate upon methylation has not been observed for other HETE isomers. 8(R/S)-HETE constitutes a good substrate for the 15-LOXs from rabbit reticulocytes and soybeans as indicated by spectrophotometric measurements (Table 5). However, its methyl ester was oxygenated at a lower rate. A similar behavior was observed with 5(R/S)-HETE and 9(R/S)-HETE (data not shown). In contrast, 12(R/S)-HETE was a very poor substrate for both enzymes. Only small amounts of 5,12-DiH(P)ETE could be detected when a 10-fold higher enzyme concentration was used. In contrast, 12(R/S)-HETE methyl ester was more effectively oxygenated. As 15S-HETE methyl ester, 15(R/S)-HETE methyl ester was also a better substrate than its corresponding free acid (not shown). These data suggest that methylation did only increase the rate of 5-lipoxygenation. However, when oxygen is introduced at C-15 during catalysis, methylation appears to be inhibitory.

Because our kinetic measurements and the alterations in the product pattern suggested that methylation of the carboxylate group appears to be important for 15-LOX-catalyzed oxygenation of 15S-HETE, we investigated the impact of more drastic modification of this part of the molecule. Oxygraphic measurements (Table 3) indicated that the propyl ester was a less effective substrate for the reticulocyte 15-lipoxygenase than the methyl ester. When an even more bulky glycerol residue was introduced at the carboxy terminus, the oxygenation rate dropped dramatically. In fact, we were unable to detect a significant oxygen uptake in the oxygraphic assay. However, HPLC analysis revealed a time-dependent decrease in the substrate concentration indicating a 15-LOX-catalyzed conversion of the 15-HETE monoglyceride (not shown). For more detailed information on the reaction products, the lipids were extracted from the incubation mixture and the extracts were hydrolyzed and analyzed by RP-HPLC. Evaluation of the chromatograms (Figure 3) indicated 14R,15S-DiH(P)ETE as the major oxygenation product (54%). In addition, various 8,15S-DiH(P)ETE isomers (30%) and 5S,15S-DiH(P)ETE (16%) were also found. This product pattern and the low oxygenation rate are similar to the corresponding characteristics of free 15S-HETE oxygenation but completely different from the reaction with 15S-HETE methyl ester.

Impact of Site-Directed Mutagenesis on 15S-HETE Oxygenation by the Rabbit 15-LOX. Crystallographic X-ray analysis of a rabbit 15-LOX/inhibitor complex (*13*) and site-directed mutagenesis studies on the human enzyme (*15*) suggested that Arg403 may form a salt bridge with the negatively charged carboxylate group of arachidonic acid and thus may fix the substrate at the active site. Mutation of this amino acid to an uncharged residue was therefore assumed to favor an inverse substrate orientation. To test this hypothesis, Arg403 of rabbit 15-LOX was replaced with leucine. We found that the mutant enzyme purified from baculovirus-infected insect cells showed a lower rate of linoleic acid oxygenation and an impaired substrate affinity. For the recombinant wild-type enzyme a linoleic acid turnover number of 12.6 s^{-1} and a K_M of 12.8 μM were determined, and these data are in fair agreement with the corresponding values for the native enzyme (*28*). In contrast, the Arg403Leu mutant exhibited a linoleic acid turnover of 1.6 s^{-1} and a K_M of 250 μM. RP-HPLC of the arachidonic acid oxygenation products indicated the formation of 15-, 12-, and 5-HPETE in a ratio of about 66:28:5. For the wild-type enzyme a 15-/12-HETE ratio of 92:8 was determined but no 5-HETE was found. 15- and 12-HETE formed by the wild-type enzyme and by the Arg403Leu mutant were predominantly in the *S*-configuration, whereas 5-HETE was a racemic mixture. These data suggested that 5-lipoxygenation by the mutant enzyme may not be completely controlled by the enzyme.

