ω -Oxidation impairs oxidizability of polyenoic fatty acids by 15-lipoxygenases: consequences for substrate orientation at the active site

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During oxygenation by 15-lipoxygenases, polyenoic fatty acids are bound at the active site in such a way that the ω -terminus of the fatty acids penetrates into the substrate binding pocket. In contrast, for arachidonic acid 5-lipoxygenation, an inverse head to tail orientation has been suggested. However, an inverse orientation may be hindered by the large energy barrier associated with burying the charged carboxylate group in the hydrophobic environment of the substrate binding cleft. We studied the oxygenation kinetics of ω -modified fatty acids by 15-lipoxygenases and found that ω -hydroxylation strongly impaired substrate affinity (higher K_m), but only moderately altered V_{max} . In contrast, ω -carboxylation completely prevented the lipoxygenase reaction; however, methylation of the additional carboxylate group restored the activity. Arg⁴⁰³ of the human 15-

lipoxygenase has been implicated in fatty acid binding by forming a salt bridge with the carboxylate group, and thus mutation of this amino acid to an uncharged residue was supposed to favour an inverse substrate orientation. The prepared Arg⁴⁰³ → Leu mutant of the rabbit 15-lipoxygenase was found to be a less effective catalyst of linoleic acid oxygenation. However, the oxygenation rate of ω -hydroxyarachidonic acid was similar when the wild-type and mutant enzyme were compared, and the patterns of oxygenation products were identical for both enzyme species. These data suggest that introduction of a polar, or even charged residue, at the ω -terminus of substrate fatty acids in connection with mutation of Arg⁴⁰³ may not alter substrate alignment at the active site of 15-lipoxygenases.

INTRODUCTION

According to the currently used nomenclature, lipoxygenases (LOXs) are classified with respect to their positional specificity of arachidonic acid oxygenation into 5- [1], 8- [2], 11- [3], 12- [4] and 15-LOXs [5]. Arachidonate 12-LOXs may be sub-classified into 2 groups: platelet-type 12-LOXs and leucocyte-type enzyme [6,7]. However, in mice there is an additional epidermal-type 12-LOX [8]. Similarly, arachidonate 15-LOXs may be differentiated into reticulocyte- [9] and epidermis-type enzymes [10]. Although the positional specificity of arachidonic acid constitutes the decisive parameter for LOX nomenclature, the mechanistic reasons for this enzyme property are not well understood. Earlier experiments with arachidonic acid isomers, suggested that optimal alignment of the substrate at the active site appears to be a precondition for a high reaction rate and for singular positional specificity [11,12]. These studies led to the development of a topological model of the substrate alignment at the active site of LOXs [12]. Recently, crystallographic X-ray structures of two plant LOXs [13–15] and of a rabbit reticulocyte 15-LOX/inhibitor complex [16] have been published. These structural data, the results of site-directed mutagenesis studies [17–19] and computer-assisted modelling [20], led to a more comprehensive understanding of enzyme–substrate interaction [21]. For the human reticulocyte-type 15-LOX a three point interaction between the enzyme and the substrate fatty acid has been suggested [18]. First, the methyl-terminus of the fatty acid substrate may interact with amino acid residues 353, 418 and 419, which form the bottom of the substrate binding cleft. The bulkiness of these residues appears to determine how deep the substrate may

penetrate into the substrate binding pocket. Secondly, the negatively charged carboxylate group of the fatty acid appears to form a salt-bridge with the positively charged Arg⁴⁰³. Mutation of this residue to an uncharged leucine resulted in an enzyme species which was less effective as a catalyst, whereas the Arg⁴⁰³ → Lys mutation had no major effect [18]. Thirdly, the aromatic ring of Phe⁴¹⁴ may interact with the double bonds of the substrates via π - π electron interaction fixing the middle part of the substrate at the active site.

The substrate alignment at the active site of mammalian 5-LOXs has recently become a matter for discussion [22,23] and at present there are two hypotheses which rationalize the positional specificity of these LOX isoforms. Firstly, the single orientation theory suggests that all lipoxygenases show a conserved ‘15-LOX-like’ substrate orientation [16]. According to this hypothesis, the methyl-end of the fatty acid substrate penetrates into the substrate binding pocket for all LOX isoforms and the positional specificity of the enzymes depends on the volume of the substrate binding cavity. Secondly, the inverse orientation hypothesis, according to which the substrate may penetrate into substrate binding cleft with its carboxylate group [24,25]. The most compelling rationale for a single, conserved substrate orientation derives from the energetic penalty associated with burying a charged carboxylate group in a hydrophobic substrate binding pocket [23]. For bacteriophage T4-ligase an energy penalty of 12–30 kJ/mol was calculated for burying a charged amino acid in the hydrophobic core of the enzyme [23,26].

