Shape and Specificity in Mammalian 15-Lipoxygenase Active Site

THE FUNCTIONAL INTERPLAY OF SEQUENCE DETERMINANTS FOR THE REACTION SPECIFICITY*

(Received for publication, June 21, 1999, and in revised form, August 26, 1999)

Sabine Borngräber‡‡, Michelle Browner‡, Sarah Gillmor§, Christa Gerth‡, Monika Anton‡, Robert Flettericks, and Hartmut Kühn¶

From the ‡Institute of Biochemistry, University Clinics (Charité), Humboldt University, Hessische Str. 3-4, 10115 Berlin, Germany, the §Inflammatory Diseases Unit, Roche Bioscience, Palo Alto, California 94304, and the ¶Department of Biochemistry and Biophysics, University of California, San Francisco, California 94143-0448

Previous mutagenesis studies along with molecular modeling using the x-ray coordinates of the rabbit 15-lipoxygenase have led to the suggestion that the size of the substrate binding pocket may play an essential role in determining the oxygenation specificity of 5-, 12-, and 15-lipoxygenases. Based on the x-ray crystal structure of rabbit 15-lipoxygenase, Ile593 appeared to be important in defining size and shape of the substrate-binding site in 15-lipoxygenases. We found that substitution of Ile593 with alanine shifted the positional specificity of this enzyme toward 12-lipoxygenation. To compare the importance of position 593 with previously defined determinants for the oxygenation specificity, we introduced small (alanine-scan) or large amino acids (phenylalanine-scan) at critical positions surrounding the putative fatty acid-binding site, so that the volume of the pocket was either increased or decreased. Enlargement or alteration in packing density within the substrate binding pocket in the rabbit 15-lipoxygenase increased the share of 12-lipoxygenase products, whereas a smaller active site favored 15-lipoxygenation. Simultaneous substitution of both large and small residues in the context of either a 15- or 12-lipoxygenase indicated that there is a functional interplay of the sequence determinants for oxygenation specificity. If the 15-lipoxygenase active site is enlarged excessively, however, no lipoxygenation was observed anymore. Together these results indicate the importance of the overall size and shape of the arachidonic acid binding pocket in defining the specificity of lipoxygenase reaction.

Lipoxygenases (LOs)1 constitute a heterogeneous family of lipid peroxidizing enzymes which catalyze the dioxygenation of free and/or esterified polyunsaturated fatty acids to their corresponding hydroperoxy derivatives (1). They are widely distributed in plants and animals (2, 3) and contain 1 mol of non-heme iron per mol of enzyme (4, 5) which is essential for the oxygenase reaction (6). According to the currently used nomenclature, LOs are categorized with respect to their positional specificity of arachidonic acid oxygenation such that they are referred to as 5-, 8-, 11-, 12-, and 15-LOs. 15-LOs have been implicated in cell maturation and differentiation (7, 8) as well as in the pathogenesis of atherosclerosis (9, 10). In mammalian cells, the biological importance of LO products has not been entirely defined, but a role in inflammation has been suggested and the involvement of 5-LO in allergic and inflammatory diseases has been confirmed (11, 12).

Our current understanding of how the positional specificity of LO isoenzymes is determined, derived from the x-ray structure of rabbit reticuloocyte 15-LO and mutagenesis data. Earlier studies with modified polyenoic fatty acids suggested that the oxygenation specificity is not an invariant enzyme characteristic but depends on the chemical structure of the substrate and its alignment within the active site (13). Based on x-ray crystallographic studies, the putative substrate binding pocket of the rabbit reticuloocyte 15-LO is defined at its base by the side chains of Phe363, Met419, Ile418, and Ile593. The walls of the binding site are lined by hydrophobic residues, except for two polar side chains, Glu537 and Gln548, adjacent to the iron center. The opening of the U-shaped active site may be capped by Arg563, which was modeled to provide an interaction with the carboxylate of the substrate.

