ELSEVIER

Chemistry & Biology 8 (2001) 779–790

Chemistry & Biology

www.elsevier.com/locate/chembiol

Research Paper

Alterations of lipoxygenase specificity by targeted substrate modification and site-directed mutagenesis

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Received 26 March 2001; revisions requested 29 May 2001; revisions received 12 June 2001; accepted 14 June 2001
First published online 28 June 2001

Abstract

Background: Mammalian lipoxygenases (LOXs) are categorised with respect to their positional specificity of arachidonic acid oxygenation. However, the mechanistic basis for this classification is not well understood. To gain a deeper insight into the structural basis of LOX specificity we determined the reaction characteristics of wild-type and mutant mammalian LOX isoforms with native and synthetic fatty acids substrates.

Results: The rabbit 15-LOX is capable of catalysing major 12-lipoxygenation when the volume of the substrate-binding pocket is enlarged. These alterations in the positional specificity can be reversed when bulky residues are introduced at the ω end of the substrate. Simultaneous derivatisation of both ends of fatty acids forces a 15-LOX-catalysed 5-lipoxygenation and this reaction involves an inverse head-to-tail substrate orientation. In contrast, for arachidonic acid 5-lipoxygenation by the human 5-LOX the substrate fatty acid may not be inversely aligned. The positional specificity of this isoenzyme may be related to its voluminous substrate-binding pocket. Site-directed mutagenesis, which leads to a reduction of active site volume, converts the 5-LOX to a 15-lipoxygenating enzyme species.

Conclusions: The positional specificity of LOXs is not an invariant enzyme property but depends on the substrate structure and the volume of the substrate-binding pocket. 15-LOX-catalysed 5-lipoxygenation involves an inverse substrate alignment but this may not be the case for 5-LOXs. Thus, both theories for the mechanistic basis of 5-lipoxygenation (straight and inverse substrate orientation) appear to be correct for different LOX isoforms.

Keywords: Atherosclerosis; Eicosanoid; Inflammation; Lipid peroxidation; Mutagenesis

1. Introduction

Lipoxygenases (LOXs) constitute a family of lipid-peroxidising enzymes, which catalyse the dioxygenation of polyenoic fatty acids to their corresponding hydroperoxides [1,2]. Mammalian LOXs are involved in the biosynthesis of mediators of anaphylactic disorders and have been implicated in cell maturation and differentiation [1,2]. Animal LOXs are classified with respect to their positional specificity of arachidonic acid oxygenation and are referred to as 5-, 8-, 12- and 15-LOXs. Although the mechanistic basis of LOX specificity is far from clear the specificity-related nomenclature is generally accepted. Our current understanding of how the positional specificity of LOX isoenzymes is determined derived from crystallographic studies [3–6] and site-directed mutagenesis [7–9]. The four available LOX crystal structures suggest a U-shaped substrate-binding cleft the bottom of which is defined by crucial amino acids. For the rabbit 15-LOX these amino acids are F353, I418, M419, and I593 and site-directed mutagenesis of these residues led to alterations in the positional specificity [7,8]. Similarly, F359, A424, N425 and A603 have been identified as sequence determinants for the human 5-LOX [9]. The walls of the substrate-binding cleft of the rabbit 15-LOX are lined by hydrophobic residues, except for two polar side chains, Glu357 and Gln548, which are adjacent to the iron centre. A simple model for substrate alignment at the active site of LOXs is

Abbreviations: LOX, lipoxygenase; 15*S*-H(P)ETE, (15*S*,5*Z*,8*Z*,11*Z*,13*E*)-15-hydro(pero)xy-5,8,11,13-eicosatetraenoic acid; GC/MS, gas chromatography/mass spectrometry; 5*S*-H(P)ETE, (5*S*,6*E*,8*Z*,11*Z*,14*Z*)-5-hydro(pero)xy-6,8,11,14-eicosatetraenoic acid; 12*S*-H(P)ETE, (12*S*,5*Z*,8*Z*,10*E*,14*Z*)-12-hydro(pero)xy-5,8,10,14-eicosatetraenoic acid; RP(SP)-HPLC, reverse phase (straight phase) high performance liquid chromatography

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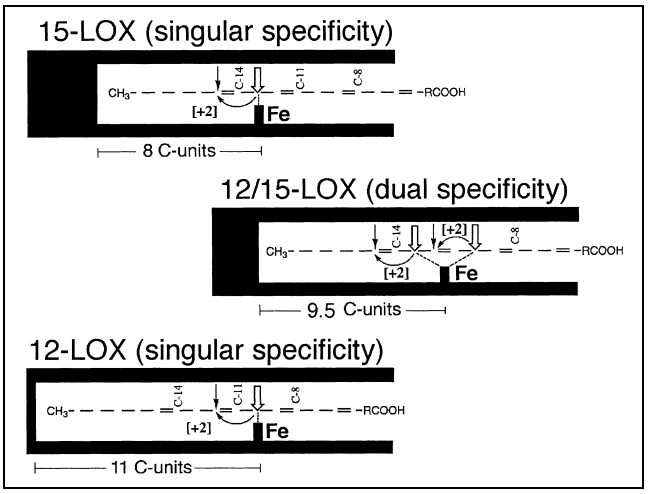
1074-5521/01/$ – see front matter
PII: S1074-5521(01)00050-3



