Alterations in Leukotriene Synthase Activity of the Human 5-Lipoxygenase by Site-Directed Mutagenesis Affecting Its Positional Specificity†

Kristin Schwarz, Christa Gerth, Monika Anton, and Hartmut Kuhn*

Institute of Biochemistry, University Clinics Charite, Humboldt University, Hessische Strasse 3-4, 10115 Berlin, Germany

Received June 23, 2000; Revised Manuscript Received September 7, 2000

ABSTRACT: The positional specificity of arachidonic acid oxygenation is currently the decisive parameter for classification of lipoxygenases. Although the mechanistic basis of lipoxygenase specificity is not completely understood, sequence determinants for the positional specificity have been identified for various isoenzymes. In this study we altered the positional specificity of the human 5-lipoxygenase by multiple site-directed mutagenesis and assayed the leukotriene A_4 synthase activity of the mutant enzyme species with (5*S*,6*E*,8*Z*,11*Z*,14*Z*)-5-hydroperoxy-6,8,11,14-eicosatetraenoic acid (5*S*-HpETE) as substrate. The wild-type 5-lipoxygenase converts 5*S*-HpETE almost exclusively to leukotriene A_4 as indicated by the dominant formation of leukotriene A_4 hydrolysis products. Since leukotriene synthesis involves a hydrogen abstraction from C_{10}, it was anticipated that the 15-lipoxygenating quadruple mutant F359W + A424I + N425M + A603I might not exhibit a major leukotriene A_4 synthase activity. Surprisingly, we found that this quadruple mutant exhibited a similar leukotriene synthase activity as the wild-type enzyme in addition to its double oxygenation activity. The leukotriene synthase activity of the 8-lipoxygenating double mutant F359W + A424I was almost twice as high, and similar amounts of leukotriene A_4 hydrolysis products and double oxygenation derivatives were detected with this enzyme species. These data indicate that site-directed mutagenesis of the human 5-lipoxygenase that leads to alterations in the positional specificity favoring arachidonic acid 15-lipoxygenation does not suppress the leukotriene synthase activity of the enzyme. The residual 8-lipoxygease activity of the mutant enzyme and its augmented rate of 5-HpETE conversion may be discussed as major reasons for this unexpected result.

Leukotrienes formed via the 5-lipoxygenase pathway of the arachidonic acid cascade are important mediators of anaphylactic diseases (*1, 2*). Lipoxygenase inhibitors and leukotriene receptor antagonists have been developed as antiasthmatic drugs and are currently available for prescription use (*3, 4*). The 5-lipoxygenase (5-LOX) constitutes the key enzyme in leukotriene biosynthesis since it catalyzes two early consecutive steps in leukotriene formation (*5, 6*): (i) dioxygenation of free arachidonic acid [(5*Z*,8*Z*,11*Z*,14*Z*)-5,8,11,14-eicosatetraenoic acid], forming (5*S*,6*E*,8*Z*,11*Z*,-14*Z*)-5-hydroperoxy-6,8,11,14-eicosatetraenoic acid (5*S*-HpETE), and (ii) dehydration of 5*S*-HpETE to 5,6-epoxy-leukotriene A_4.

Mammalian LOXs constitute a heterogeneous family of lipid-peroxidizing enzymes that are categorized with respect to their positional specificity of arachidonic acid oxygenation (*7, 8*) into various subfamilies: 5-LOXs, 8-LOXs, 12-LOXs, and 15-LOXs. Although sequence determinants of the positional specificity have been identified for several isoforms, the structural reasons for positional specificity remain a matter of discussion (*9*−*13*). For 12*S*- and 15*S*-LOXs, the amino acids that align with F353, I418, M419, and I593 of the rabbit reticulocyte LOX are important for the enzyme specificity (*14*−*17*). Y603 and H604 of the murine epidermis 8*S*-LOX were identified as sequence determinants, and mutation of these amino acids to the residues present at these positions in the human epidermis-type 15-LOX converted the murine enzyme to a 15-lipoxygenating species (*18*). In contrast, previous attempts to convert mammalian 12- and 15-LOXs to a 5-LOX were not successful (*14, 15*); in these cases inactive enzyme species were always created.

For the present study we transformed the human 5-LOX to a 15-lipoxygenating enzyme species by multiple site-directed mutagenesis targeting the above-mentioned sequence determinants. Since 15-LOXs are not capable of catalyzing C-10 hydrogen abstraction, the resulting 15-lipoxygenating mutants were expected to lack a sizable LTA_4 synthase activity with 5*S*-HpETE as substrate. Surprisingly, we found that the mutant enzyme converted 5*S*-HpETE to LTA_4 with a similar effectiveness as the wild-type enzyme. This was mainly due to the augmented conversion rate of 5*S*-HpETE by the mutant enzyme.

MATERIALS AND METHODS

Chemicals. The chemicals used were from the following sources: arachidonic acid [(5*Z*,8*Z*,11*Z*,14*Z*)-eicosa-5,8,11,-14-tetraenoic acid], 5(*S*)-H(p)ETE [(5S,6*E*,8*Z*,11*Z*,14*Z*)-5-

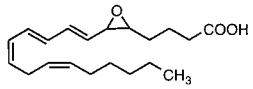
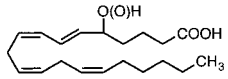
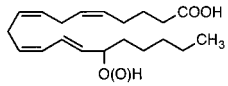
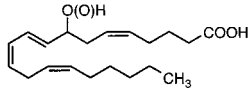
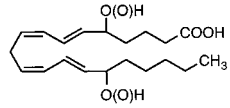
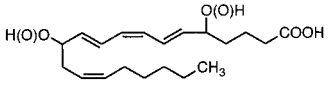
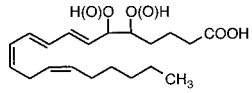
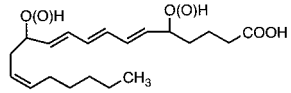
† Financial support for this study was provided by the Deutsche Forschungsgemeinschaft (Ku 961/7-1) and by the European Commission (BMH4-CT98-3191).