The recombinant wild-type 15-LOX and its Arg403Leu mutant oxygenated 15S-HETE at similar rates. When identical linoleic acid oxygenase activities were adjusted, the wild-type enzyme consumed 6.2 nmol of 15S-HETE/mL

Table 4. Product Composition of 15S-HETE Oxygenation by the Native 15-LOXs[a]

	lipoxygenases from			
	soybean		rabbit reticulocytes	
product	free 15S-HETE (%)	methyl 15S-HETE (%)	free 15S-HETE (%)	methyl 15S-HETE (%)
14,15-DiH(P)ETE	<1	<1	49	<1
8,15-DiH(P)ETE	34	5	28	10
5,15-DiH(P)ETE	65	90	21	69
lipoxin B	<1	5	2	21
ratio of hydrogen removal from C-10/C-7[b]				
	35:65	5:95	77:23	10:90

[a] The LOXs were incubated with the substrates as described in the legend to Figure 1. Product preparation and RP-HPLC were carried out as reported under Materials and Methods. The structures of the compounds have been identified by HPLC and GC/MS of the trimethylsilyl ethers. [b] Kinetic studies indicated that the formation of lipoxin B by the reticulocyte lipoxygenase requires the intermediate formation of 5,15-DiH(P)ETE (*30*). Therefore, the hydrogen removal from C7 is the first step in its formation.

Table 5. Oxygenation of Various Hydroxy Fatty Acids by 15-LOXs[a]

		rate (nmnol/mL min)		
lipoxygenase	substrate	methyl ester	free acid	major product
reticulocyte	8-HETE	2.3	3.0	8,15-DiH(P)ETE
	12-HETE	0.2	0	5,12-DiH(P)ETE
soybeans	8-HETE	1.3	3.6	8,15-DiH(P)ETE
	12-HETE	0.9	0	5,12-DiH(P)ETE

[a] Lipoxygenases (comparable arachidonate oxygenase activities) were incubated with racemic HETE isomers (28 μM) in the presence of 8 μM 13-hydroperoxylinoleic acid for 5 min at room temperature. Reaction rates were determined spectrophotometrically by measuring either the increase in absorbance at 270 nm or the decrease in absorbance at 235 nm. Reaction products were identified by HPLC and GC/MS.

Table 6. Quantification of the Product Pattern of 15S-HETE Oxygenation by Recombinant 15-LOX Species[a]

	DiH(P)ETE isomers formed (%)		
enzyme species	5S,15S-DiHETE	8S,15S-DiHETE	14R,15S-DiHETE
wild-type	44.2[b]	4.4	51.4[b]
Arg403Leu mutant	32.3	25.8	41.9

[a] Arg403Leu mutation of the rabbit 15-LOX was carried out as described under Materials and Methods. The wild-type and mutant enzyme species were overexpressed in the baculovirus insect cell system and were purified to apparent homogeneity; 2 nkat (linoleic acid oxygenase activity) of wild-type and mutant 15-LOX were incubated with 30 μM 15S-HETE in the presence of 5 μM linoleic acid for 15 min. After reduction of the hydroperoxy lipids with sodium borohydride, the sample was acidified, the lipophilic products were extracted with ethyl acetate, and the oxygenation products were analyzed by RP-HPLC as described in the legend to Figure 2. [b] The 14R,15S-DiHETE/5S/15S-DiHETE ratio obtained in this experiment with the recombinant 15-LOX (1.2:1) was somewhat lower than the corresponding ratios obtained with the native enzyme (ranging from 2:1 to 10:1), but the reason for this behavior has not been elucidated.

during a 15 min incubation period, whereas the Arg403Leu mutant converted 4.6 nmol/mL. HPLC analysis indicated that 14R,15S-DiH(P)ETE, 5S,15S-DiH(P)ETE, and 8S,15S-DiH(P)ETE were the major reaction products. As expected from the analysis of the product pattern formed by the native 15-LOX, no lipoxin isomers were detected. However, the composition of the product patterns was different when the wild-type enzyme was compared with the Arg403 mutant (Table 6). If one calculates the 5S,15S-DiH(P)ETE + 8S,15S-DiH(P)ETE/14R,15S-DiH(P)ETE quotient, which may