There are no experimental data available to date, determining whether LOXs tolerate polar or charged fatty acid residues inside the hydrophobic substrate binding cavity. To approach this

Abbreviations used: (15S)-HPETE, (15S)-hydroperoxy-5,8,11,13-eicosatetraenoic acid; LOX(s), lipoxygenase(s).

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problem various ω -hydroxylated and ω -carboxylated fatty acids were synthesized and tested as substrates for wild-type arachidonic acid 15-LOXs and for a mutant enzyme species in which the positively charged Arg⁴⁰³, apparently involved in substrate binding, was changed to a neutral leucine. Our data indicated that although the ω -hydroxylated fatty acids were well oxygenated by the wild-type and mutant 15-LOXs, substrate binding was severely hampered. In contrast, dicarboxylic fatty acids were found to be poor substrates, suggesting steric hindrance of the LOX reaction by a second charged carboxylate group.

MATERIALS AND METHODS

Chemicals

Soybean LOX-1 (grade I) and bis(trimethylsilyl)trifluoroacetamide were from Serva; arachidonic acid, linoleic acid, and sodium borohydride were from Merck; *N*-nitroso-*N*-methylurea was from Sigma; 2,2'-azobis-(2-methylpropionamidine) dihydrochloride was from Aldrich and ω -hydroxyarachidonic acid was from Cayman Chemicals. The other modified fatty acids used as substrates for LOX were synthesized in the laboratories of the Moscow Academy of Fine Chemical Technology [27]. All solvents were of HPLC grade and were purchased from Merck.

Preparations

Rabbit 15-LOX was purified from rabbit reticulocyte-rich blood cell suspensions as described previously [28]. For structural elucidation of LOX products, the hydroperoxy fatty acids formed were reduced by the addition of a molar excess of sodium borohydride. The samples were then acidified to pH 3 and the lipids were extracted with 2 volumes of ethyl acetate. The solvent was evaporated, the remaining lipids were reconstituted in 0.2 ml of methanol and aliquots were analysed by HPLC. In order to prepare racemic mixtures of fatty acid oxygenation products, the substrate fatty acids (final concentration 800 μ M) were emulsified in 1 ml of 0.1 M sodium phosphate buffer, pH 7.4. 2,2'-Azobis-(2-methylpropionamidine) dihydrochloride (50 mM final concentration) was then added and the mixture was incubated for 1 h at 37 °C. After acidification and lipid extraction, the positional isomers of the oxygenation products were separated by straight-phase HPLC and their enantiomer composition was determined by chiral-phase HPLC. Methylation of the free hydroxy fatty acids was carried out with diazomethane in ether. Hydroxy groups were silylated by reacting with bis(trimethylsilyl)trifluoroacetamide in pyridine.

Assays for LOX activity

LOX activity was determined spectrophotometrically by measuring the time-dependent increase in absorbance at 235 nm using a Shimadzu UV-160A spectrophotometer at 15 °C. The assay mixtures were prepared by adding 10 μ l of a methanolic stock solution of the fatty acid substrates to 1 ml of either 0.1 M sodium phosphate buffer, pH 7.4, containing 0.2 % (w/v) sodium cholate (for the rabbit enzyme), or 0.1 M borate buffer, pH 9.0 (for the soybean enzyme). The assay mixture (final substrate concentration 200 μ M) was then sonicated with a Braun tip-sonifier for 20 s. The reaction was started by the addition of 10 μ l of LOX solution containing either 1 mg/ml soybean LOX-1 or 0.5 mg/ml rabbit reticulocyte 15-LOX.

HPLC and GC/MS analysis

Reverse-phase HPLC was carried out on a Shimadzu LC-6A liquid chromatograph connected to a Hewlett-Packard 1040A HPLC-diode array detector. Separation of the fatty acid derivatives was performed on a Nucleosil C-18 column (Macherey-Nagel, Düren, Germany; 250 $\times$ 4 mm, 5 μ m particle size) coupled to a guard-column (30 $\times$ 4 mm, 5 μ m particle size). A solvent system of methanol/water/acetic acid (850:150:1, by vol.) and a flow rate of 1 ml/min were used. Products characterized by a conjugated-diene chromophore were collected, the solvent evaporated and the residues reconstituted in *n*-hexane. Straight-phase HPLC was performed on a Shimadzu LC-9A liquid chromatograph connected to a SPD M 6A photodiode array UV-VIS detector. The hydroxy fatty acid isomers were analysed on a Nucleosil 100-7 column (Macherey-Nagel, Düren, Germany; 250 $\times$ 4 mm, 5 μ m particle size) using the solvent system hexane/isopropanol/acetic acid (1000:100:1, by vol.) and a flow rate of 1 ml/min. Chiral-phase HPLC was performed on a Chiralcel OD column (purchased from Baker, Deventer, The Netherlands; 250 $\times$ 5 mm, 5 μ m particle size) using the solvent system hexane/isopropanol/acetic acid (1000:100:1, by vol.) and a flow rate of 1 ml/min.