* Correspondence should be addressed to this author at the Institute of Biochemistry, University Clinics Charité, Humboldt University, Hessische Str. 3-4, 10115 Berlin, Germany. Tel. +49-30-2093 7539; fax +49-30-2093 7300; e-mail hartmut.kuehn@charite.de.

[1] Abbreviations: 5*S*-HpETE, (5*S*,6*E*,8*Z*,11*Z*,14*Z*)-5-hydroperoxy-6,8,11,14-eicosatetraenoic acid; RP-HPLC, reverse-phase high-performance liquid chromatography; LOXs, lipoxygenases; LT A_4, leukotriene A_4; GC/MS, gas chromatography/mass spectrometry; TRA, triethanolamine; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

10.1021/bi001447n CCC: $19.00
Published on Web 11/02/2000

Table 1: Chemical Structures of the Hydroxyeicosanoids

compound	biosynthesis	chemical structure
LTA_4	LTA_4-synthase	
5-H(p)ETE	oxygenation	
15-H(p)ETE	oxygenation	
8-H(p)ETE	oxygenation	
5,15-DiH(p)ETE	double oxygenation	
5,12-DiH(p)ETE(E,Z,E)	double oxygenation	
5,6-DiH(p)ETE	LTA_4-hydrolysis/ double oxygenation	
5,12-DiH(p)ETE(E,E,E)	LTA_4-hydrolysis	

hydro(pero)xy-6,8,11,14-eicosatetraenoic acid], $CaCl_2$, EDTA, ATP, dipalmitoylphosphatidylcholine, and sodium borohydride were from Serva (Heidelberg, Germany); ampicillin was from Gibco (Eggenstein, Germany); isopropyl β-D-thiogalactopyranoside (IPTG) and ATP−Sepharose were from Sigma−Aldrich (Deisenhofen, Germany); leukotriene A_4 methyl ester, 12*R*-HETE [(12*R*/*S*,5*Z*,8*Z*,10*E*,14*Z*)-12-hydroxy-5,8,10,14-eicosatetraenoic acid], 12*S*-HETE [(12*S*,-5*Z*,8*Z*,10*E*,14*Z*)-12-hydroxy-5,8,10,14-eicosatetraenoic acid], 5*S*,6*S*-diHETE [(5*S*,6*S*,7*E*,9*E*,11*Z*,14*Z*)-5,6-dihydroxy-7,9,-11,14-eicosatetraenoic acid], 5*S*,6*R*-diHETE [(5*S*,6*R*,7*E*,9*E*,-11*Z*,14*Z*)-5,6-dihydroxy-7,9,11,14-eicosatetraenoic acid], 5*S*,-15*S*-diHETE [(5*S*,15*S*,6*E*,8*Z*,11*Z*,13*E*)-5,15-dihydroxy-6,8,-11,13-eicosatetraenoic acid] from Cayman Chem. (distributed by Alexis GmbH, Grünberg, Germany); 5*S*,12*S*-diHETE-(*E*,*E*,*E*) [(5*S*,12*S*,6*E*,8*E*,10*E*,14*Z*)-5,12-dihydroxy-6,8,10,-14-eicosatetraenoic acid] and 5*S*,12*R*-diHETE(*E*,*E*,*E*) [(5*S*,12*R*,6*E*,8*E*,10*E*,14*Z*)-5,12-dihydroxy-6,8,10,14-eicosatetraenoic acid] from Biomol (Hamburg, Germany). The chemical structures of the hydroxyeicosanoids used are summarized in Table 1. HPLC solvents were obtained from Merck (Darmstadt, Germany). Restriction enzymes were purchased from New England Biolabs (Schwalbach, 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). The human 5-LOX cDNA (cloned into the Bluescript SK+ cloning vector) was a kind gift of Dr. A. Habenicht (Heidelberg).

Bacterial Expression of 5-LOX and Site-Directed Mutagenesis. To express the human 5-LOX as a nonfusion protein, its cDNA was subcloned into the expression plasmid pKK233-2. For this purpose we introduced a *Nco*I restriction site at the starting ATG and a *Hin*dIII site just behind the stop codon. The *Nco*I/*Hin*dIII restriction fragment was then ligated into the expression vector, and bacteria (HB 101) were transformed with the recombinant plasmid. Site-directed mutagenesis of the human 5-LOX was carried out by the PCR overlap extension technique with mismatching synthetic oligonucleotides. The PCR products containing the mutations were digested with appropriate restriction enzymes and inserted into the wild-type expression plasmid. Transformed bacteria were replated, and for each mutation 20−30 clones were screened for the expression of the mutant 5-LOX by restriction mapping and activity assay. Several 5-LOX-positive clones were sequenced. For the final activity assay one sequenced clone was replated, and five well-separated colonies were picked and cultured at 37 °C in 5 mL of LB medium containing 0.1 mg/mL ampicilin to an optical density at 600 nm of 0.5. Then LOX expression was induced by addition of IPTG (1 mM final concentration). After 12 h at 30 °C the bacteria were spun down, washed with phosphate-buffered saline (PBS), resuspended in 0.5 mL of 0.1 M phosphate buffer, pH 7.4, containing 1 mM EDTA, and kept on ice for 10 min. The cells were lyzed by sonication with a tip sonifier (Braun, Melsungen, Germany), cell debris was removed by centrifugation (4000*g*), and the lysis supernatant was used for activity assay.