A simple model for the alignment of arachidonic acid is that the hydrophobic base of the substrate-binding site, in concert with the charged Arg453 at the opening of the pocket, position substrate fatty acids appropriately for 5-, 8-, 11-, 12-, or 15-lipoxygenation (14). Structural amino acid alignments suggest that the general theme of a deep hydrophobic pocket that includes the catalytic iron center and a positively charged residue at the entrance may be conserved among LO isomers. However, the critical question of which structural features control the precise registration of arachidonic acid so as to position one doubly allylic methylene adjacent to the iron center remains unanswered. Molecular modeling of critical residues that differ among various LO isomers using the three-dimensional structure of the rabbit 15-LO, suggested that the volume at the base of the substrate binding pocket could be important for the positional specificity. The 12-LO cavity was predicted to be slightly larger (6%) than that of the rabbit 15-LO, whereas the 5-LO pocket appears to be significantly (20%) larger (15, 16). These predictions assume that the protein backbones defining the substrate-binding site coincide for these LOs.

To examine the importance of the pocket volume in determining the specificity of oxygenation, we added or deleted mass at the bottom of the pocket in 15-LO to test whether such
changes may contribute to adjusting the substrate position with respect to the catalytic iron. Amino acid residues 353, 417, 418, 419, and 593 were replaced with Ala, singly and in combination, to increase the volume of the pocket. Likewise, these residues were replaced with Phe to reduce the size of the binding site. The assumption was that these amino acids may control how deeply arachidonic acid can bind and thus, which bisallylic methylene is positioned near the catalytic iron (17–22). The results of these mutagenesis studies show that the sequence determinants for the positional specificity functionally interact and that enlargement of the substrate binding pocket in rabbit 15-LO correlates with an increased share of 12-lipoxygenation.

MATERIALS AND METHODS

Chemicals—The chemicals used were obtained from the following sources: arachidonic acid (5Z,8Z,11Z,14Z-eicosatetraenoic acid), HPLC standards of 5S-HETE, 12S-HETE, 15S-HETE, and sodium borohydride from Serva (Heidelberg, Germany), ampicillin from Life Technologies, Inc. (Eggenstein, Germany), isopropyl-β-D-thiogalactopyranoside from Sigma-Aldrich (Deisenhofen, Germany), HPLC solvents 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 Roche Molecular Biochemicals (Mannheim, Germany) and Escherichia coli strain HB 101 was purchased from Invitrogen (San Diego, CA). Oligonucleotide synthesis was carried out by Tib-Molbiol (Berlin, Germany).

Site-directed Mutagenesis and Bacterial Expression—Site-directed mutagenesis was performed by polymerase chain reaction overlap extension using mismatching synthetic oligonucleotides. The polymerase chain reaction products containing each mutation were digested with appropriate restriction enzymes and inserted into the wild-type plasmid. For each mutation, 20–30 clones were screened by restriction mapping, activity assays, and immunoblot analysis to identify LO-positive clones, and mutants were confirmed by sequencing. The plasmid pKK232-2 containing the wild-type coding region of the rabbit reticulocyte-type 15-LO cDNA (23) or the mutated coding sequence was used for bacterial expression. Enzyme expression and activity assays were performed as described previously (22). Briefly, bacterial clones were cultured at 37 °C in 1–3 ml of LB medium containing 0.1 mg/ml ampicillin to an optical density at 600 nm of 0.5. Lipoxygenase expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (1 mM final concentration). After 12 h of incubation, bacteria were centrifuged, washed, and resuspended in 0.5 ml of phosphate-buffered saline. Activity assays were performed by adding 0.1 mM arachidonic acid down, washed, and resuspended in 0.5 ml of phosphate-buffered saline. The hydroperoxy fatty acids formed were reduced to their more stable hydroxy derivatives by addition of sodium borohydride. After acidification to pH 3, 0.5 ml of methanol was added and the samples were kept on ice for 10 min. Protein precipitate was spun down and aliquots of the clear supernatant were directly analyzed by reverse-phase HPLC for quantification of the LO products.