Scheme 1. Substrate alignment at the active site of LOXs with different positional specificity. Arachidonic acid is aligned at the active site of 15-LOXs (singular positional specificity) in such a way that the bisallylic C_{13} is located in close proximity to the enzyme's hydrogen acceptor (probably the non-haem iron). 12-LOXs (singular positional specificity) have a deeper substrate-binding pocket so that the substrate may slide in farther. Thus, the bisallylic C_{13} is displaced from the hydrogen acceptor whereas C_{10} approaches it favouring hydrogen abstraction from this pro-chiral centre. LOXs with dual positional specificity (12/15-LOXs) are capable of abstracting hydrogen from different bisallylic methylenes (C_{10} and C_{13}).

that the hydrophobic base of the substrate-binding cage, in concert with the charged residue at the opening of the pocket, positions substrate fatty acids appropriately for 5-, 8-, 11-, 12- or 15-lipoxygenation [10]. For 12- and 15-LOXs the substrate fatty acid slides into the active site with its methyl terminus ahead [11,12]. In contrast, the substrate alignment for arachidonic acid 5-lipoxygenation has recently become a matter of discussion [13,14] and for the time being there are two hypotheses rationalising the positional specificity of 5-lipoxygenation. (i) The orientation theory [13]: this hypothesis suggests that for 5-lipoxygenation fatty acids may slide into the substrate-binding cleft with the carboxylate group ahead. The possibility of such an inverse substrate orientation was first concluded during mechanistic studies on corn and soybean LOXs [15]. More recently, an inverse substrate alignment was suggested for 15-HETE methyl ester oxygenation by the rabbit 15-LOX [16] and when the human epidermis-type 15-LOX was mutated to an 8*S*-lipoxygenating enzyme species [17]. With this hypothesis the stereochemistry of 5- and 8-lipoxygenation can be explained perfectly. However, its major problem is the energetic penalty associated with burying the polar carboxylate in the hydrophobic environment of the substrate-binding pocket [14]. In fact, for burying a hydroxy group at the active site of LOXs an energy barrier of 5.4 kJ/mol (soybean LOX) or 7.8 kJ/mol (rabbit LOX) was calculated [18] and for the more polar carboxylate the energy penalty is expected to be even higher. (ii) The volume theory [5,14]: according to this hypothesis the substrate alignment is similar for all LOX isoforms (methyl end slides into the active site). The positional specificity depends on the volume of the substrate-binding cavity. A structural model of the active site of 12-LOXs suggested a cavity which is about 6% larger than that of 15-LOX and 5-LOX has a 20% bigger substrate-binding cage [14]. The major disadvantage of the volume hypothesis is that the stereochemistry of arachidonate 5-lipoxygenation is not straightforward. However, considering the structural flexibility of fatty acids it appears possible that arachidonic acid may adopt a steric conformation suitable for pro-*S* hydrogen abstraction from C_7.

The present study was aimed at testing the two hypotheses of LOX specificity using two mammalian LOX isoforms (rabbit 15-LOX and human 5-LOX). To achieve this goal a combination of targeted substrate modification and site-directed mutagenesis was used and we found that both theories may be applicable under certain experimental conditions. Thus, they may not exclude each other.

2. Results

2.1. *Oxygenation of modified arachidonic acid derivatives by the pure rabbit 15-LOX*

Free polyenoic fatty acids slide into the substrate-binding pocket of 12/15-LOX with their methyl end ahead [11,12]. For optimal binding the substrate's hydrogen do-

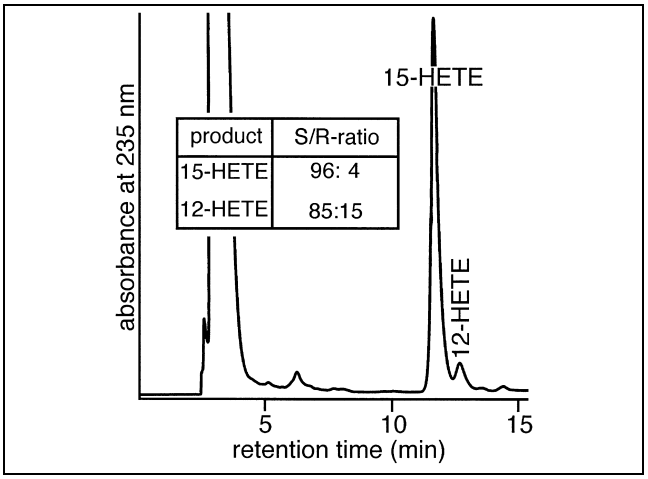
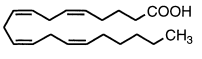
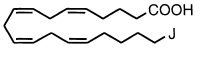
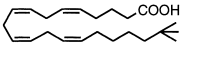
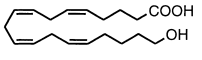
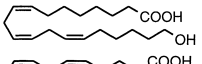
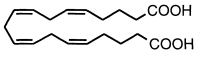