Activity Assays. To determine the arachidonic acid oxygenase activity of the recombinant 5-LOX species, HPLC base activity assays were carried out. For this purpose aliquots of the bacterial lysate supernatants or of the purified enzyme preparations were incubated (10−15 min) at room temperature with 0.1 mM arachidonic acid in the presence of 0.4 mM $CaCl_2$, 0.1 mM EDTA, 40 μg/mL dipalmitoylphosphatidylcholine, and 0.1 mM ATP (final volume of the assay mixture 0.5 mL). The hydroperoxy compounds formed were reduced with sodium borohydride to the corresponding hydroxy derivatives, the mixture was acidified to pH 3, and 0.5 mL of ice-cold methanol was added. The protein precipitate was spun down and aliquots of the clear supernatant were injected for reverse-phase HPLC quantification of the LOX products.

Enzyme Purification. The recombinant 5-LOX species were purified from the bacterial lysis supernatant by ATP−Sepharose affinity chromatography on an open-bed column with a gel volume of 3 mL. This column was washed with 10 mL of 50 mM TRA/HCl buffer, pH 7.3, containing 2 mM EDTA, 10 mM mercaptoethanol, and 1 M NaCl to remove unspecifically bound proteins, and the 5-LOX was then eluted with 50 mM TRA/HCl buffer, pH 7.3, containing 2 mM EDTA, 10 mM mercaptoethanol, 100 mM NaCl, and 15 mM ATP. Fractions of 2 mL were collected and the LOX activity was assayed. With this one-step purification procedure the enzyme was purified roughly 500-fold but we did not reach electrophoretic homogeneity. As indicated by SDS−PAGE, the recombinant enzyme species contributed 10−30% to the total protein content of the final enzyme preparation. Attempts to further purify the enzyme species failed since we experienced severe losses in enzyme activity.

Analysis. RP-HPLC was carried out on a Shimadzu system connected to a Hewlett-Packard diode array detector 1040. Analytes were separated on a Nucleosil C-18 column

(Macherey-Nagel, KS system, 250 × 4 mm, 5 μm particle size) coupled with a guard column (30 × 4 mm, 5 μm particle size). Solvent systems consisting of methanol/water/acetic acid (different compositions) were used and a flow rate of 1 mL/min was adjusted. The absorbencies at 235 nm (conjugated dienes), 242 nm (double-conjugated dienes of 5*S*,15*S*-diHETE), 270 nm (conjugated trienes such as 8*S*,-15*S*-diHETE 5*S*,12*S*-diHETE, and 5*S*,12*R*-diHETE), and 301 nm (lipoxin isomers) were recorded simultaneously. Calibration curves (five-point calibrations) for conjugated dienes, double-conjugated dienes, and conjugated trienes were established.

GC/MS was performed with the Shimadzu QP-2000 system equipped with a capillary RSL-150 column (polydimethylsiloxane, 0.25 μm coating thickness; 30 m × 0.32 mm; Research Separation Labs, Belgium).

Miscellaneous Methods. Reference compounds of 5*S*,12*S*-diHETE(*E*,*Z*,*E*) [(5*S*,12*S*,6*E*,8*Z*,10*E*,14*Z*)-5,12-dihydroxy-6,8,10,14-eicosatetraenoic acid] and 5*S*,12*R*-diHETE(*E*,*Z*,*E*) [(5*S*,12*R*,6*E*,8*Z*,10*E*,14*Z*)-5,12-dihydroxy-6,8,10,14-eicosatetraenoic acid] were prepared from 12*S*-HETE and 12*R*-HETE, respectively, with the recombinant human 5-LOX.

For GC/MS the hydroxy fatty acids prepared were methylated with diazomethane, repurified on HPLC, and silylated with bis(trimethylsilyl) trifluoroacetamide in the presence of dry pyridine. For more informative mass spectra, catalytic hydrogenation of the hydroxy fatty acid methyl esters was carried out (palladium asbestos as catalyst in ethanolic solutions). LTA_4 hydrolysis products were prepared by incubating 5 μg of LTA_4 methyl ester for 2 h at pH 2. Then KOH was added to a final concentration of 0.5 M and the methyl esters of the resulting diol isomers were hydrolyzed to the free acids. After acidification to pH 3, the hydrolysis mixture was directly injected for RP-HPLC and the hydrolysis products were prepared. Anaerobiosis was achieved by bubbling argon through the reaction mixture at 2 °C for 60 min. Then the sample was allowed to warm to room temperature under an argon cushion. For basic kinetic characterization of the wild-type and mutant enzyme species, their LOX activity was assayed at five different substrate concentrations and the linear part of the Lineweaver−Burk plot was evaluated.