For GC-MS analysis a Shimadzu GC-MS QP-2000 system equipped with a capillary column (12 m $\times$ 0.3 mm; coating thickness 0.33 μ m) was used. An injector temperature of 270 °C, ion source temperature of 180 °C and an electron energy of 70 eV were used. The derivatized hydroxylated fatty acids were eluted isothermally at 180 °C.

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. The R403L (Arg⁴⁰³ $\rightarrow$ Leu) substitution was carried out with the PCR mediated overlap extension technique using mismatching synthetic oligonucleotides. The cDNA construct was sequenced to verify the primary structures. Bacteria transformed with the mutant plasmid expressed the mutant 15-LOX; however, the level of expression was not sufficient for a large scale enzyme preparation. Thus we decided to overexpress the mutant enzyme in the baculovirus/insect cell system. The mutant 15-LOX cDNA was excised from the pKK 233-2 vector and sub-cloned into the baculovirus transfer vector pVL1393. The resulting recombinant transfer plasmid pVL-LOX-L403 was amplified in *Escherichia coli*. Wild-type *Autographa californica* nuclear polyhedrosis virus DNA (1 μ g) was mixed with 2 μ g of the pVL-LOX-L403 transfer plasmid and co-transfected into *Trichoplusia ni* insect cells (HighFive[™], Invitrogen, San Diego, CA, U.S.A.) using the lipofectin procedure. Recombinant baculovirus clones were isolated and purified by 2–3 rounds of viral plaque purification and viral stocks with titres of about 3×10^8 plaque-forming units/ml were produced. HighFive[™] cells were cultured in spinner flasks at 27 °C. Cells were grown in Grace's medium supplemented with 10 % (v/v) heat-inactivated fetal-calf serum and penicillin/streptomycin (final concentration 100 μ g/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 Bistris buffer, pH 6.8, containing 2 mM EDTA and the proteinase inhibitors PMSF (40 μ g/ml), leupeptin (1 μ g/ml) and pepstatin A (1 μ g/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 injected directly onto a semipreparative Mono Q HR 10/10 FPLC column. Proteins were eluted with a step gradient of sodium chloride as described previously [28]. Fractions (2 ml) were collected and the 15-lipoxygenase activity was assayed spectrophotometrically. The resulting enzyme preparation was approx. 70 % pure and exhibited a linoleic acid oxygenase turnover rate of 1.6 s⁻¹.

Calculation of the energy barriers for binding and lipoxygenation of ω -modified fatty acids

Differences in the basic kinetic parameters (K_m and V_{max}) for lipoxygenation of linoleic acid and its ω -modified derivatives can be translated into energy barriers by making the assumption that the lipoxygenase reaction follows the energy profile of an irreversible two-step catalytic mechanism [29]. The energy barriers for substrate binding (ΔG_1) and lipoxygenation (ΔG_2) can be related to the three elementary rate constants k_1 (formation of the enzyme-substrate complex), k_{-1} (decomposition of the enzyme-substrate complex) and k_2 (catalysis) by the following equations:

$$K_m \approx \frac{k_{-1}}{k_1} = \frac{k_r}{k_d} \exp\left(\frac{\Delta G_1}{RT}\right) \quad (1)$$

and:

$$k_2 = k_m \exp\left(-\frac{\Delta G_2}{RT}\right) \quad (2)$$

Here k_r , k_d and k_m represent the maximal values for the rate constants depending on the diffusion coefficient of the ligand and the viscosity of the solvent [29]. From eqn. (1) and eqn. (2) the changes in the energy barriers for binding and lipoxygenation of two different fatty acid substrates can be estimated according to:

$$\Delta(\Delta G_1) = \Delta G_1^* - \Delta G_1 = RT \ln\left(\frac{K_m^*}{K_m}\right) \quad (3)$$

and:

$$\Delta(\Delta G_2) = \Delta G_2^* - \Delta G_2 = RT \ln\left(\frac{k_2^*}{k_2}\right) \quad (4)$$

where * indicates the kinetic values of the ω -modified fatty acids.