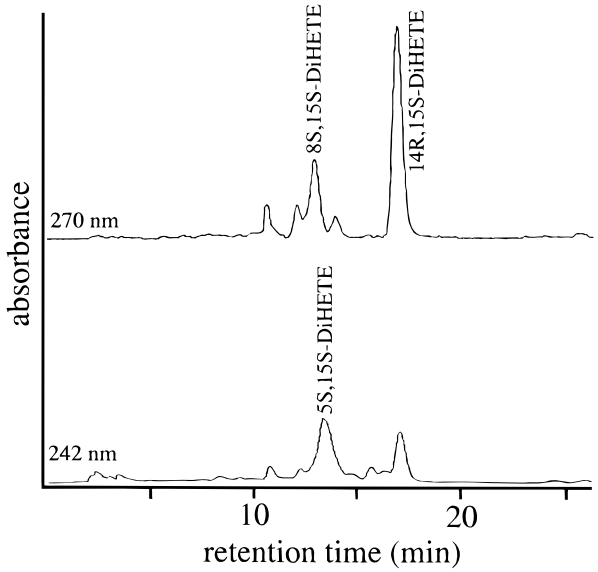


FIGURE 3: Oxygenation products formed from 2-[15S-HETE]-monoglyceride by the reticulocyte 15-LOX. 2-[15S-HETE]-monoglyceride (28 μM) was incubated with the rabbit reticulocyte LOX in the presence of 8 μM 13-hydroperoxylinoleic acid. After 15 min, the reaction was stopped by the addition of sodium borohydride and the products were extracted with ethyl acetate. After evaporation of the solvent, the products were subjected to alkaline hydrolysis in a mixture of methanol/5 N KOH (4:1, by vol) for 30 min at 60 °C under argon. The hydrolysis mixture was acidified with acetic acid, and aliquots were directly injected to RP-HPLC on an Altex ODS column (5 × 250 mm, 5 μm particle size). The solvent system was methanol/water acetic acid (70:30:0.1), flow = 1 mL/min. The arrows indicate the retention times of the authentic standards. The UV spectra (not shown) taken during the run are supportive for the chemical structure of the compounds.

reflect the shares of inversely bound versus arachidonic acid-like bound substrate, ratios of 1.4:1 for the mutant enzyme and 0.9:1 for the recombinant wild-type LOX are obtained. These data suggested that the inversely bound substrate appears to be more efficiently oxygenated by the Arg403Leu mutant than by wild-type enzyme. When 15S-HETE methyl ester was used as substrate, we analyzed a very similar product pattern for the wild-type 15-LOX and for the Arg403Leu mutant with 5S,15S-DiHETE and LX B_4 being the major reaction products.[2]

[2] These experiments were carried out with recombinant enzyme species expressed in *E. coli*.

Table 7. Quantification of the Product Pattern Formed from 15-HETE Methyl Ester by 15-LOX Mutants[a]

	reaction products formed (%)				
	arachidonic acid oxygenase[b]		15S-HETE methyl ester oxygenase[c]		
enzyme species	12-H(P)ETE	15-H(P)ETE	8S,15S-DiH(P)ETE	5S,15S-DiH(P)ETE	14R,15S-DiHP)ETE
recombinant wild-type	4.9	95.1	17.0	83.0	0
Phe353Leu mutant	69.4	30.6	31.2	62.8	0
Ile418Ala mutant	92.8	7.2	89.8	9.3	0.9

[a] Twenty-five milliliter cultures were grown overnight, and the bacteria were spun down and resuspended in 4 mL of phosphate-buffered saline. The cells were disrupted by sonication in the presence of 2 mM EDTA, and the 20000*g* supernatant was used as source of the enzyme; 1 mL of the supernatant was used for the experiments. The supernatant was incubated at 37 °C for 15 min with 0.1 mM arachidonic acid. After reduction with sodium borohydride, the mixture was acidified to pH 3 and the lipids were extracted twice with ethyl acetate. The combined extracts were dried, the lipids were reconstituted in 0.2 mL of methanol, and aliquots were analyzed by RP-HPLC with the solvent system methanol/water/acetic acid (75:25:0.1; by vol) at a flow rate of 1 mL/min. For determination of the specificity of 15-HETE methyl ester oxygenation, 3 mL of the 20000*g* supernatant was incubated for 15 min at room temperature with the substrate (30 μM final concentration) in the presence of 5 μM linoleic acid. The data represent the means of duplicate measurements of two independent bacterial cultures.