RESULTS

(1) LTA_4 Synthase Activity of the Recombinant Wild-Type 5-LOX. 5*S*-HpETE, the primary arachidonic acid oxygenation product of the 5-LOX pathway, can be metabolized by LOXs via two alternative routes: (i) the LTA_4 synthase pathway and (ii) the double oxygenation route. We confirmed that the native human leukocyte 5-LOX converts 5*S*-HpETE mainly to LTA_4 via its intrinsic leukotriene synthase activity (*6*). When similar experiments were carried out with the recombinant human enzyme we observed the formation of four conjugated triene isomers, which cochromatographed with authentic standards of 5*S*,12*R*/*S*-diHETE(*E*,*E*,*E*) and 5,6-diHETE isomers (Figure 1). We only detected minor amounts of the double oxygenation products 5*S*,15*S*-diH(p)ETE, 5*S*,12*S*-diH(p)ETE(*E*,*Z*,*E*), and 5*S*,12*R*-diH(p)ETE-(*E*,*Z*,*E*). A very similar product pattern was found when the reaction was carried out under anaerobic conditions (data not shown). Nonenzymatic hydrolysis of LTA_4 revealed an almost identical product pattern (inset to Figure 1). However, careful quantification of the products formed during enzymatic 5*S*-HpETE turnover indicated that the amounts of the early-eluting 5,6-diHETE isomer, which coeluted with 5*S*,6*R*-diHETE, were always higher than those of the late-eluting triene (cochromatographed with 5*S*,6*S*-diHETE). In contrast, during nonenzymatic hydrolysis always equal amounts of both products were detected. Since 5*S*,6*R*-diHETE can be formed during LTA_4 hydrolysis but also via double oxygenation of 5*S*-HpETE (after reduction of the primary hydroperoxy derivative), one may conclude that the preponderance of the early-eluting 5,6-diHETE may be due to a small share (less than 10%) of the double oxygenation pathway. These data indicate that the LTA_4 synthase activity is dominating when the recombinant wild-type human 5-LOX reacts with 5*S*-HpETE. In contrast, the double oxygenation pathway is dominant when the rabbit 15-LOX reacted with 5*S*-HpETE (data not shown). Upon HPLC analysis, 5*S*,15*S*-diHETE was identified as the major product (after borohydride reduction of the primary hydroperoxy derivatives) and we also found small amounts of 5*S*,12*S*-diHETE originating from the intrinsic 12-LOX activity of the enzyme.

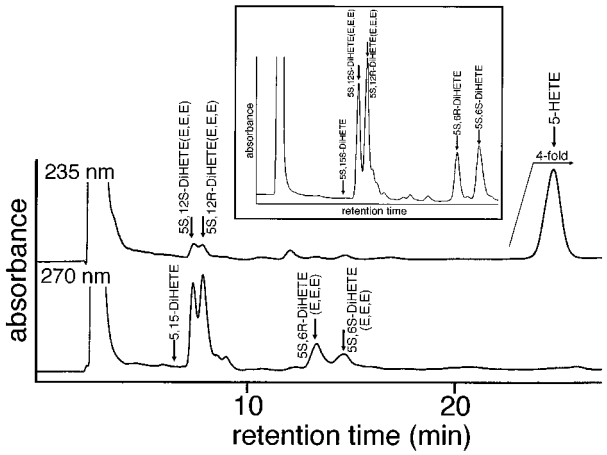


FIGURE 1: Conversion of 5*S*-HpETE by the recombinant human 5-LOX. The wild-type human 5-LOX was overexpressed in *E. coli* and purified from the bacterial lysis supernatant as described under Materials and Methods. The purified enzyme preparation (0.5 mL; corresponds to the enzyme content of a 10 mL liquid culture) was incubated in 0.1 M Tris-HCl buffer, pH 7.4, with 25 μM 5*S*-HpETE in the presence of 0.4 mM $CaCl_2$, 0.1 mM EDTA and 40 μg/mL dipalmitoylphosphatidylcholine, for 5 min at room temperature. Product preparation and RP-HPLC analysis was performed as described under Materials and Methods. A solvent system consisting of methanol/water/acetic acid 75:25:0.1 (v/v/v) was used, and the absorbencies at 235, 270, and 300 nm were recorded. At 300 nm no conjugated tetraenes were detected, and thus, this chromatogram is not shown. Elution of unconverted 5*S*-H(p)ETE was recorded at a 4-fold less sensitive scale. Inset: Nonenzymatic hydrolysis of LTA_4 (see Materials and Methods); the absorbance at 270 nm was recorded. The retention times of authentic standards are indicated by the arrows above the traces.

(2) Site-Directed Mutagenesis of the Recombinant Human 5-LOX. To find out whether the LTA_4 synthase activity and the double oxygenation activity of the human 5-LOX are altered when its positional specificity is modified, we mutated critical amino acids at the active site of the human 5-LOX and quantified both catalytic activities. As target amino acids for the mutagenesis (Figure 2), we selected those residues forming the bottom of the putative substrate binding pocket (F359, A424, N425, and A603). These amino acids have

	359	424 425	603
wild-type 5-LOX	SDFHV	DKANAT	GAV
F359W+A424I	SD**W**HV	DK**I**NAT	GAV
F359W+A424I+ N425M+A603I	SD**W**HV	DK**IM**AT	G**I**V

FIGURE 2: Partial amino acid alignment of the wild-type human 5-LOX and of the mutants created for this study. The one-letter code for the amino acids is used. The mutants introduced are indicated in boldface type.