RESULTS

15-Lipoxygenase-catalysed oxygenation of ω -modified polyenoic fatty acids

To determine whether the introduction of polar residues at the methyl-terminus of polyenoic fatty acids influences the LOX reaction, we compared basic kinetic parameters (K_m and V_{max}) for the oxygenation of various linoleic acid derivatives by different 15-LOXs. From Table 1 it can be seen that under our experimental conditions, K_m and V_{max} for linoleic acid oxygenation were comparable for the 15-LOXs from soybean (isoenzyme-1) and rabbit reticulocytes. Introduction of a polar hydroxy group at the methyl-terminus resulted in a large decrease in substrate affinity (9-fold higher K_m for the soybean LOX and 25-fold higher K_m for the rabbit reticulocyte enzyme) but only a moderate decrease in V_{max} . When the polar ω -hydroxy group was oxidized to a charged carboxylate group, the dicarboxylic fatty acid was not accepted as substrate by either of the two enzymes.

Table 1 Kinetic parameters for the oxygenation of modified polyenoic fatty acids by the arachidonate 15-LOXs from rabbit reticulocytes and soybeans

The LOXs were incubated with the different substrates under standard assay conditions as described in the Materials and methods section. The substrate concentration varied between 5 and 200 μ M and the oxygenation was assayed spectrophotometrically by measuring the increase in absorbance at 235 nm. K_m and V_{max} were determined from Lineweaver-Burk plots and each data point given in the Table represents 5–7 activity assays. The relative energy barriers in relation to linoleic acid oxygenation, given in kJ/mole, are in parenthesis. * Monomethylation of dicarboxylic fatty acid was achieved by incubating the free acid with stoichiometric amounts of diazomethane. Monomethylated derivatives were purified by straight-phase HPLC. Monomethylation of the dicarboxylic fatty acids increased the chain length of the substrate from C₁₈ to C₁₉ thus altering the distance of the doubly allylic methylene from the ω -terminus of the substrate. To keep this distance in the same range as in linoleic acid, the monomethylester of 17-carboxy-5(Z), 8(Z)-heptadecadienoic acid was used.

Substrate	Soybean lipoxygenase		Rabbit reticulocyte lipoxygenase	
	V_{max} (μ mol/mg·min)	K_m (μ M)	V_{max} (μ mol/mg·min)	K_m (μ M)
	1.04	10.5	0.95	8.5
	0.87 (0.4)	91.3 (5.4)	0.28 (3.0)	201 (7.8)
	no activity		no activity	
	0.59 (1.4)	1.87 (7.1)	0.36 (2.4)	84.0 (5.7)

Methylation of the ω -carboxylate group restored the LOX activity; however, the K_m values for the monomethylated substrates remained high. GC-MS analysis of the oxygenation products indicated an almost exclusive 13-lipoxygenation of all the substrates listed in Table 1 (results not shown). These data suggest that the ω -hydroxylated fatty acids are aligned at the active site in a similar way to polyenoic fatty acids. We did not obtain any evidence to suggest an inverse 'head-to-tail' substrate orientation. From the K_m and V_{max} values the relative energy barriers for the binding and oxygenation of the ω -hydroxylated fatty acid derivatives (in comparison to linoleic acid oxygenation) were calculated (Table 1). As expected from the kinetic parameters, the energy barrier for both LOXs was higher for substrate binding (ΔG_1) than for the oxygenation reaction (ΔG_2), suggesting that burying the polar hydroxy group in the hydrophobic substrate-binding pocket appears to be more severely hindered than the oxygenation reaction itself.

The introduction of a hydroxy group at the methyl-terminus of linoleic acid not only alters the polarity of this part of the substrate molecule but also increases the distance of the doubly allylic methylene (C-11) from the modified methyl-end. Thus the changes observed in K_m and V_{max} may be due to either of these factors. In order to determine whether the distance of the doubly allylic methylene from the modified methyl-terminus is important for substrate oxygenation, we tested a set of ω -hydroxylated derivatives of linoleic acid differing in the positions of their double bonds (Figure 1). As expected from the data from similar experiments using arachidonic acid isomers [18], the soybean LOX-1 preferred the substrate with a n-8 doubly allylic methylene (highest reaction rate), where n-8 refers to the eighth carbon atom, counting from the methyl end of the fatty acid. A shift of the double-bond system in either direction led to an impaired rate of oxidation. Interestingly, the substrate affinity (K_m values) was not very different for all the substrates tested. The rabbit reticulocyte 15-LOX oxygenated the substrates containing a n-8, n-9 and n-10 doubly allylic methylene with

Figure 1 Oxygenation of various ω -hydroxylated fatty acid isomers by LOXs

The various ω -hydroxylated fatty acid isomers, which differed from each other with respect to the distance of the doubly allylic methylene from the ω -terminus of the substrate (n-7, n-8 etc.), were incubated at different concentrations with soybean LOX-1 (A) and rabbit reticulocyte 15-LOX (B). The oxygenation rates were determined spectrophotometrically by measuring the increase in absorbance at 235 nm. K_m and V_{max} were determined from Lineweaver–Burk plots; each data point represents 5–7 activity assays.