Phe353Leu mutation of rabbit 15-LOX was shown to alter the product pattern of arachidonic acid oxygenation, favoring 12-lipoxygenation (Table 7), although considerable amounts of 15S-H(P)ETE were also formed (*9*). It was suggested that this mutation increases the volume of the substrate binding pocket approaching the doubly allylic methylene C-10 of the substrate molecule to the enzyme-bound non-heme iron. Similarly, Ile418Ala exchange in the rabbit 15-LOX led to a 12-lipoxygenating enzyme species, but in this case only trace amounts of 15-H(P)ETE were found. It may be hypothesized that the Ile418Ala exchange enlarges the volume of the substrate binding pocket more drastically than Phe353Leu mutation so that C-10 is localized much more closely to the non-heme iron. If 15-HETE methyl ester is inversely aligned at the active site, a bigger volume of the substrate binding pocket was supposed to lead to an augmented formation of 8S,15S-DiHETE, which was detected only in very small amounts with the native rabbit 15-LOX. Moreover, this effect should be more pronounced with the Ile418Ala mutation when compared with the Phe353Leu exchange. In contrast, assuming an arachidonic acid-like orientation, no increase in 8S,15S-DiH(P)ETE formation would be expected. As indicated in Table 7, very small amounts of 8S,15S-DiHETE were formed during 15S-HETE methyl ester oxygenation by the wild-type enzyme. In contrast, 8S,15S-DiHETE was a major product of 15S-HETE methyl ester oxygenation by the two mutant LOX species. Interestingly, the Ile418Ala mutant produced more 8S,15S-DiH(P)ETE than the Phe353Leu mutant, and the potential reasons for this behavior were explained above.

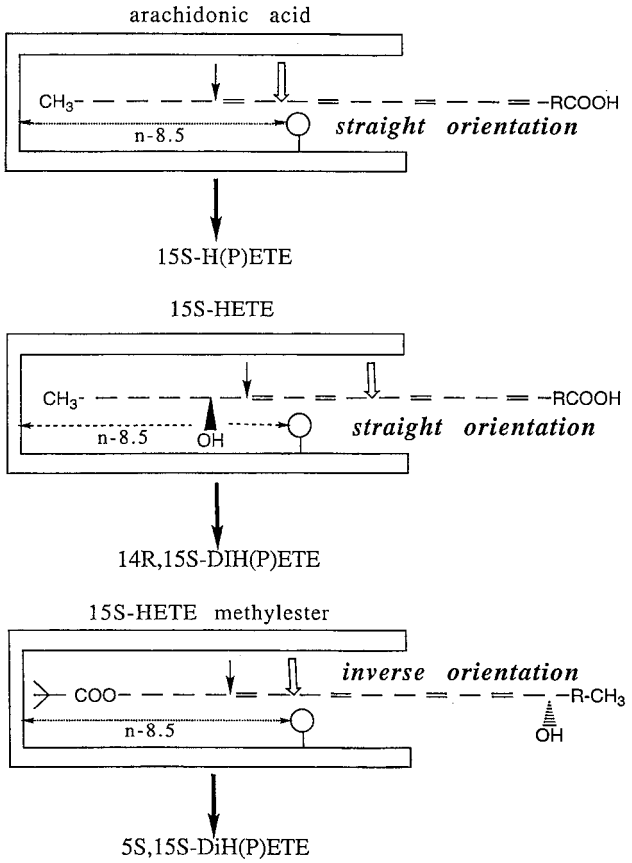
DISCUSSION

The positional specificity of LOXs, which is the basis of the currently used nomenclature for these enzymes, depends on the alignment of the substrate at the active site. Several factors such as substrate structure, substrate concentration, pH, and reaction temperature (*4*) may influence the specificity of the LOX reaction. Soybean LOX-1, which exhibits a singular positional specificity with arachidonic acid [exclusive 15-H(P)ETE formation], oxygenates 15-HPETE to 8S, 15S- and 5S,15S-DiH(P)ETE (*22*). Thus, it exhibits a dual positional specificity with 15S-HPETE as substrate, and the stereochemistry of this reaction is consistent with an inverse head to tail substrate orientation. We found that the 15-LOX of rabbit reticulocytes oxygenates 15S-HETE to a mixture of 14R,15S- and 5S,15S-DiH(P)ETE. Methylation of the carboxylate group strongly stimulated this reaction and augmented the relative share of 5S-lipoxygenation.