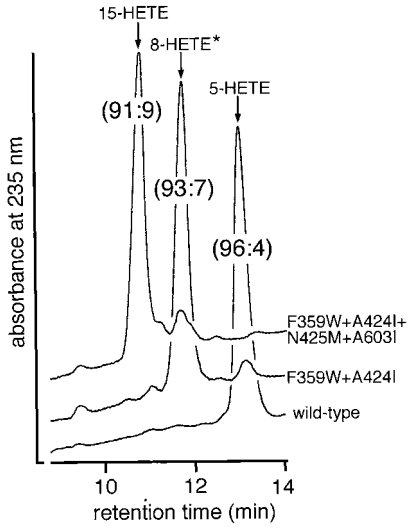


FIGURE 3: Positional specificity of arachidonic acid oxygenation by 5-LOX mutants. The 5-LOX mutants were expressed in *E. coli* and purified as described under Materials and Methods. The pooled LOX active fractions (1500 μL), which represented the LOX content of about 10 mL of a bacterial liquid culture, were incubated with arachidonic acid, the reaction products were prepared and analyzed by RP-HPLC (see Materials and Methods). A solvent system consisting of methanol/water/acetic acid 80:20:0.1 (v/v/v) was used, and the absorbance at 235 nm was recorded. The *y*-axis was scaled for each sample to the absorbance of the highest peak. Since 12- and 8-HETE were not well separated under our chromatographic conditions the 8-HETE peak (asterisk) was reanalyzed by SP-HPLC to confirm its chemical structure. The *S/R* ratio of the products is given in parentheses.

been identified previously as sequence determinants for the positional specificity of other LOX isoforms (*11*, *16*, *17*). The goal of our mutagenesis experiments was to reduce the volume of the substrate binding pocket as much as possible to force a substrate alignment that may be optimal for 15-lipoxygenation (*11*, *12*). Since single mutants did not show major effects on the positional specificity (data not shown), we created a double and a quadruple mutant and tested their positional specificity of arachidonic acid oxygenation. From Figure 3 it can be seen that the F359W + A424I double mutant exhibits an 8-lipoxygenating activity with arachidonic acid as substrate. In contrast, the quadruple mutant F359W + A424I + N425M + I603A was a major 15-LOX with 8*S*-HpETE being a minor side product. Basic kinetic characterization of the 5-LOX species indicated that the mutant isoforms exhibit a somewhat reduced substrate affinity (63 μM for the F359W + A424I double mutant, 57 μM for the F359W + A424I + N425M + I603A quadruple mutant) when compared with the wild-type enzyme (35 μM).

(3) LTA_4 Synthase Activity of Mutant 5-LOX Species. When the 8-lipoxygenating double mutant was used as catalyst for 5-HpETE conversion (Figure 4, trace B) the

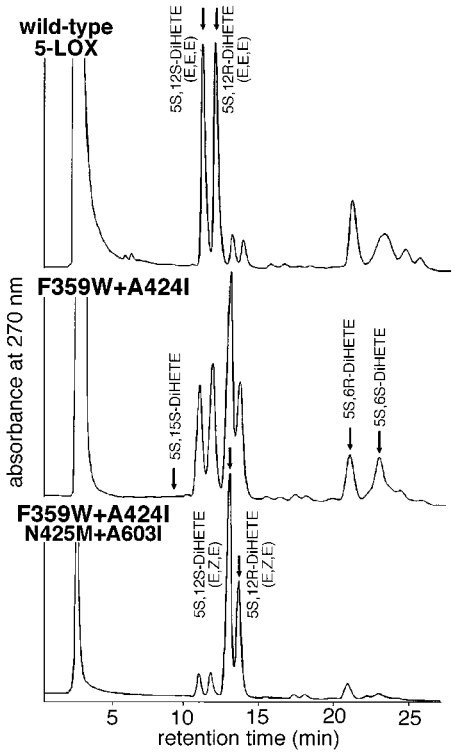


FIGURE 4: Conversion of 5*S*-HpETE by 5-LOX mutants exhibiting different positional specificity of arachidonic acid oxygenation. Wild-type and mutant 5-LOX species were expressed in *E. coli* and purified as described under Materials and Methods. The pooled LOX active fractions (0.5−2 mL), which represent the LOX content of about 10−40 mL of a bacterial liquid culture, were incubated for 15 min at room temperature with 25 μM 5*S*-HpETE in 2 mL of 0.1 M Tris-HCl buffer, pH 7.4, containing 0.4 mM $CaCl_2$, 0.1 mM EDTA and 40 μg/mL dipalmitoylphosphatidylcholine, for 15 min. After reduction of the hydroperoxy compounds with sodium borohydride, the lipids were extracted twice with 2 mL of ethyl acetate, the solvent was removed, and the residue was reconstituted in 0.5 mL of methanol. Water (0.5 mL) containing 1% acetic acid was added, and aliquots of this solution were injected for RP-HPLC with the solvent system methanol/water/acetic acid (70:30:0.1 v/v/v). The retention times of authentic standards are indicated by the arrows above the traces. This experiment was carried out twice with different preparations of 5-LOX mutants, and representative chromatograms for the different enzyme species are shown. The *y*-axis was scaled for each sample to the absorbance of the highest peak in the diHETE region.

product mixture was somewhat more complex than that obtained with the wild-type enzyme (Figure 4, trace A). In addition to the LTA_4 hydrolysis products indicating a share of LTA_4 formation, we observed two other compounds eluting in the 5,12-diHETE region. After borohydride reduction of the formed hydroperoxy fatty acids, we found that the early-eluting product cochromatographed with an authentic standard of 5*S*,12*S*-diHETE(*E*,*Z*,*E*) and the late-eluting triene with a standard of 5*S*,12*R*-diHETE(*E*,*Z*,*E*). Both products, which showed similar UV and mass spectra, contained an *E*,*Z*,*E*-triene chromophore as indicated by the characteristic UV spectrum (Figure 5). This spectrum was distinct from that of the *E*,*E*,*E*-triene present in the 5,12-diHETE(*E*,*E*,*E*) isomers, which were formed during nonenzymatic hydrolysis of LTA_4. When the reaction was carried out under anaerobic conditions the formation of the *E*,*Z*,*E*-trienes was largely suppressed, suggesting their double oxygenation origin. Quantification of the HPLC profiles indicated that LTA_4 synthase and double oxygenation activity