Fig. 1. Reaction specificity of the recombinant rabbit 15-LOX with arachidonic acid as substrate. The recombinant rabbit reticulocyte LOX was expressed in *Escherichia coli* and aliquots of the LOX-active Ni-agarose fractions were incubated in the standard assay system (see Section 5) for 15 min at room temperature (total assay volume 1 ml). After reduction of the hydroperoxy products (borohydride) and acidification to pH 3 proteins were precipitated with an equal volume of ice-cold methanol, the debris was spun down and aliquots of the clear supernatant were injected to RP-HPLC with the solvent system methanol/water/acetic acid (80:20:0.1, v/v). The chemical structure of the reaction products was concluded from co-injections with authentic standards and from GC/MS of the native product and its hydrogenated derivatives. Inset: Enantiomer composition of the two reaction products.

Table 1
Structures of synthetic LOX substrates

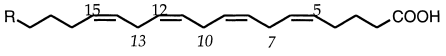
substrate	structure	distances from methyl terminus (Å)	
		C-13	C-10
18-CH_2-CH_3-C20:Δ5,8,11,14 (arachidonic acid)		8.63	9.55
18-CH_2-J-C19:Δ5,8,11,14		9.21	10.26
18-CH_2-C-$(CH_3)_3$-C23:Δ5,8,11,14		9.93	10.79
18-CH_2-OH-C19:Δ5,8,11,14		8.42	9.37
18-CH_2-CH_2-OH-C20:Δ8,11,14		9.64	10.46
18-COOH-C19:Δ5,8,11,14		8.46	9.74

For calculations of the distances the most distant atom at the ω end was used.

nor site (bisallylic methylene) is localised in close proximity to the enzyme's hydrogen acceptor, most probably the non-haem iron. Thus, optimal substrate alignment requires a defined distance between the bisallylic methylene and the methyl end of the substrate molecule (Scheme 1). If this distance is short a dual positional specificity results since two bisallylic methylenes are located in proximity to the iron centre (Scheme 1). The native rabbit 15-LOX oxygenates arachidonic acid predominantly at C_{15} [19] and here we confirmed this finding for the recombinant enzyme (Fig. 1). The selective formation of 15*S*-H(P)ETE suggests an almost optimal substrate alignment at the active site so that a hydrogen abstraction from C_{10} (oxygenation at C_{12}) may only be a minor site reaction. To disturb this optimal alignment we modified the structure of the fatty acid substrate in three different ways (Table 1). (i) Introduction of more space-filling residues [–$(CH_3)_3$ or J] at the methyl end of arachidonic acid. This modification was predicted to displace the bisallylic methylene C_{10} from the enzyme's hydrogen acceptor reducing the enzyme's capability of 12-lipoxygenation. (ii) Replacement of the substrate's ω-terminal CH_3 group by a polar OH. An arachidonic acid-like alignment of this substrate should be hindered because of the energetic barrier associated with burying the polar OH group in the hydrophobic environment of the substrate-binding pocket. Thus, this substrate should be prone to an inverse orientation. (iii) Methylation of the polar carboxylate. To force an inverse substrate orientation the energy penalty associated with burying the polar carboxylate in the hydrophobic milieu of the substrate-binding pocket should be reduced and this can be accomplished by carboxylate methylation.

When arachidonic acid (18-CH_2-CH_3-C20:Δ5,8,11,14), its 18-CH_2-J-C19:Δ5,8,11,14 and its 18-CH_2-C-$(CH_3)_3$-C23:Δ5,8,11,14 derivatives were oxygenated by the pure rabbit 15-LOX we observed the formation of one major conjugated diene in reverse phase and/or straight phase high performance liquid chromatography (RP- or SP-HPLC) (data not shown). These products contributed some 90% to the mixture of oxidation products for each substrate (Table 2) and were identified as the corresponding 15-hydro(pero)xy derivatives by gas chromatography/mass spectrometry (GC/MS) (Table 3). Interestingly, we did not detect any 12-lipoxygenation products with either 18-CH_2-J-C19:Δ5,8,11,14 or its 18-CH_2-C-$(CH_3)_3$-C23:Δ5,8,11,14 derivative. These data are plausible if one considers the fact that in these synthetic substrates the bisallylic methylene C_{10} is more distant from the ω end when compared with arachidonic acid (Table 1). Substitution of an OH group for the terminal CH_3 of arachidonic acid caused a dramatic change in the positional specificity of the lipoxygenase reaction (Table 2). Using 18-CH_2-OH-C19:Δ5,8,11,14 as substrate we observed a major 12-lipoxygenation (Fig. 2). Since the distance of C_{10} from the ω end of this fatty acid was only slightly shorter than in arachidonic acid we expected only a minor shift towards 12-lipoxygenation. The more drastic alterations observed indicate that other factors such as polarity of the ω residue may also influence the substrate alignment at the active site. Interestingly, we did not detect any 5-lipoxygenation products suggesting that introduction of an ω-terminal OH group is not sufficient to force an inverse substrate orientation. When ω-OH arachidonic acid was used as substrate an exclusive 15-lipoxygenation was observed