comparable rates. The substrate affinity was augmented with increasing distances of the doubly allylic methylene from the modified methyl-terminus. Surprisingly, the hydroxy fatty acid with the n-7 doubly allylic methylene was not accepted as a substrate by this enzyme and the mechanistic reason for this behaviour remains unclear. HPLC analysis of the reaction products indicated that both enzymes formed an almost identical pattern of oxygenation products. Each substrate was converted into one major early-eluting conjugated diene and a minor, late-eluting product (Figure 2). The UV spectra (inset to Figure 2) of both compounds (the conjugated-diene chromophore of the late eluting peak has an absorbance maximum at 230 nm, whereas the maxima for the early eluting peak is at 235 nm) and the almost identical mass spectra suggest that the early-eluting peaks represent the *cis, trans* isomers and the minor late-eluting peaks represent the *trans,trans* isomers of the oxygenated fatty acids. GC–MS analysis of the major products (Table 2) indicated that oxygen was introduced at the carbon atom which was localized two C-units distant from the site of hydrogen removal, in the direction of the modified methyl-terminus (+2 oxygen insertion [24]). Chiral-phase HPLC of the major products revealed a single peak with all substrates, indicating a high degree of chirality (results not shown). These data suggest that all

Figure 2 HPLC analysis of the major oxygenation products formed from ω -hydroxylated fatty acids

LOXs were incubated with the different ω -hydroxylated fatty acids (see Figure 1) as described in the Materials and methods section. The reaction was stopped by the addition of sodium borohydride, the mixture was acidified to pH 3, and the lipophilic products were extracted twice with an equal volume of ethyl acetate and analysed by straight-phase HPLC (see the Materials and methods section). Partial chromatograms are shown for (A) rabbit reticulocyte 15-LOX and (B) soybean LOX-1. The inset shows the UV-spectra of the 12-hydroxy derivatives formed by the soybean LOX-1 from the C-9 substrate. Similar spectra were recorded for the products formed from the other substrate fatty acids.

ω -hydroxylated fatty acids are aligned at the active site in a ‘linoleic acid-like’ way. Thus introduction of a polar hydroxy group at the methyl-terminus of substrate fatty acids, in connection with alterations in the distance of the doubly allylic methylene from the modified methyl-terminus, did not significantly alter the substrate alignment at the active site.

In order to further increase the polarity of the ω -terminus we synthesized different ω -carboxylic fatty acids (Figure 3) and tested them as 15-LOX substrates. Considering the fact that both lipoxygenases examined prefer fatty acids with a n-8 (soybean) or n-9 (rabbit reticulocyte) doubly allylic methylene, we expected the highest reaction rates with substrates II or III (Figure 3). However, when the enzymes were incubated with the dicarboxylic fatty acids, conjugated-diene-containing products were only formed with substrate V and not with any of the other dicarboxylic fatty acids. Substrate V constitutes a fairly symmetric molecule and thus may be oriented in two different ways at the active site of LOXs. HPLC analysis (Figure 4) of the oxygenation products formed by the two enzymes indicated the formation of two products containing a conjugated-diene chromophore with an absorbance maximum at 234 nm. Interestingly, the late-eluting compound, product (II), was the major oxygenation product formed by the rabbit reticulocyte enzyme, whereas the early-eluting compound, product (I), was dominant for the soybean LOX. For more detailed structural elucidation both compounds were prepared and analysed by GC–MS. The mass spectra (Figure 5) identified product (I) as 20-carboxy, 12-hydro(pero)xy-8,10-eicosadienoic acid and product (II) as 20-carboxy, 8-hydro(pero)xy-9,11-eicosadienoic acid. Analysis of the enantiomer composition of 20-carboxy, 12-hydro(pero)xy-8,10-eicosadienoic acid formed by the soybean

Table 2 GC–MS data of the major oxygenation products formed from ω -hydroxylated fatty acids

LOXs were incubated with the different ω -hydroxylated fatty acids (see Figure 1) as described in the Materials and methods section. The reaction products were prepared as described in the legend of Figure 2 and GC–MS analysis was carried out after suitable derivatization as described in the Materials and methods section. The mass spectra for the rabbit and soybean enzymes were indistinguishable.