Purification of LO Mutants Expressed in E. coli—500 ml of LB medium were inoculated with an aliquot of a preculture and the bacteria were grown to an optical density at 600 nm of 0.5. Protein expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (final concentration) and the culture was incubated for another 12 h. Cells were spun down, washed with phosphate-buffered saline, resuspended in 10 ml of 10 mM BisTris buffer, pH 6.8, containing 1 mM EDTA and sonicated 3 times for 20 s with a Labsonic U-tip sonifier. The cell debris was spun down, the lysis supernatant desalted on an Econo-Pac 10 DG desalting column (Bio-Rad), and subsequently loaded onto a semi-preparative Mono Q column (Amersham Pharmacia Biotec). The chromatogram was developed with a NaCl step gradient in 10 mM BisTris buffer, pH 6.8, with a flow rate of 2 ml/min (23). If the eluting fractions contained many impurities, the sample was re-chromatographed on an analytical Mono Q column at a flow rate of 0.5 ml/min.

Analytes—High pressure liquid chromatography was performed on a Shimadzu system equipped with a Hewlett-Packard diode array detector at 280 nm. Reversed-phase HPLC was carried out on a Nucleosil C18 column (Macherey-Nagel, Düren, Germany; KS-system, 250 × 4 mm, 5-µm particle size) coupled with a guard column (30 × 4 mm, 5-µm particle size). A solvent system of methanol/water/acetic acid (83/17/0.1, by volume) was used at a flow rate of 1 ml/min. Straight phase HPLC was performed on a Zorbax-SIL column (250 × 4 mm, 5-µm particle size) with a solvent system of n-hexane, 2-propanol, acetic acid (100/20/0.1, by volume) at a flow rate of 1 ml/min. For chiral-phase HPLC, a Chiralcel OD column (250 × 4 mm, 5-µm particle size) and a solvent system consisting of n-hexane, 2-propanol, and acetic acid (100/5/0.1, by volume) were used at a flow rate of 1 ml/min.

Molecular Modeling—Insight II from Molecular Simulations, Inc. was used in all modeling experiments.

RESULTS

Amino Acid 593 Is Important for the Positional Specificity of 12/15-LOs—The crystal structure of the rabbit 15-LO predicted that Ile593, sitting near the base of the substrate binding pocket, may play a role in the alignment of arachidonic acid at the active site and thus, may influence the positional specificity of the enzyme (15). Replacing Ile593 with Ala was assumed to increase the volume of the substrate binding pocket favoring arachidonic acid 12-lipoxygenation. This assumption was confirmed by site-directed mutagenesis. We found that the major reaction products of arachidonic acid oxygenation by the I593A mutant, 15S-H(P)ETE and 12S-H(P)ETE were formed in a ratio of 57/43, compared with 97/3 for the wild-type enzyme (Fig. 1). Both products were shown to be chiral with strong preponderance of the S-isomer indicating that the oxygenation reaction was completely enzyme-controlled. Importantly, the I593A mutant had a similar arachidonic acid oxygenase activity as the recombinant wild-type LO (Table I). These results suggest that substitution of the branched Ile with the smaller Ala enlarges the pocket, presumably allowing the substrate to bind deeper. Consequently, arachidonic acid aligns such that hydrogen can be removed from both C-13 and C-10, so that almost equal amounts of 12- and 15-H(P)ETE are formed.

Functional Interactions of the Specificity Determinants—Previous mutagenesis studies on the human and rabbit 15-LOs indicated that Gln417, Ile418, and Met419 (17, 18) as well as Phe455 (22) may constitute primary determinants for the positional specificity of 12/15-LOs. In these experiments, the 15-LO was changed to a 12-lipoxygenating enzyme by introducing the counterparts of 12-LOs. We investigated the role of the specificity determinants in defining the size of the arachidonic acid-binding site in the rabbit 15-LO by replacing each of these amino acids with Ala (Ala-scan). This experiment examines the relative importance of each of these residues in affecting the volume of the pocket and thus, the positional specificity of the lipoxygenase reaction. As seen from Table I, the I418A mutation has the most dramatic effect on the oxygenation specificity. This substitution completely converted the rabbit 15-LO to a 12-lipoxygenating species, whereas the alanine substitutions at positions 417 and 419 did not have significant effects. The second largest change in positional specificity was achieved by replacement of Phe455 with Ala (Table I), but the relative activity of this mutant was decreased to about 25%. As described above, I593A mutation led to equal 12/15-lipoxygenation.