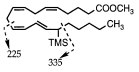
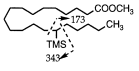
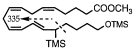
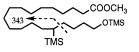
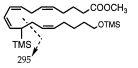
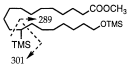
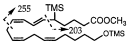
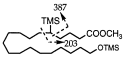
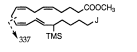
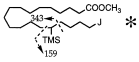
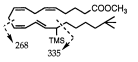
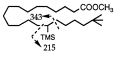
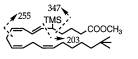
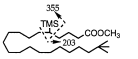
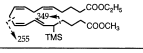
Table 2
Oxygenation of arachidonate derivatives modified at the methyl terminus



residue R	K_M-value (μM)	V_{max} (s^{-1})	oxygenation ratio (C_{15}/ C_{12})
-CH_2-CH_3	6.5	22.8	93/7
-CH_2-J	43	22.5	99/1
-CH_2-C$(CH_3)_3$	41	3.1	99/1*
-CH_2-OH	153	3.7	21/79
-COOH	n.d.	<0.1	n.d

The various arachidonate derivatives were oxygenated by the pure rabbit reticulocyte 15-LOX (0.4 μg/ml) at pH 7.4 in the standard assay system (Section 5). Apparent K_M and V_{max} values were extracted from Lineweaver–Burk plots (measurements at five different substrate concentrations). V_{max} values are given as turnover number under V_{max} conditions (substrate saturation). The ratio of C_{12} and C_{15} oxygenation was calculated from the RP- and SP-HPLC analyses. The chemical structures of the products were concluded from GC/MS data (Table 3) obtained with the derivatised native and hydrogenated compounds. n.d., not determined because of the low reaction rate. *In addition to the 15-lipoxygenation products about 10% of 5-lipoxygenation was observed.

Table 3
Mass spectral data of lipoxygenase products

substrate residue	product	most informative ions (m/z) native	most informative ions (m/z) hydrogenated
$-CH_3$	15-OH	225 (81), 335 (11) 	173 (72), 343 (23) 
-OH	15-OH	480 (12 [M$^+$]), 335 (20) 	473 (5 [M$^+$-15]), 343 (100) 
-OH	12-OH	295 (100) 	301 (100), 289 (65), 473 (7 [M$^+$-15]) 
-OH	5-OH	480 (3.5 [M$^+$]), 203 (60), 255 (14) 	203 (100), 387 (10), 473 (30 [M$^+$-15]) 
-J	15-OH	518 (4 [M$^+$]), 337 (100) 	382 (1 [M$^+$-15]), 343 (25), 159 (100)  *
$-C(CH_3)_3$	15-OH	448 (16 [M$^+$]), 268 (75), 335 (8) 	441 (1 [M$^+$-15]), 215 (100), 343 (40) 
$-C(CH_3)_3$	5-OH	448 (16 [M$^+$]), 203 (56), 255 (60), 347 (24) 	441 (1, [M$^+$-15]), 203 (100), 355 (40) 
$-COCH_3$	15-OH	450 (15 [M$^+$]), 255 (50), 349 (25)  **	

In parentheses relative abundances in %. *CH_2-J was reduced to CH_3 during hydrogenation, **the free carboxylic group was ethylated with diazoethane (numbering of the carbon atoms as for the other substrates).

(data not shown). Since the methyl end of this substrate is one CH_2 unit longer than that of the 18-CH_2-OH-C19:Δ5,8,11,14 this result was actually expected.

Comparing the kinetic constants of fatty acid oxygenation (Table 2) one may conclude that among the substrates tested arachidonic acid is the best one. The low K_M indicates a strong binding affinity and the high turnover rate suggests an optimal substrate alignment for C_{13} hydrogen abstraction (hydrogen abstraction is the rate-limiting step of the LOX reaction). The iodine-substituted fatty acid was oxygenated with a similar V_{max}, but substrate binding was less effective. The highest K_M was determined for the ω-hydroxylated fatty acid and this may reflect the energetic penalty associated with burying the polar OH group in the hydrophobic milieu of the substrate-binding pocket. The dicarboxylic derivative was not oxygenated which may be due to similar energetic constraints.