Substrate	Products	Key ions observed
$C_{18}:7(Z),10(Z)$	11-OH(<i>Z,E</i>) 11-OH(<i>E,E</i>)	470 ^a , 283 ^b , 289 ^c , 341 ^d
$C_{18}:8(Z),11(Z)$	12-OH(<i>Z,E</i>) 12-OH(<i>E,E</i>)	470 ^a , 297 ^b , 275 ^c , 327 ^d
$C_{18}:9(Z),12(Z)$	13-OH(<i>Z,E</i>) 13-OH(<i>E,E</i>)	470 ^a , 311 ^b , 313 ^d
$C_{18}:10(Z),13(Z)$	14-OH(<i>Z,E</i>) 14-OH(<i>E,E</i>)	470 ^a , 325 ^b , 247 ^c

Figure 3 Structures of the dicarboxylic fatty acids tested as LOX substrates

LOX-1 (Figure 6A) indicated a high degree of chirality. Similar data were obtained for the 20-carboxy, 8-hydro(pero)xy-9,11-eicosadienoic acid formed by the rabbit reticulocyte 15-LOX (results not shown). When both enzymes were adjusted to a comparable linoleic acid oxygenase activity, the rabbit reticulocyte 15-LOX was a more effective catalyst than the soybean LOX-1 (approx. reaction-rate ratio 2:1, rabbit:soybean enzyme; results not shown). When compared with linoleic acid, the oxygenation rate of the dicarboxylic fatty acid V was approx. 1%. From the pattern of oxygenation products one can conclude that this dicarboxylic fatty acid may be aligned at the active site of the rabbit reticulocyte 15-LOX in the same orientation as linoleic acid. In contrast, for the soybean enzyme an inverse orientation may be concluded. Unfortunately, no detailed kinetic studies could be carried out because of the extremely low reaction rates.

Impact of the R403L mutation on positional specificity

Arg⁴⁰³ of the human 15-LOX may be involved in fatty acid binding by forming a salt bridge with the carboxylate group of

Figure 4 HPLC analysis of oxygenation products formed from the dicarboxylic fatty acid substrate V

A 3.6 μ l aliquot from a methanolic stock solution (55 mM) of substrate V (see Figure 3) was mixed with 4 μ l of 53 mM NaOH. Sodium cholate solution [30 μ l, 10% (w/v)] and 963 μ l of phosphate buffer, pH 7.5, were added and the reaction was started with 15 μ l of enzyme solution (1 mg/ml soybean LOX-1 or 0.5 mg/ml rabbit reticulocyte 15-LOX). After 1 h at 18 °C, sodium borohydride was added to reduce the hydroperoxy lipids, the mixture was acidified and the lipophilic products were extracted twice with 1 ml of ethylacetate. The organic phases were combined, the solvent was evaporated, the residue was reconstituted in 0.2 ml of hexane and aliquots were analysed by straight-phase HPLC as described in the Materials and methods section using the solvent system n-hexane/isopropanol/acetic acid (1000:100:1, by vol.). Partial chromatograms are shown for (A) soybean LOX-1, (B) rabbit reticulocyte 15-LOX.

the substrate [16,18]. Thus mutation of this amino acid to an uncharged residue was assumed to favour an inverse substrate orientation. The R403L mutation was carried out on rabbit reticulocyte 15-LOX and it was found that the mutant enzyme, which was purified from the baculovirus/insect cell system, showed an impaired rate of linoleic acid oxygenation (turnover number of 12.6 s⁻¹ for the recombinant wild-type enzyme and 1.6 s⁻¹ for the recombinant R403L mutant). Structural elucidation of the reaction products indicated mainly 13-lipoxygenation of linoleic acid (results not shown). A similar difference in the kinetic characteristics was also observed when arachidonic acid was used as a substrate (results not shown). In contrast to the wild-type 15-LOX, which preferred arachidonic acid over ω -hydroxyarachidonic acid, the R403L mutant oxygenated both arachidonate derivatives with comparable rates (formation of 10 nmol of conjugated dienes/min for arachidonic acid versus 8.3 nmol of conjugated dienes/min for ω -hydroxyarachidonic acid). With both substrates the main oxygenation products were identified as 15-hydro(pero)xy derivatives (Figure 7). Taken together, these data suggest an 'arachidonic acid-like' orientation of ω -hydroxylated fatty acids at the active site of both the wild-type 15-LOX and its R403L mutant.

The product patterns of 15-LOX-catalysed oxygenation of the dicarboxylic fatty acid V (Figure 3) suggested a different orient-

Figure 5 GC-MS analysis of the oxygenation products formed from dicarboxylic fatty acid V

Incubation, product preparation and straight-phase HPLC analysis were carried out as described in the legend to Figure 4. Products I and II were prepared, the carboxylate groups were methylated with diazomethane, the hydroxy groups were silylated and aliquots were analysed by GC-MS as described in the Materials and methods section. (A) The major oxygenation product (II) formed by rabbit reticulocyte 15-LOX. (B) The major oxygenation product (I) formed by soybean LOX-1.

ation of the substrate at the active sites of the soybean LOX-1 and the rabbit reticulocyte enzyme. These data prompted us to study the oxygenation of this substrate by the R403L mutant. When comparable linoleic acid oxygenase activities were adjusted, the oxygenation catalysed by the R403L mutant was approx. one order of magnitude slower than that of the wild-type species (formation of $9.1 \mu\text{g}$ of conjugated dienes/h with the wild-type enzyme versus $0.8 \mu\text{g}$ of conjugated dienes/h with the R403L mutant). However, the pattern of oxygenation products was almost identical for the two enzyme species (results not shown); in both cases dioxygen was introduced at C-8. These data suggest that the R403L mutation may not favour an inverse orientation of the dicarboxylic fatty acid.