Next we tested the ability of the 15-LO to accommodate added bulk to the substrate binding pocket by substituting the same residues with Phe. Since a smaller pocket may favor 15-lipoxygenation, we used the F353L mutant of the rabbit 15-LO which exhibits a major 12-LO activity as template for mutagenesis (22). Replacement of Ile418 with Phe in this 12-lipoxygenating mutant significantly shifted the oxygenation specificity back toward 15-lipoxygenation (Table II). Thus, introduction of a large Phe at position 418 reversed the effect arising from a small Leu at 353. Similarly, Phe at position 419 did compensate for Leu at 353. Here again, the product ratio of 15/12-HETE was almost 75:25 (Table II). These results show that decreasing the size of the arachidonic acid-binding site favored 15-HETE formation whereas increasing it augmented 12-lipoxygenation.

Interestingly, replacement of Ile593 with Phe in the wild-type
Enzyme expression was induced by adding isopropyl-β-D-thiogalactopyranoside (1 mM) and the cultures were grown overnight. The bacteria were spun down, washed with phosphate-buffered saline, and resuspended in 10 mM of fast protein liquid chromatography loading buffer. The cells were sonicated and the lysis supernatant was injected to fast protein liquid chromatography (see "Materials and Methods"). The LO containing fractions were pooled and aliquots were used for oxygenation of arachidonic acid. Activity assays and HPLC analysis were carried out as described under "Materials and Methods."

Site-directed mutagenesis, activity assays and HPLC analysis were performed as described in the legend of Fig. 1. The enzymatic activity is expressed as micrograms of HETE formation/ml culture fluid for 15 min incubation with arachidonic acid.

| Enzyme species | Enzymatic activity | Positional specificity |
|----------------|--------------------|-----------------------|
|                | µg HETE/ml 15 min  | 15-HETE/12-HETE ratio in % |
| Wild-type      | 5.8                | 97/3                  |
| Q417A          | 5.2                | 88/12                 |
| I418A          | 5.3                | 89/92                 |
| M419A          | 1.8                | 88/12                 |
| F353A          | 1.3                | 20/80                 |
| I593A          | 6.0                | 55/45                 |

Table I
Alanine scan of the sequence determinants for the positional specificity of rabbit 15-LO

Site-directed mutagenesis, activity assays and HPLC analysis were performed as described in the legend of Fig. 1. The enzymatic activity is expressed as micrograms of HETE formation/ml culture fluid for 15 min incubation with arachidonic acid.

To obtain independent evidence for the functional interaction of the sequence determinants, we employed a triple mutagenesis strategy. As indicated above, the F353L mutant of rabbit 15-LO creates an enzyme species that formed significant amounts of 12-lipoxygenase product (12-15-H(P)ETE ratio of 29:71 compared with the wild-type enzyme ratio of 3:97) and the possible mechanistic reasons for this unexpected behavior will be discussed below. In this context, it was not surprising that introduction of phenylalanine at 593 in the F353L mutant augmented the share of 12-LOX products (Table II).

To determine the extent to which the volume of the substrate binding pocket can be increased, we combined the single Ala mutations at positions 353, 418, and 593. The background for this experiment came from the hypothesis that 15-LOs might be converted to 5-lipoxygenating enzyme species. However, the active triple mutant of the rabbit 15-LO converted arachidonic acid almost exclusively to 12-H(P)ETE and the enzyme activity remained unchanged (Table III). However, the activity of the triple mutant of the rabbit 15-LO was rather low (about 15% of the wild-type activity). The three amino acid residues that were altered contact a total of 22 side chain atoms. It is possible that these changes have caused significant perturbations in the protein structure, thus changing the structure of the active site, and strongly impairing catalysis. Not surprisingly, the quadruple mutant F353L/I418A/M419A/I593A had no detectable activity. Similar results were obtained when residue 403 was included in the mutagenesis strategy. Position 403 is Arg in 15- and 12-LOs, but Lys in 5-LOs. The greater flexibility of Lys was suggested to be important for a deeper binding of arachidonic acid (15), as it may be the case in 5-LOs. Previous mutagenesis of Arg to Lys in the human 15-LO did not affect the activity of the enzyme (14). We substituted Lys for Arg and created the following mutants: F353L/I418A/M419A/R403K and F353L/I418A/M419A/I593A/R403K. Again, no substrate oxygenation was detectable, although the mutant enzymes were expressed at similar levels as the wild-type species (immunoblots not shown). These results suggest that there is a limit for allowable manipulations in the substrate-binding site of the rabbit 15-LO. This enzyme obviously tolerates enlargements of the binding pocket to a certain extent creating an active 12-LO. A more dramatic increase in the volume of the binding pocket leads to poorly registered substrate and, thus, to an impaired catalytic activity. It is important to note that we never observed the formation of significant amounts of chiral 5S-HETE in any of the