When compared with the free acid, arachidonic acid methyl ester is less well oxygenated. Under our experimental conditions V_{max} was only about 50% of that of the free acid and the binding affinity was lower by one order of magnitude (Table 4). The pattern of reaction products was almost identical; with both substrates 15-H(P)ETE was identified as the major product. These data suggest that methylation of the carboxylate did not lead to major alterations in substrate alignment. In contrast, methylation of 18-CH_2-OH-C19:Δ5,8,11,14 drastically altered the product pattern (Fig. 2). Although the 12-oxygenation product was still detectable (33%) the major reaction catalysed was 5-lipoxygenation (67%). Moreover, the binding affinity of the methyl ester was several-fold higher than that of the free acid, which contrasts with the data of methyl arachidonate oxygenation. Although the mechanistic reasons for this effect have not been studied in detail it may be assumed that the energetic barrier associated with burying the methyl ester group in the hydrophobic environment of the active site may be smaller than that for the OH group and thus, an inverse orientation may be favoured. When the $C_5=C_6$ double bond was removed from the substrate molecule we did not observe any 5-lipoxygenation (Table 4). This finding was straightforward since lack of the bisallylic C_7 prevented this reaction. An even more pronounced alteration in the positional specificity was observed when 18-CH_2-C-$(CH_3)_3$-C23:Δ5,8,11,14 was

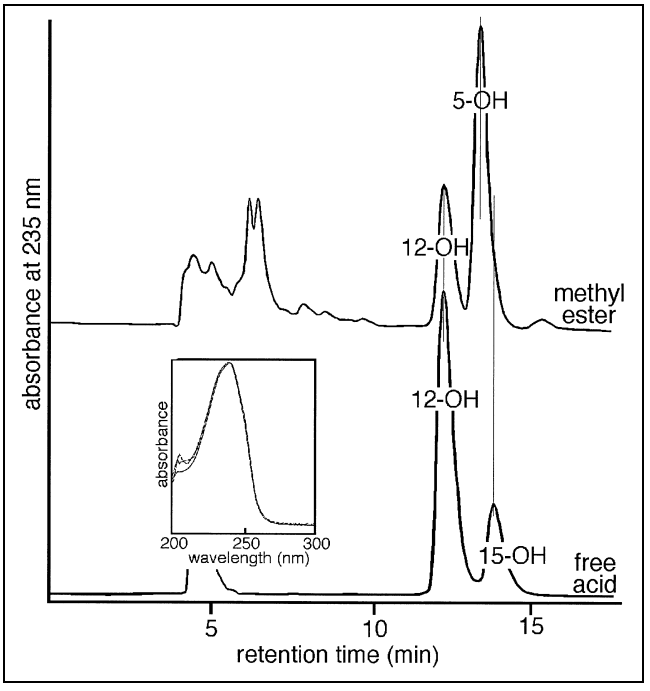


Fig. 2. Oxygenation products formed from 18-CH_2-OH-C19:Δ5,8,11,14 by the native rabbit 15-LOX. The pure rabbit 15-LOX (4.2 μg/ml) was incubated in the standard assay (1 ml assay volume) in the presence of 100 μM 18-CH_2-OH-C19:Δ5,8,11,14 (free acid and methyl ester). The hydroperoxy products formed were reduced with sodium borohydride and methylated. The samples were acidified and the proteins were precipitated with 1 ml ice-cold methanol. Debris was spun down and aliquots of the clear supernatant were injected to RP-HPLC (see Section 5) carried out with the solvent system methanol/water/acetic acid (70/30/0.1, v/v). The chemical structures of the compounds were concluded from GC/MS data obtained for the derivatised native compounds and their hydrogenated derivatives (Table 3). Inset: The UV spectra of the major reaction products are very similar and indicate the presence of a conjugated diene chromophore.

used as the substrate. Methylation of this fatty acid led to a complete shift of the positional specificity from 15- towards 5-lipoxygenation (Fig. 3). With the free acid we observed the predominant formation of the corresponding 15-hydro(pero)xy derivatives (Table 4). In contrast, 5-lipoxygenation was the major process when the methyl ester was used. In chiral phase HPLC both the 5- and 15-oxygenation products gave a single peak suggesting a high degree of chirality (data not shown). Comparison of the kinetic constants for the 18-CH_2-$C(CH_3)_3$-C23:Δ5,8,11,14 derivatives did not reveal major differences (Table 4).

As indicated in Table 2 the dicarboxylic acid 18-HOOC-C19:Δ5,8,11,14 was not oxygenated by the rabbit 15-LOX. As a possible reason for the lacking activity energetic constraints associated with burying the polar carboxylate in the hydrophobic environment of the substrate-binding pocket were discussed. To reduce this energy barrier but to keep a free carboxylate the dicarboxylic acid was monomethylated. Surprisingly, we found that the monomethyl ester was rapidly oxygenated (16.8 s^{-1}) although its binding affinity was rather low (K_M = 166 μM). RP-HPLC revealed a single oxygenation product and GC/MS data indicated that dioxygen was introduced close to the monomethylated carboxylate (Table 3). These data suggest that the methylated carboxylate may penetrate into the substrate-binding pocket and the free carboxylate is kept outside the hydrophobic environment.