DISCUSSION

For most LOXs the stereochemistry of fatty acid oxygenation is well characterized; however, there are no direct experimental

Figure 6 Chiral-phase HPLC of the major oxygenation product (I) formed from substrate V by soybean LOX-1

Incubation, product preparation and straight-phase HPLC analysis were carried out as described in the legend to Figure 4. Product I was prepared by straight-phase HPLC and its enantiomer composition was determined by chiral-phase HPLC as described in the Materials and methods section. (A) Product (I) formed by soybean LOX-1. (B) racemic standard formed by autooxidation of substrate V and subsequent reverse-phase HPLC preparation.

data on the alignment of the substrates at the active site of the enzymes. Co-crystallization of LOXs with substrate fatty acids has not been carried out and it remains unclear whether the orientation of the inhibitor used to crystallize the rabbit 15-LOX mirrors the alignment of fatty acid substrates. For arachidonate 15-lipoxygenation, the methyl-terminus of the fatty acid appears to constitute the signal for initial hydrogen removal [11,12]. In contrast, for the 5-LOX of wheat the distance of the doubly allylic methylene from the carboxylate group appeared to be of higher priority [30], suggesting an inverse 'head-to-tail' orientation of the substrate fatty acid at the active site of this particular LOX isoform. An inverse orientation has also been suggested as the mechanistic reason for (5*S*)-lipoxygenation of (15*S*)-hydroperoxy-5,8,11,13-eicosatetraenoic acid [(15*S*)-HPETE] by 15-LOXs [31,32], for (9*S*)-oxygenation of linoleic acid by soybean LOX-1 at neutral pH [33] and for the oxygenation of linolealaidic acid by the same enzyme [34]. In the present study we attempted to force an inverse substrate orientation by introducing hydrophilic groups at the methyl-terminus of polyenoic fatty acids and by mutating a critical amino acid involved in substrate binding. The introduction of a polar hydroxy group at the methyl-terminus of the fatty acid strongly impaired substrate affinity (lower K_m value) but only resulted in a moderate decrease in V_{max} . These data and the calculated energy barriers (Table 1) indicate that substrate binding is more severely hampered than oxygenation. However, we did not obtain any

Figure 7 Reverse-phase HPLC analysis of the oxygenation products formed during the reaction of the wild-type and mutant rabbit reticulocyte 15-LOXs with ω -hydroxylated arachidonic acid

The wild-type (A) and R403L mutant (B) 15-LOXs of rabbit reticulocytes were incubated with ω -hydroxyarachidonic acid (0.16 mM final concentration) at room temperature for 10 min and the reaction was followed spectrophotometrically. The hydroperoxy derivatives formed were reduced with sodium borohydride, the mixture was acidified, the lipids were extracted and the conjugated dienes formed were analysed by reverse-phase HPLC using the solvent system methanol/water/acetic acid (750:250:1, by vol.). The UV-spectra of the major product formed by the two enzyme species were identical (inset) and we were not able to separate the major oxygenation product into different positional and/or geometric isomers in normal phase HPLC. 15,20-DiHETE, 15,20-dihydroxy-5,8,11,13-eicosatetraenoic acid.

evidence to suggest an inverse 'head-to-tail' orientation for the modified substrates, even after mutagenesis of Arg⁴⁰³.