FIG. 1. Alteration of the positional specificity of the rabbit reticulocyte-type 15-LO by I593A mutation. Site-directed mutagenesis was performed by the polymerase chain reaction-overlap extension technique using mismatching synthetic oligonucleotides as described under "Materials and Methods." 500-ml bacterial cultures were grown up to an optical density at 600 nm of about 0.5. Enzyme expression was induced by adding isopropyl-β-D-thiogalactopyranoside (1 mM) and the cultures were grown overnight. The bacteria were spun down, washed with phosphate-buffered saline, and resuspended in 10 mM of fast protein liquid chromatography loading buffer. The cells were sonicated and the lysis supernatant was injected to fast protein liquid chromatography (see "Materials and Methods"). The LO containing fractions were pooled and aliquots were used for oxygenation of arachidonic acid. Activity assays and HPLC analysis were carried out as described under "Materials and Methods."

Enlargement of the Arachidonic Acid-binding Site by Multiple Amino Acid Replacements—To determine the extent to which the volume of the substrate binding pocket can be increased, we combined the single Ala mutations at positions 353, 418, and 593. The background for this experiment came from the hypothesis that 15-LOs might be converted to 5-lipoxygenating enzyme species if one enlarges the 15-LO pocket sufficiently. The 15-LO double mutants F353L/I593A and F353A/I418A converted arachidonic acid almost exclusively to 12-H(P)ETE and the enzyme activity remained unchanged (Table III). However, the activity of the triple mutant F353A/I418A/I593A was rather low (about 15% of the wild-type activity). The three amino acid residues that were altered contact a total of 22 side chain atoms. It is possible that these changes have caused significant perturbations in the protein structure, thus changing the structure of the active site, and strongly impairing catalysis. Not surprisingly, the quadruple mutant F353L/I418A/M419A/I593A had no detectable activity. Similar results were obtained when residue 403 was included in the mutagenesis strategy. Position 403 is Arg in 15- and 12-LOs, but Lys in 5-LOs. The greater flexibility of Lys was suggested to be important for a deeper binding of arachidonic acid (15), as it may be the case in 5-LOs. Previous mutagenesis of Arg to Lys in the human 15-LO did not affect the activity of the enzyme (14). We substituted Lys for Arg and created the following mutants: F353L/I418A/M419A/R403K and F353L/I418A/M419A/I593A/R403K. Again, no substrate oxygenation was detectable, although the mutant enzymes were expressed at similar levels as the wild-type species (immunoblots not shown). These results suggest that there is a limit for allowable manipulations in the substrate-binding site of the rabbit 15-LO. This enzyme obviously tolerates enlargements of the binding pocket to a certain extent creating an active 12-LO. A more dramatic increase in the volume of the binding pocket leads to poorly registered substrate and, thus, to an impaired catalytic activity. It is important to note that we never observed the formation of significant amounts of chiral 5S-HETE in any of
Site-directed mutagenesis, activity assays, and HPLC analysis were carried out as described in the legend of Fig. 1.