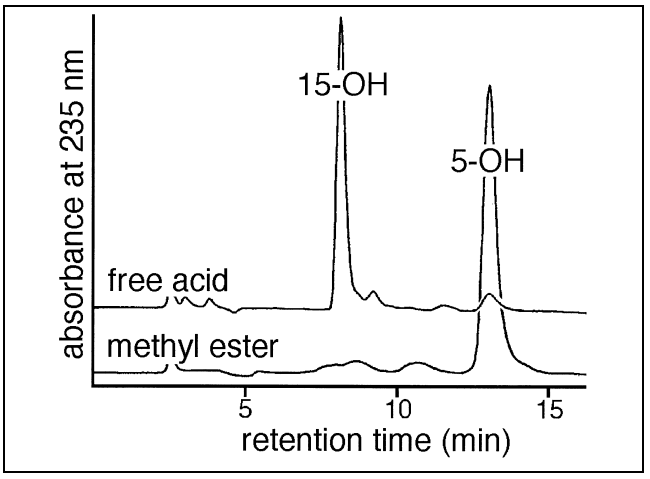
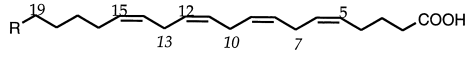


Fig. 3. Oxygenation products formed from 18-CH_2-$C(CH_3)_3$-C23:Δ5,8,11,14 by the native rabbit 15-LOX. The purified native rabbit 15-LOX (4.2 μg/ml) was incubated in the standard assay (1 ml assay volume) in the presence of 100 μM of 18-CH_2-$C(CH_3)_3$-C23:Δ5,8,11,14 (free acid and methyl ester). The hydroperoxy products formed were reduced with sodium borohydride, the sample was acidified and the lipids were extracted. The solvent was evaporated, the free carboxylate was methylated, the lipids were reconstituted in 100 μl methanol and aliquots were injected to RP-HPLC purification. Conjugated dienes were collected and further analyzed by SP-HPLC with the solvent system *n*-hexane/2-propanol/acetic acid (100:1:0.1, v/v). The chemical structures of the products were concluded from GC/MS data obtained for the derivatised native product and its hydrogenated derivatives (Table 3).

Table 4
Effects of carboxylate methylation on the oxygenation characteristics of arachidonate derivatives by the pure reticulocyte 15-LOX



R	free acid		methyl ester		oxygenation at carbon	
	rel. K_M	rel. V_{max}	rel. K_M	rel. V_{max}	Free acid	Methyl ester
-CH_3	1	100	9.2	52.5	15 (95%) 12 (5%)	15 (90%) 12 (10%)
-OH	23.5	16.2	4.2	55.0	15 (20%) 12 (80%)	12 (33%) 5 (67%)
-CH_2OH*	28.2	30.6	21.2	25.5	15 (98%)	15 (98%)
-$C(CH_3)_3$	6.3	13.4	8.7	23.6	15 (93%) 5 (7%)	5 (>98%)

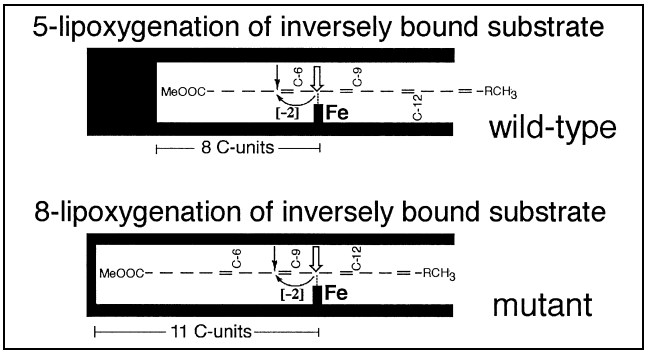
The various arachidonate derivatives were oxygenated by the rabbit reticulocyte 15-LOX (0.4 μg/ml) at pH 7.4 in the standard assay system (see Section 5). Substrates were methylated with diazomethane and repurified by RP-HPLC. Apparent K_M and V_{max} values were extracted from Lineweaver–Burk plots (measurements at five different substrate concentrations). The patterns of the oxygenation products for each substrate were compared by RP-HPLC after complete methylation. GC/MS data indicating the structure of the oxygenation products are summarised in Table 3. To calculate the relative kinetic parameters the apparent K_M for arachidonic acid (6.5. μM) was set at 1 and V_{max} (22.8 s^{-1}) was set at 100. *This substrate lacks the $C_5 = C_6$ double bond, no 5-lipoxygenation is possible.

Summarising our data on the oxygenation of arachidonic acid derivatives by the native rabbit 15-LOX one may conclude that neither derivatisation of the carboxylate nor modification of the ω terminus of fatty acids may lead to major 5-lipoxygenation. However, if both substrate ends are modified simultaneously 5-lipoxygenation may be forced.