It should be stressed that our data do not exclude the possibility that with other substrates, and/or with other LOX isoforms, an inverse substrate orientation may occur. The 15-LOXs from soybeans and rabbit reticulocytes are capable of oxygenating (15*S*)-HPETE to a mixture of double oxygenation products which includes (5*S*,15*S*)-dihydroxy-6,8,11,13-eicosatetraenoic acid [(5*S*,15*S*)-DiHETE] [31,32]. In other words, with this substrate both enzymes can catalyse (5*S*)-lipoxygenation. At the present time there are two hypotheses which rationalize the positional specificity of 5-lipoxygenation [22,23]: firstly, the single orientation theory, and secondly, the inverse orientation hypothesis. According to the single orientation hypothesis the volume of the substrate binding cavity may be decisive for the positional specificity of LOXs. 15-LOXs have a small cavity and thus polyenoic fatty acids are oxygenated close to the methyl-terminus [16,21]. In contrast, more space may be available in the substrate binding pocket of 5-LOXs thus allowing the fatty acid to slide farther into the cavity which may then lead to oxygenation close to the carboxylate-terminus [16,21]. Model calculations suggest that the substrate binding cavity of mammalian 5-LOXs is approx. 20% larger than those of 15-LOXs [16]. If one applies this postulate to 15-LOX-catalysed (5*S*)-lipoxygenation of (15*S*)-HPETE, additional assumptions must be made in order to explain the experimental data. Firstly, binding of (15*S*)-HPETE at the active site may induce conformational changes in the enzyme which may lead to an increase in the volume of the

substrate binding cavity. Although such an induced fit cannot be ruled out, it appears unlikely that it would lead to a 20% increase in the volume of the substrate binding cavity. Secondly, (15*S*)-HPETE may adopt a more 'condensed' configuration at the active site than arachidonic acid. In this case the substrate would require less volume for binding and may slide farther into the binding pocket thus favouring 5-lipoxygenation. To address this point we carried out computer simulations of (15*S*)-H(P)ETE binding at the active site of the rabbit reticulocyte 15-LOX using the X-ray co-ordinates of the enzyme [16]. However, we failed to pinpoint a thermodynamically-most-probable configuration for the substrate because of the flexibility of the substrate molecule. On the other hand, the inverse orientation hypothesis can explain the experimental data for 15-HPETE oxygenation without the need for additional major assumptions. The low binding affinities of (15*S*)-HPETE for the 15-LOXs of soybeans (K_m of 440 μ M versus 8.5 μ M for arachidonic acid) and of rabbit reticulocytes (K_m of 95 μ M versus 5 μ M for arachidonic acid), may account for the energetic barrier associated with burying the charged carboxylate group in the hydrophobic environment of the substrate binding pocket.

With our ω -modified fatty acid derivatives we did not find any evidence to suggest an inverse 'head-to-tail' substrate orientation. However, for (5*S*)-oxygenation of 15-HPETE such an orientation may be possible. Since both 15-HPETE and the ω -modified substrates contain a polar residue at, or close to, their methyl-terminus, a similar binding behaviour may be expected. On the other hand, there is an important difference in the chemical structure of both types of LOX substrates which has to be considered for the interpretation of the experimental data. In contrast to the ω -modified fatty acids 15-HPETE does not contain a *n*-8 doubly allylic methylene. Consequently, the majority of 15-HPETE may be bound at the active site in a 'linoleic acid-like' way but, since oxygenation is sterically hindered under these conditions it is not reflected in the level of the reaction products (abortive substrate binding). In contrast, the small share of 15-HPETE which is bound inversely, may be oxygenated to double oxygenation products including (5*S*, 15*S*)-dihydro(peroxy)-6,8,11,13-eicosatetraenoic acid [(5*S*,15*S*)-DiH(P)ETE].

The principal possibility of an inverse substrate orientation at the active site of 15-LOXs, which was suggested by the (5*S*)-lipoxygenation of (15*S*)-HPETE by 15-LOXs, does not mean that arachidonic acid may be aligned inversely at the active site of 5-LOXs. Computer-assisted structural modelling and structure-based sequence alignment suggest that the substrate-binding cleft of the human 5-LOX does not contain any positively charged residues which could potentially interact with the carboxylate group of the substrate [16,23]. If this is true, an inverse orientation would be hindered by a large energy barrier which should be reflected by a strongly reduced substrate affinity. However, the basic kinetic characteristics (K_m and V_{max}) of arachidonic acid oxygenation by 5-LOXs are very similar to those of the 15-LOX reaction and thus an inverse orientation may not be likely. If, on the other hand, the volume of the active site is decisive for the positional specificity, it should be possible to convert a 15-LOX into a 5-LOX by mutating amino acids, with space filling side-chains, to smaller residues. We created single, double, triple and quadruple mutations, aimed at increasing the volume of the substrate-binding pocket of the rabbit reticulocyte 15-LOX, without obtaining any indication for the sizeable formation of chiral 5-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE). These data suggest that an increase in the volume of the substrate-binding pocket may not be sufficient to convert a 15-LOX into a 5-LOX.

This work was carried out at the Institute of Biochemistry, University Clinics Charité, Humboldt University and was supported in part, by a DAAD fellowship to I.I. (A/96/28729) and by a grant of Deutsche Forschungsgemeinschaft (Ku 961/2-2) to H.K. We thank Dr. M. Browner for providing the X-ray co-ordinates for the rabbit reticulocyte 15-LOX/inhibitor complex.

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