**DISCUSSION**

The positional specificity of fatty acid oxygenation by LOs is not an invariant enzyme property but depends on several factors, such as structure of LO substrate and/or reaction conditions (24, 25). The alignment of the substrate fatty acid at the active site and its spatial relation to the non-heme iron are crucial for the specificity of the oxygenase reaction. Although no direct experimental data on the enzyme/substrate interaction are currently available, experiments with modified LO substrates (13), structural modeling (15, 26, 27), and site-directed mutagenesis studies (17–22) provided insight into how substrate alignment occurs at the active site. Molecular modeling based on the three-dimensional structure of mammalian 15-LO suggested that the size of the substrate binding pocket may be important for the positional specificity (15) and indeed most mutagenesis data supported this hypothesis. 12-LOs have a substrate binding pocket which is predicted to be only slightly larger than that of 15-LO. By introducing small residues into the 15-LO pocket, this enzyme can easily be converted to a 12-lipoxygenating enzyme. Replacement of Ile residue and Met at positions 418 and 419 with Ala did not alter the 15/12-HETE product ratio of arachidonic acid oxygenation. In contrast to the substitution of branched amino acid side chains, replacement of the flexible side chain did not change product specificity. This result presumably reflects less influence of an unconstrained amino acid side chain in defining the overall volume of the substrate-binding site. It was somewhat surprising that substitution of Met with Ala did not have a significant effect on the positional specificity of the rabbit 15-LO, in particular because previous double mutations with Ile and Met of the human enzyme had suggested that this residue may play a role in determining product specificity (17). Substitution of the Gln side chain, which does not line the substrate-binding site, had no effect on the specificity of the oxygenase reaction.

Replacement of either Gln or with Ala did not alter the 15/12-HETE product ratio of arachidonic acid oxygenation. In contrast to the substitution of branched amino acid side chains, replacement of the flexible side chain did not change product specificity. This result presumably reflects less influence of an unconstrained amino acid side chain in defining the overall volume of the substrate-binding site. It was somewhat surprising that substitution of Met with Ala did not have a significant effect on the positional specificity of the rabbit 15-LO, in particular because previous double mutations with Ile and Met of the human enzyme had suggested that this residue may play a role in determining product specificity (17). Substitution of the Gln side chain, which does not line the substrate-binding site, had no effect on the specificity of the oxygenase reaction.

We also applied an opposite mutagenesis strategy, decreasing the pocket size of 15-LO by introducing Phe at critical positions. To do this, we first mutated Phe to Leu and used the resulting 12-lipoxygenating enzyme as the template for the phenylalanine scan. Introduction of Phe at either position 418 or 419 regained 15-LO activity. Thus, one may suggest that in general a 15-LO can be converted to a 12-LO by introducing small residues at the base of the pocket, whereas a 12-LO can become more like a 15-LO by incorporating large residues. In contrast to this general observation, replacement of residue 593 with Phe (either alone or in combination with F353L) produced mutant enzymes forming increased shares of 12-HETE. Modeling of the three-dimensional structure of the mutants suggests that most reasonable rotamer for a phenylalanine at this position would point the side chain in defining the overall volume of the substrate-binding site. It was somewhat surprising that substitution of Met with Ala did not have a significant effect on the positional specificity of the rabbit 15-LO, in particular because previous double mutations with Ile and Met of the human enzyme had suggested that this residue may play a role in determining product specificity (17). Substitution of the Gln side chain, which does not line the substrate-binding site, had no effect on the specificity of the oxygenase reaction.

Replacement of either Gln or with Ala did not alter the 15/12-HETE product ratio of arachidonic acid oxygenation. In contrast to the substitution of branched amino acid side chains, replacement of the flexible side chain did not change product specificity. This result presumably reflects less influence of an unconstrained amino acid side chain in defining the overall volume of the substrate-binding site. It was somewhat surprising that substitution of Met with Ala did not have a significant effect on the positional specificity of the rabbit 15-LO, in particular because previous double mutations with Ile and Met of the human enzyme had suggested that this residue may play a role in determining product specificity (17). Substitution of the Gln side chain, which does not line the substrate-binding site, had no effect on the specificity of the oxygenase reaction.

We also applied an opposite mutagenesis strategy, decreasing the pocket size of 15-LO by introducing Phe at critical positions. To do this, we first mutated Phe to Leu and used the resulting 12-lipoxygenating enzyme as the template for the phenylalanine scan. Introduction of Phe at either position 418 or 419 regained 15-LO activity. Thus, one may suggest that in general a 15-LO can be converted to a 12-LO by introducing small residues at the base of the pocket, whereas a 12-LO can become more like a 15-LO by incorporating large residues. In contrast to this general observation, replacement of residue 593 with Phe (either alone or in combination with F353L) produced mutant enzymes forming increased shares of 12-HETE. Modeling of the three-dimensional structure of the mutants suggests that most reasonable rotamer for a phenylalanine at this position would point the side chain away from the substrate-binding site and therefore I593F substitution could create a larger pocket.