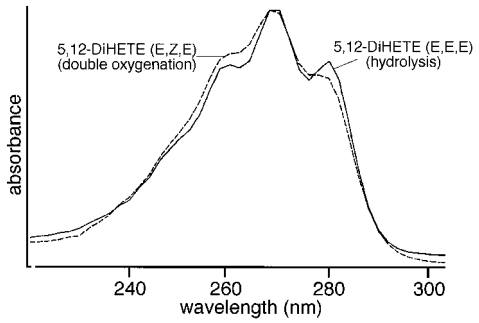


FIGURE 5: UV spectra of 5*S*,12*S*-diHETE isomers formed via LTA_4 hydrolysis and 5*S*-HpETE double oxygenation. The UV spectra were recorded with the diode array detector during analysis of the 5*S*-HpETE conversion products by the 8-lipoxygenating F359W + A424I double mutant (see Figure 4, middle panel).

of the F359W + A424I mutant contributed similar shares (50% each) to 5-HpETE conversion.

In contrast, the 15-lipoxygenating quadruple mutant F359W + A424I + N425M + I603A converted 5*S*-HpETE predominantly via the double oxygenation pathway. From Figure 4, trace C, it can be seen that the major reaction products (after borohydride reduction of the primary hydroperoxy derivatives) cochromatographed with 5*S*,12*S*-diHETE(*E*,*Z*,*E*) and 5*S*,12*R*-diHETE(*E*,*Z*,*E*). In addition, small amounts of 5*S*,6*R*-diHETE and of LTA_4 hydrolysis products were detected. Surprisingly, we only found minor amounts of the double oxygenation product 5*S*,15*S*-diH(p)ETE (data not shown).

Summarizing the information provided by the product patterns, one may conclude that the wild-type 5-LOX converts 5*S*-HpETE almost exclusively via the LTA_4 synthase pathway. In contrast, the 15-lipoxygenating quadruple mutant mainly catalyzes the double oxygenase reaction. It only exhibits a minor LTA_4 synthase activity as indicated by the small amounts of LTA_4 hydrolysis products. The 8-lipoxygenating double mutant F359W + A424I catalyzes both reactions to similar extents.

(4) Quantification of LTA_4 Synthase and Double Oxygenase Pathways. The relative amounts of LTA_4 hydrolysis products shown in Figure 4 do not reflect the overall LTA_4 synthase capacity of the various 5-LOX mutants since they do not consider the reaction rates. From Table 2 it can be seen that the wild-type 5-LOX converts arachidonic acid with a higher rate than 5*S*-HpETE, indicating that arachidonic acid is a better substrate for this enzyme species (5*S*-HpETE conversion/arachidonic acid oxygenase = 0.7). In contrast, the 5-LOX mutants prefer 5*S*-HpETE over arachidonic acid. When normalized to an equal arachidonic acid oxygenase activity, the 15-lipoxygenating quadruple mutant converts 5*S*-HpETE with a 6-fold higher rate than the wild-type enzyme (5*S*-HpETE conversion/arachidonic acid oxygenase = 4.4). For the 8-lipoxygenating double mutant a 3-fold higher rate of 5*S*-HpETE conversion was calculated (5*S*-HpETE conversion/arachidonic acid oxygenase = 2.2). When the LTA_4 synthase activity of the various LOX species was related to their 5*S*-HpETE-conversion rate, the wild-type enzyme was most effective. However, when normalized to an equal arachidonic acid oxygenase activity, the 8-lipoxygenating double mutant F359W + A424I was more effective in synthesizing LTA_4 than the wild-type 5-LOX and the F359W + A424I + N425M + I603A quadruple mutant, which did not significantly differ from each other. These data indicate that the LTA_4 synthase activities of the wild-type human 5-LOX and its 15-lipoxygenating quadruple mutant are comparable despite the fact that the latter enzyme mainly catalyzes double oxygenation of 5*S*-HpETE.

DISCUSSION

Leukotrienes are the major metabolites of the 5-LOX pathway and have been identified as mediators in anaphylactic and inflammatory diseases (*19*, *20*). The parent compound for cysteinyl leukotrienes (LTC_4, LTD_4, and LTE_4) on one hand and for LTB_4 on the other is 5,6-epoxy-LTA_4, the formation of which involves two consecutive reactions (*21*): (i) 5-lipoxygenation of arachidonic acid to 5*S*-HpETE, which involves a stereoselective hydrogen abstraction from C_7 and stereospecific introduction of molecular dioxygen at C_5, and (ii) conversion of 5*S*-HpETE to the 5,6-epoxy-LTA_4; according to the radical mechanism, this reaction involves a stereoselective hydrogen abstraction from C_{10} and a homolytic cleavage of the peroxy bond. The resulting bis-radical stabilizes by epoxide formation. It should be stressed that both reactions, 5-lipoxygenation and 5,6-epoxide formation, are catalyzed by 5-LOXs. In contrast, mammalian 15-LOXs are not capable of converting 5*S*-HpETE to 5,6-epoxy-LTA_4. In this case substrate oxygenation to 5*S*,15*S*-diHpETE is the major metabolic route. Thus, the quadruple 5-LOX mutant F359W + A424I + N425M + A603I, which converts arachidonic acid mainly to 15*S*-HpETE, was not expected to exhibit a major LTA_4 synthase activity. Surprisingly, we detected considerable amount of LTA_4 hydrolysis products when this mutant reacted with 5*S*-HpETE. Although the double oxygenase pathway was clearly dominant, the quadruple mutant exhibited a similar LTA_4 synthase activity as the wild-type enzyme when normalized to an equal arachidonic acid oxygenase activity. The major reason for the effective LTA_4 formation by the 15-lipoxygenating mutant was its augmented rate of 5*S*-HpETE conversion (Table 2). We found that the rates of 5*S*-HpETE conversion by the mutant 5-LOX species were higher than their arachidonic acid oxygenase activities, indicating that 5*S*-HpETE constitutes a better substrate for these enzymes. From Figure 3 it can be seen that the 15-lipoxygenating quadruple mutant exhibited a residual 8-lipoxygenase activity, which amounted to about 10% of the total arachidonic acid oxygenase activity. This residual 8-LOX activity may be responsible for LTA_4 formation since it is capable of catalyzing C_{10} hydrogen abstraction.