2.2. Oxygenation of modified arachidonic acid derivatives by mutant 15-lipoxygenase species

The data obtained with the synthetic arachidonate derivatives indicate that 15-LOXs are capable of catalysing 5-lipoxygenation if fatty acids are suitably derivatised. It should be stressed at this point that the 5-lipoxygenated substrates contain a bisallylic C_{13} so that, in principle, 15-lipoxygenation was possible. However, the enzyme favoured 5-lipoxygenation and as a mechanistic reason for this observation an inverse head-to-tail substrate orientation may be suggested. To test this hypothesis we mutated the rabbit 15-LOX at critical positions and analysed the oxygenation products formed from the substrates, which are 5-lipoxygenated by the wild-type enzyme. The I418A mutant of the rabbit 15-LOX oxygenates arachidonic acid predominantly to 12-H(P)ETE [8]. Since this amino acid is localised at the bottom of the substrate-binding pocket the Ile → Ala exchange deepens the pocket so that the methyl

terminus of the fatty acid may slide in farther approaching the bisallylic methylene C_{10} to the enzymes hydrogen acceptor (Scheme 1). If one assumes an inverse substrate orientation for the 5-lipoxygenated fatty acids the I418A mutant should catalyse 8-lipoxygenation (Scheme 2). To test this prediction we prepared the I418A mutant and determined its positional specificity when arachidonic acid, 18-CH_2-C(CH_3)$_3$-C23:Δ5,8,11,14, 18-CH_2-OH-C19:Δ5,8,11,14 and their methyl esters were used as substrates. For this purpose the wild-type rabbit 15-LOX and its I418A mutant were overexpressed in *Escherichia coli* and aliquots of the purified enzyme preparations (see Section 5) were used as enzyme sources. When analysing the product pattern of arachidonic acid oxygenation we found that the wild-type enzyme produced mainly 15-HPETE (12-/15-HPETE ratio 4:96) whereas the mutant 15-LOX preferred 12-lipoxygenation (12-/15-HPETE ratio of 89:11). The free acid of 18-CH_2-C(CH_3)$_3$-C23:Δ5,8,11,14 was oxygenated by the wild-type recombinant enzyme at C_{15} and small amounts of the corresponding 5-hydro(pero)xy derivatives were also detected (Table 5). Using the methylated substrate an exclusive 5-lipoxygenation was observed. These data are in line with the product pattern obtained with the native enzyme (Fig. 3). The I418A mutant converted this substrate to a more complex product mixture (Table 5) which can be explained as follows. The mutant enzyme has a deeper substrate-binding pocket. Since the 18-CH_2-C(CH_3)$_3$-C23:Δ5,8,11,14 substrate contains a bulky trimethyl group at its methyl end the 15-LOX → 12-LOX conversion achieved by the mutation was predicted to be incomplete with this substrate. In fact, 15-lipoxygenation was identified as the major oxygenation process and we only detected some 20% of 12-oxygenation products. The 5-hydro(pero)xy derivative, which amounted to about one third of the oxygenation products, may originate from an inverse head-to-tail substrate orientation. To obtain experimental evidence for such an inverse alignment the oxygenation products of the methyl ester were analysed. The wild-type LOX catalysed exclusively 5-lipoxygenation (Table 5) and our model for an inverse substrate alignment predicted an 8-lipoxygenation when the I418A mutant was used as the catalyst (Scheme 2). Analysing the product pattern of 18-CH_2-C(CH_3)$_3$-C23:Δ5,8,11,14 methyl ester oxygenation by the I418A mutant we did indeed detect significant amounts of 8-lipoxygenation products (Fig. 4).

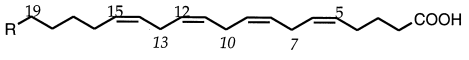


Scheme 2. Effect of a deeper substrate-binding pocket on the oxygenation specificity of inversely bound substrates. 15-LOX-catalysed 5-lipoxygenation involves an inverse head-to-tail substrate orientation. The major consequence of an inverse substrate alignment is that the bisallylic C_7 is localised in close proximity to the enzyme's hydrogen acceptor. Deepening of the substrate-binding pocket allows the substrate to slide in farther so that C_7 is displaced from the hydrogen acceptor whereas C_{10} approaches it. Such an alignment is predicted to lead to an increased share of 8-lipoxygenation.

When the recombinant wild-type 15-LOX was reacted with 18-CH_2-OH-C19:Δ5,8,11,14 the results were similar to those obtained with the native enzyme. With the free acid 12-lipoxygenation was the major process. In contrast, the methyl ester was mainly oxygenated at C_5 and this may be due to an inverse substrate orientation. To check for such an inverse substrate alignment we analysed the product pattern obtained with the I418A mutant. As discussed for the 18-CH_2-C(CH_3)$_3$-C23:Δ5,8,11,14 methyl ester the large amounts of the corresponding 8-hydro(pero)xy products suggest an inverse orientation of the substrate.

Similar results were also obtained when the free acids and the methyl esters of 18-CH_2-C(CH_3)$_3$-C23:Δ5,8,11,14 were oxygenated by the F353L mutant. This mutant catalyses major arachidonic acid 12-lipoxygenation and we also observed a shift from 5- towards 8-lipoxygenation with the methyl esters as substrate.