In order to engineer an effective arachidonate 5-LO, we attempted to increase the volume of the substrate-binding site as much as possible. A combination of Ala substitutions in the rabbit 15-LO (double mutations F353A/I418A and F353I/I593A) was tolerated by the enzyme without impaired activity and resulted in arachidonic acid 12-lipoxygenation. However, when we changed residues Phe, Ile, and Leu to Ala, the resulting triple mutant exhibited only a residual activity of

Table II: Phenylalanine scan of sequence determinants for the positional specificity of the rabbit 15-LO

| Amino acid at position | Enzymatic activity | Positional specificity |
|------------------------|-------------------|------------------------|
|                        | µg HETE/ml 15 min | 15-HETE/12-HETE %   |
| 353 Leu                 | 23                | 28/72                 |
| 353 Phe                 | 5.8               | 97/3                  |
| 418 Leu                 | 3.9               | 75/25                 |
| 418 Phe                 | 7.0               | 73/27                 |
| 419 Leu                 | 1.8               | 21/79                 |
| 419 Met                 | 5.8               | 97/3                  |
| 593 Leu                 | 3.9               | 75/25                 |
| 593 Phe                 | 7.0               | 73/27                 |
| 593 Met                 | 1.8               | 21/79                 |

**FIG. 2. Multiple active site mutations in rabbit 15-LO.** Switching positional specificity from 15- to 12-LO and vice versa. Site-directed mutagenesis, activity assays, and HPLC analysis were carried out as described in the legend to Fig. 1.

The mutants studied, suggesting that other features may contribute to defining the positional specificity of 5-LO.
about 15%. This result may suggest that the pocket became too large to properly align the fatty acid substrate. In addition, we replaced Arg\textsuperscript{403} with Lys in several mutants having a large binding pocket, because Arg\textsuperscript{403} was suggested to interact with the substrate’s carboxylate and Lys may provide a higher degree of flexibility which may be crucial for a deep arachidonic acid binding. Unfortunately, none of these mutants formed significant amounts of chiral 8- or 5-HETE. All multiple mutations beyond the triple F353A/I418A/I593A mutant were inactive. These data illustrate that excessive re-engineering of the lipoxygenase active site may not be possible without a severe loss in the functionality of the enzyme. We conclude that the volume of the substrate binding pocket is not the only parameter to be considered in creating a 5-lipoxygenating enzyme from a 15-LO.

Currently, there are two hypotheses which rationalize the positional specificity of 5-LO. (i) The conserved orientation hypothesis suggesting that arachidonic acid substrate is bound in the same orientation in all lipoxygenase isoforms. The product specificity is determined by the volume of the substrate binding pocket. (ii) The inverse orientation hypothesis (27–30) according to which there may be an inverse head to tail substrate alignment for effective 5S-lipoxygenation. With this hypothesis the stereochemistry of 5S-lipoxygenation can easily be explained but the most compelling argument against it is that substrate binding may be severely hampered by the large energy barrier associated with burying the polar carboxylate in the hydrophobic environment of the substrate binding pocket (15). This energy barrier should be reflected by a strongly increased $K_m$ for arachidonic acid, but basic kinetic characterization revealed similar $K_m$ values for 5- and 15-LOs (31, 32). An inverse substrate orientation would be energetically favored, if a charged amino acid was present in the substrate binding pocket. Molecular modeling of the enzyme/substrate interaction of the human 5-LO suggested that the closest potential positive charge would be His\textsuperscript{354} which is located at a distance of more than 6 Å from the position of the modeled substrate carboxylate group and this distance is too large for an effective interaction (16). However, it should be stressed that no structural data on 5-LOs are currently available and that modeling studies must be interpreted with care.