As indicated in Figure 4, 5*S*,12*S*-diH(p)ETE was identified as major product of 5*S*-HpETE conversion by the 5-LOX quadruple mutant. Under anaerobic conditions the formation of this product was strongly reduced, suggesting its double oxygenation origin. Since arachidonic acid is mainly oxygenated at C-15 by this mutant enzyme species we specifically looked for the formation of 5*S*,15*S*-diH(p)ETE but could only detect small amounts (<5% of the total product mixture). Since the apparent lack of 5*S*,15*S*-diH(p)ETE formation could be due to a rapid secondary oxidation of this double oxygenation metabolite, we looked for the formation of triple oxygenation products. However, upon recording the HPLC chromatograms at 300 nm, we only detected a minimal formation of lipoxin isomers. These data indicate an interesting mechanistic difference between the oxygenation of 5*S*-

Table 2: Quantification of Enzymatic Activities of Various 5-LOX Species[a]

enzyme species	arachidonate oxygenase activity[b] (μg of HETE/sample)	5*S*-HpETE conversion[c] (μg/sample)	LTA_4 synthase[d] (μg/sample)	LTA_4 synthase/ 5*S*-HpETE conversion	LTA_4 synthase/ arachidonate oxygenase
wild-type 5-LOX	3.2 ± 0.6	2.3 ± 0.9	2.1 ± 0.8	0.91	0.66
F359W + A424I	1.3 ± 0.2	2.9 ± 2.7	1.6 ± 1.3	0.55	1.23
F359W + A424I + N425M + A603I	1.1 ± 0.7	4.9 ± 0.4	0.8 ± 0.2	0.14	0.64

[a] Wild-type and mutant 5-LOX species were expressed in *E. coli* as described under Materials and Methods. Aliquots of the lysis supernatant were incubated with the corresponding substrates for 10 min at room temperature. [b] One sample corresponds to a 10 mL bacterial liquid culture. [c] Assayed by HPLC as disappearance of 5*S*-HpETE. [d] Determined as sum of LTA_4 hydrolysis products. The data represent the means of two independent experiments carried out with two different enzyme preparations. The activity ratios were calculated from the mean values.

HpETE and of arachidonic acid. Lipoxygenation of 5*S*-HpETE [formation of 5*S*,12*R*/*S*-diH(p)ETE(*E*,*Z*,*E*)] is initiated by a hydrogen removal from C_{10}, whereas arachidonic acid oxygenation [15*S*-HpETE formation] involves C_{13} hydrogen abstraction. Although the structural reasons for this difference remain to be elucidated, it may be concluded that 5*S*-HpETE is differently aligned at the active site. The additional OH group close to the substrate's carboxylate and/or the conjugated diene system of 5*S*-HpETE may alter the substrate binding in such a way that C_{10} approaches the enzymes hydrogen acceptor whereas C_{13} may be dislocated.

Another point of mechanistic interest is the relative lack of stereoselectivity of 5*S*-HpETE oxygenation by the two 5-LOX mutants examined. From Figure 4 it can be seen that the major oxygenation product, 5*S*,12*S*-diH(p)ETE, is always accompanied by a considerable share of the corresponding 12*R* isomer. Thus, 12-lipoxygenation of 5*S*-HpETE by the 5-LOX mutants exhibits a reduced degree of enantioselectivity when compared with arachidonic acid oxygenation by the same enzyme species (Figure 3). However, the regioselectivity of the double oxygenation reaction was quite high since 5,15-diHpETE was almost absent (no C-13 hydrogen removal). These data suggest that the first step of the LOX reaction, the initial hydrogen removal, appears to be largely enzyme-controlled. This is an important finding since hydrogen abstraction is the rate-limiting step of the overall LOX reaction (*22*). On the other hand, the share of stereorandom oxygenation products suggests that the mutant enzyme species may have lost control over dioxygen insertion in case of 5*S*-HpETE oxygenation. It should be mentioned at this point that a loss of stereochemical control is not unusual in the LOX field. When the soybean LOX-1 oxygenates linoleic acid at neutral pH, its stereospecificity is impaired (*23*). A large share of stereorandom oxygenation products is also formed when the rabbit 15-LOX oxygenated biomembranes at relatively high concentrations. In contrast, at lower substrate concentrations a highly specific product pattern was found (*24*). Several plant LOX exhibit a high reaction specificity with C-18 fatty acids (linoleic acid, linolenic acid) but oxygenate arachidonic acid to a complex mixture of stereorandom oxygenation products (*25*, *26*). Although the mechanistic reasons for these changes have not been investigated in detail, alterations in the substrate alignment at the active site and/or dissociation of reaction intermediates may be involved.