At the present time there are two hypotheses that rationalize the positional specificity of arachidonic acid 5-lipoxygenation (*17*, *18*): (i) The single-orientation theory (*18*) suggests that both 15S- and 5S-lipoxygenation involve a single conserved substrate orientation with the fatty acid methyl terminus penetrating into the substrate binding pocket. The charged carboxylate group may form a salt bridge to Arg403, fixing the carboxy terminus of the substrate. It should be stressed that it is somewhat problematic to explain the stereochemistry of 5S-lipoxygenation with the single-orientation theory. However, given the conformational flexibility of arachidonic acid derivatives, it appears possible to position the pro-S hydrogen at C-7 near the active site iron (*18*). According to this concept the volume of the substrate binding cavity may be decisive for the positional specificity of the LOX reaction. 15-LOXs have a small cavity and, thus, polyenoic fatty acids are oxygenated close to the methyl terminus. In contrast, more space may be available in the substrate binding cleft of 5-LOXs. In this case the fatty acid substrates may slide farther into the cavity, leading to an oxygenation close to the carboxylic terminus. In fact, sequence-based structural modeling suggested that the substrate binding cavity of mammalian 5-LOXs is ~20% larger than that of 15-LOXs (*13*). (ii) The inverse orientation theory (*17*) suggests that for 5-lipoxygenation the substrate is bound inversely at the active site, so that the carboxylate group may penetrate into the substrate binding pocket. According to the inverse orientation hypothesis, the stereochemistry of 5S-lipoxygenation is straightforward and there is no need to postulate conformational changes of the substrate.

If one applies the single orientation hypothesis to 15-LOX-catalyzed 5S-lipoxygenation of 15S-H(P)ETE or its methyl ester, additional assumptions must be made to explain the experimental data: (a) Binding of 15S-HPETE at the active site may induce conformational changes of the enzyme leading to an increased volume of the substrate binding cavity. Although such an induced fit cannot be ruled out, it appears unlikely that it may lead to a 20% increase in the volume of the substrate binding cavity. (b) 15S-H(P)ETE adopts a more "condensed" configuration at the active site than arachidonic acid. In this case the substrate would require less volume for binding and, thus, may slide farther into the binding pocket, favoring 5-lipoxygenation. To

Scheme 1. Orientation of Different Substrates at the Active Site of the Rabbit 15-LOX[a]



[a] The "horse-shoe-like" structure symbolizes the hydrophobic substrate binding pocket of the enzyme. The circles represent the hydrogen acceptor (non-heme iron). The estimated distance of the hydrogen acceptor from the bottom of the hydrophobic pocket is given in methyl groups. Solid arrows, oxygen insertion; open arrows, hydrogen abstraction.

address this point, we carried out computer simulations of 15S-H(P)ETE binding at the active site of the rabbit 15-LOX using the X-ray coordinates of rabbit 15-LOX/inhibitor complex. However, we failed to pinpoint a thermodynamically most probable configuration of the substrate because of the flexibility of the molecule. On the other hand, the experimental data on oxygenation of 15S-HETE and its methyl ester by 15-LOX can easily be explained with the inverse orientation hypothesis (Scheme 1). The large energy barrier associated with burrying the polar carboxylate group in the hydrophobic environment of the substrate binding pocket is reflected by the high K_M values of 15-LOX for 15-H(P)ETE (Table 2).

In this paper we present the following experimental evidence for an inverse orientation of 15S-HETE during 15-LOX-catalyzed oxygenation and, thus, for the principal possibility of an inverse head to tail orientation of the substrate at the active site of LOXs. (i) Methylation of 15S-HETE strongly stimulated its oxygenation rate (Table 1). This may be explained by the fact that methylation increases the hydrophobicity of the carboxy terminus, reducing the energy barrier associated with burying the former carboxylate group in the hydrophobic substrate binding pocket. In this context it is of particular interest that the oxygenation rates of the substrates which are not oxygenated at C-15 (arachidonic acid, 5-HETE, 8-HETE) were not augmented upon methylation (Table 5). (ii) 15-HETE methylation reduced 14R- but favored 5S-lipoxygenation by the rabbit 15-LOX (Figure 2), suggesting an increased share of the inversely aligned substrate. However, this share was found to be variable for different enzyme preparations. (iii) Introduction of the bulky and hydrophilic glycerol moiety at the carboxy terminus reversed the effects of methylation (Figure 3). This may be explained by the fact that the bulky glycerol moiety prevents an inverse substrate orientation. (iv) Phe353Leu and Ile418Ala mutation in rabbit 15-LOX led to a strong increase in the formation of 8S,15S-DiH(P)ETE (Table 7). Similarly, the Arg403Leu mutant of the rabbit enzyme, which catalyzed 12- and 15-lipoxygenation of arachidonic acid, oxygenated 15-HETE to 8S,15S-DiH(P)ETE. However, this product was formed only in small amounts by the wild-type enzyme. (v) Site-directed mutagenesis of Arg403, which was supposed to interact with the carboxylate group of the substrate, to an uncharged leucine favors an inverse substrate orientation as indicated by an increased 5S,15S-DiH(P)ETE + 8S,15S-DIH(P)ETE/14R,15S-DIH(P)ETE ratio during 15S-HETE oxygenation. If free 15S-HETE cannot be fixed any more at the active site via a salt bridge between its carboxylate group and Arg403, an increased share of inversely aligned substrate becomes plausible.