For the time being, site-directed mutagenesis influencing the positional specificity of arachidonic acid oxygenation has mainly been carried out on mammalian 12- and 15-LOs. We recently studied potential sequence determinants for the specificity of linoleic acid oxygenation by the cucumber lipid body LO and found that mutation of His\textsuperscript{608}, which aligns with Met\textsuperscript{419} of the rabbit enzyme, to a Val effectively converted the linoleic acid 13-LO to a 9-lipoxygenating species (33). Here an inverse head to tail substrate orientation may be possible (33). Moreover, we found that mutation of the corresponding amino acids of the human 5-LO altered the positional specificity toward arachidonic acid 8-lipoxygenation. However, more work is needed to obtain detailed information on sequence determinants for the positional specificity of this pharmacologically most relevant mammalian LO isomerase.
11. Hay, D. W., Torphy, T. J., and Undem, B. J. (1995) *Trends Pharmacol. Sci.* **16**, 304–309
12. Lewis, R. A., Austen, A. F., and Soberman, R. J. (1990) *N. Engl. J. Med.* **323**, 645–655
13. Kuhn, H., Sprecher H., and Brash, A. R. (1990) *J. Biol. Chem.* **265**, 16300–16305
14. Gan, Q. F., Browner, M. F., Sloane, D. L., and Sigal, E. (1996) *J. Biol. Chem.* **271**, 25412–25418
15. Gillmor, S. A., Villasenor, A., Fletterick, R., Sigal, E., and Browner, M. F. (1997) *Nat. Struct. Biol.* **4**, 1003–1009
16. Browner, M., Gillmor, S. A., and Fletterick, R. (1998) *Nat. Struct. Biol.* **5**, 179
17. Sloane, D. L., Leung, R., Craik, C. S., and Sigal, E. (1991) *Nature* **354**, 149–152
18. Sloane, D. L., Leung, R., Barnett, J., Craik, C. S., and Sigal, E. (1995) *Protein Eng.* **8**, 275–282
19. Chen, X. S., and Funk, C. D. (1993) *FASEB J.* **7**, 694–701
20. Suzuki, H., Kishimoto, K., Yoshimoto, T., Yamamoto, S., Kanai, P., Ebina, Y., Miyatake, A., and Tanabe, T. (1994) *Biochim. Biophys. Acta* **1210**, 308–316
21. Watanabe, T., and Haeggstrom, J. Z. (1993) *Biochem. Biophys. Res. Commun.* **192**, 1023–1029
22. Borngraber, S., Kuban, R. J., Anton, M., and Kuhn, H. (1996) *J. Mol. Biol.* **264**, 1145–1153
23. Kuhn, H., Thiele, R. J., Ostarreck-Lederer, A., Stender, H., Suzuki, H., Yoshimoto, T., and Yamamoto, S. (1993) *Biochim. Biophys. Acta* **1168**, 73–78
24. Gardner, H. W. (1989) *Biochim. Biophys. Acta* **1001**, 274–281
25. Prigge, S. T., Boyington, J. C., Gaffney, B. J., and Amzel, L. M. (1996) *Proteins* **24**, 275–291
26. Funk, C. D., and Loll, P. J. (1997) *Nat. Struct. Biol.* **4**, 966–968
27. Prigge, S. T., Gaffney, B. J., and Amzel, L. M. (1998) *Nat. Struct. Biol.* **5**, 178–179
28. Egmond, M. R., Veldink, G. A., Vliegenthart, J. F. G., and Boldingh, J. (1973) *Biochem. Biophys. Res. Commun.* **54**, 1178–1184
29. Kuhn, H., Schewe, T., and Rapoport, S. M. (1986) *Adv. Enzymol.* **58**, 273–311
30. Lehmann, W. D. (1994) *Free Rad. Biol. Med.* **16**, 241–253
31. Ludwig, P., Holzhutter, H. G., Colosimo, A., Silvestrini, M. C., Schewe, T., and Rapoport, S. M. (1987) *Eur. J. Biochem.* **168**, 325–337
32. Aharony, D., and Stein, R. L. (1986) *J. Biol. Chem.* **261**, 11512–11519
33. Hornung, E., Walthner, M., Kuhn, H., and Feussner, I. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 4192–4197