It has been reported before for the rabbit 15-LOX that the amino acids F353, I418, M419, and I593 constitute sequence determinants for the positional specificity of this enzyme (*16*, *17*). X-ray structural studies suggested that these residues may form the bottom of the substrate binding pocket and thus, may be crucial for enzyme−substrate interaction (*16*, *17*). From structural modeling of other LOX isoforms it was concluded that the mechanistic basis for the different positional specificity of 5- and 15-LOXs (type 1) might be the volume of the substrate binding cavity (*11*, *12*). 5-LOXs were suggested to have a bigger active site, which may allow a substrate alignment optimal for 5-lipoxygenation. In contrast, the substrate binding cage of 15-LOXs (type 1) may not be as deep so that the substrate fatty acids cannot slide in farther. Thus, the substrate molecules are oxygenated close to their methyl terminus (*11*, *12*, *13*). To obtain experimental evidence for this space-based hypothesis, we targeted the amino acids of the human 5-LOX, which align with the sequence determinants of the rabbit 15-LOX, by multiple site-directed mutagenesis. To reduce the volume of the substrate binding pocket, more space-filling amino acids were introduced. According to the space-based hypothesis (*11*, *12*), we expected alterations in the positional specificity favoring arachidonic acid 15-lipoxygenation. Although previous attempts failed to alter the positional specificity of this pharmacologically most relevant enzyme (*14*, *15*), we succeeded in creating active 5-LOX mutants. Since single point mutants had only minor effects (data not shown), we were forced to create multiple mutants. It should be stressed at this point that multiple mutations are always critical since they may cause major alterations in the three-dimensional structure. In principle, the effects reported here might be due not only to a reduction of the active-site volume but also to more drastic alterations in the overall structure. However, dramatic overall changes appear to be not very likely since the mutant enzyme species remain catalytically active. Moreover, we did not experience any loss in the stereochemical control of fatty acid oxygenation, which would be expected in case of dramatic structural alterations.

REFERENCES

1. Samuelsson, B., Dahlen, S. E., Lindgren, J. A., Rouzer, C. A., and Serhan, C. N. (1987) *Science 237*, 1171−1176.
2. Drazen, J. M., Israel, E., and O'Byrne, P. M. (1999) *N. Engl. J. Med. 340*, 197−206.
3. Wenzel, S. E., and Kamada, A. K. (1996) *Ann. Pharmacother. 30*, 858−864.
4. O'Byrne, P. M., Israel, E., and Drazen, J. M. (1997) *Ann. Intern. Med. 127*, 472−480.
5. Shimizu, T., Radmark, O., and Samuelsson, B. (1984) *Proc. Natl. Acad. Sci. U.S.A.* 81, 689−693.
6. Rouzer, C. A., Matsumoto, T., and Samuelsson, B. (1986) *Proc. Natl. Acad. Sci. U.S.A.* 83, 857−861.
7. Brash, A. R. (1999) *J. Biol. Chem. 274*, 23679−23682.
8. Kuhn, H., and Thiele, B. J. (1999) *FEBS Lett. 449*, 7−11.
9. Prigge, S. T., Boyington, J. C., Gaffney, B. J., and Amzel, L. M. (1996) *Proteins: Struct., Funct., Genet. 24*, 275−291.

10. Prigge, S. T., Gaffney, B. J., and Amzel, L. M. (1998) *Nat. Struct. Biol. 5*, 178−179.
11. Browner, M., Gillmor, S. A., and Fletterick, R. (1998) *Nat. Struct. Biol. 5*, 179.
12. Gillmor, S. A., Villasenor, A., Fletterick, R., Sigal, E., and Browner, M. F. (1997) *Nat. Struct. Biol. 4*, 1003−1009.
13. Funk, C. D., and Loll, P. J. (1997) *Nat. Struct. Biol. 4*, 966−968.
14. Sloane, D. L., Leung, R., Craik, C. S., and Sigal, E. (1991) *Nature 354*, 149−152.
15. Chen, X. S., and Funk, C. D. (1993) *FASEB J. 7*, 694−701.
16. Borngräber, S., Kuban, R. J., Anton, M., and Kühn, H. (1996) *J. Mol. Biol. 264*, 1145−1153.
17. Borngräber, S., Browner, M., Gillmor, S., Gerth, C., Anton, M., Fletterick, R., and Kühn, H. (1999) *J. Biol. Chem. 274*, 37345−37350.
18. Jisaka, M., Kim, R. B., Boeglin, W. E., and Brash, A. R. (2000) *J. Biol. Chem. 275*, 1287−1293.
19. Hay, D. W., Torphy, T. J., and Undem, B. J. (1995) *Trends Pharmacol. Sci. 16*, 304−309.
20. Lewis, R. A., Austen, A. F., and Soberman, R. J. (1990) *N. Engl. J. Med. 323*, 645−655.
21. Brash, A. R., Yokoyama, C., Oates, J. A., and Yamamoto, S. (1989) *Arch. Biochem. Biophys. 273*, 414−422.
22. Glickman, M. H., and Klinman, J. P. (1995) *Biochemistry 34*, 14077−14092.
23. Gardner, H. W. (1989) *Biochim. Biophys. Acta 1001*, 274−281.
24. Kühn, H., Belkner, J., Wiesner, R., and Brash, A. R. (1990) *J. Biol. Chem. 265*, 18351−18361.
25. Regdel, D., Schewe, T., and Rapoport, S. M. (1985) *Biomed. Biochim. Acta 44*, 1411−1428.
26. Feussner, I., and Kuhn, H. (1995) *FEBS Lett. 367*, 12−14.

BI001447N