These findings suggest the ability of 15-LOXs to tolerate both the methyl end and the carboxy terminus of fatty acid substrates in the substrate binding pocket. With a defined substrate there may be a binding equilibrium between the arachidonic acid-like orientation (methyl terminus slides into the binding pocket) and the inverse alignment (carboxylate group penetrates into the pocket), and this equilibrium may be influenced by functional groups on either end of the fatty acid substrates. With polyenoic fatty acids the methyl end of the fatty acid slides into the binding pocket. Introduction of an OH group at C-15 or C-12 may shift the equilibrium toward an inverse orientation, although the formation of 14R,15S-DiH(P)ETE from 15S-HETE by the rabbit enzyme suggested that a large share of the substrate appears to be bound in an arachidonic acid-like way. Methylation of the carboxylate group of 15S-HETE shifted the equilibrium further toward an inverse orientation. In contrast, introduction of a bulky and polar glycerol residue may shift the equilibrium back, favoring an arachidonic acid-like orientation. It should be stressed that the lack of 14R,15S-DiH-(P)ETE by the soybean lipoxygenase does not exclude an arachidonic acid-like substrate orientation. It may be possible that only the inversely aligned substrate is oxygenated by the enzyme. If the hydrogen abstraction is sterically hindered, for instance by a large distance of the doubly allylic methylene from the non-heme iron, the share of 15S-HETE that is bound in an arachidonic acid-like way may not be oxygenated, although it was bound at the active site. Thus, the lack of 14R,15S-DiH(P)ETE formation by the soybean LOX does not exclude that a certain share of the substrate is bound in an arachidonic acid-like way. Similarly, the formation of 5S,15S-DiH(P)ETE from 15S-HETE methyl ester by the reticulocyte enzyme does not exclude an arachidonic acid-like substrate alignment because abortive substrate binding may interfere with product formation.

The data on the oxygenation of 15S-HETE and its methyl ester by plant and mammalian 15-LOX strongly suggest that substrate fatty acids may be oriented in either direction

(straight or inverse orientation) at their active site. Moreover, we found that the chemical structure of the substrate is important for its orientation. Thus, it may not be possible to predict the orientation (straight or inverse) of other LOX substrates from the data obtained with 15S-HETE and/or arachidonic acid. Moreover, from our data it cannot be concluded how polyenoic fatty acids may be aligned at the active site of other LOX isoforms. Other LOXs, in particular those exhibiting a low degree of amino acid identity such as archidonate 5- or 8-LOXs, may behave differently.

An inverse substrate orientation would strongly be favored if a positively charged amino acid would be present at the bottom of the active site which may interact with the carboxylate group. However, structure-based sequence alignments did not provide any evidence for a charged group at the active site of the human 5-LOX (*13*). If this is true, an inverse orientation would be hindered by a large energy barrier (*18*), which should be reflected by a strongly reduced substrate affinity. However, the oxygenation kinetics (K_M and V_{max}) of arachidonic acid by 5-LOXs are similar to those of the 15-LOX reaction and, thus, an inverse orientation may not be likely. Moreover, methylation of arachidonic acid should, in theory, attenuate the energy barrier and thereby increase the substrate affinity of the enzyme. However, methyl arachidonate is hardly oxygenated by 5-LOXs. If, on the other hand, the volume of the active site would be decisive for the positional specificity, it should be possible to convert a 15-LOX to a 5-lipoxygenating enzyme by mutating amino acids carrying space-filling side chains to smaller residues. Collaborative work is in progress in our laboratory to address this specific question.

For the time being experimental data on the substrate alignment are available only for arachidonate 12- or 15-LOXs (*6*−*9*). For other LOX isoforms no comparable experiments have been carried out. Preliminary mutagenesis studies on the human 5-LOX suggested that this enzyme shares several features with the human or rabbit 15-LOXs. However, more work is needed to pinpoint primary determinants for the positional specificity of this pharmacologically most relevant enzyme.

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