Table 5
Composition of the product pattern formed from arachidonic acid derivatives by the recombinant wild-type and mutant (I418A) rabbit 15-LOX



R	oxygenation at carbon	wild-type (%)		I418A (%)	
		free acid	methyl ester	free acid	methyl ester
-C(CH_3)$_3$	15	84	0	42	0
	12	0	0	22	0
	8	0	0	0	40
	5	16	100	36	60
-OH	15	27	0	15	0
	12	73	31	85	11
	8	0	0	0	67
	5	0	69	0	22

Mutant and wild-type 15-LOX were expressed in *E. coli* and aliquots of the purified enzyme preparations exhibiting a comparable arachidonate oxygenase activity were used for incubations (see Section 5). The oxygenation products were isolated by RP-HPLC, purified by SP-HPLC and their structures were identified by GC/MS (Table 3). Lysates obtained from bacteria transformed with the wild-type plasmid did not oxygenate any of the substrates.

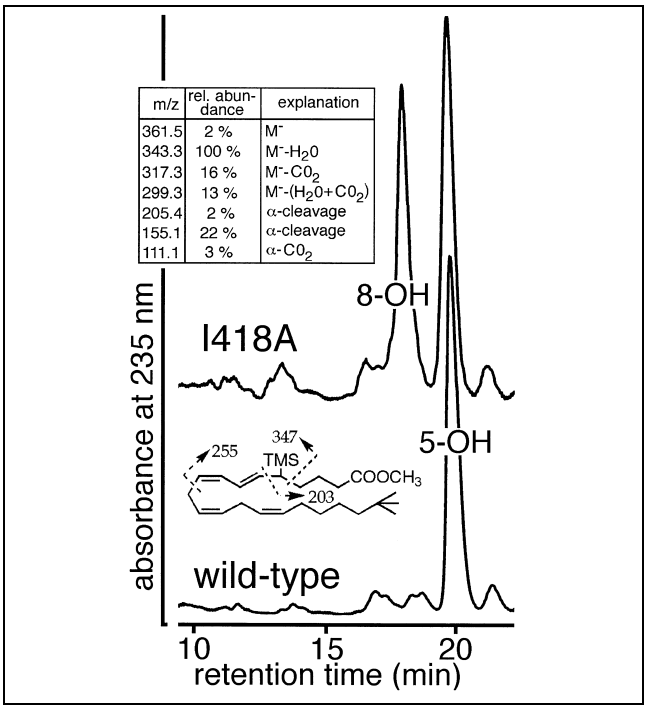


Fig. 4. Oxygenation products formed from the methyl ester of 18-CH_2-$C(CH_3)_3$-C23:Δ5,8,11,14 by the recombinant wild-type rabbit 15-LOX and its I418A mutant. The wild-type and mutant 15-LOX species were expressed in *E. coli*. Aliquots of the purified LOX preparation (Ni-agarose column) exhibiting comparable arachidonic acid oxygenase activities were incubated for 15 min at room temperature in the standard 15-LOX assay system in the presence of 100 μM 18-CH_2-$C(CH_3)_3$-C23:Δ5,8,11,14 methyl ester (1 ml assay volume). The hydroperoxy products formed were reduced with sodium borohydride, the sample was acidified and proteins were precipitated with 1 ml ice-cold methanol. Debris was spun down and aliquots of the clear supernatant were injected to RP-HPLC carried out with solvent system methanol/water/acetic acid (75/25/0.1, v/v). Lower inset: Key ions observed in GC/MS and fragmentation pattern of the major oxygenation product formed by the wild-type enzyme. Upper inset: Informative key ions obtained in electrospray MS for the early eluting compound formed by the I418 mutant. These data indicate the structure as the corresponding 8-hydroxy derivatives.

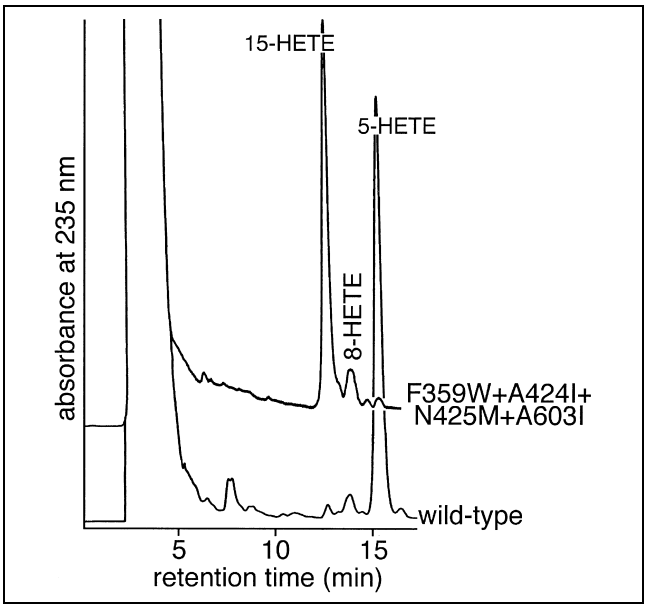


Fig. 5. Oxygenation products formed from arachidonic acid by wild-type and mutant 5-LOX (F359W+A424I+N425M+A603I). The enzyme species were expressed in *E. coli* and purified on ATP Sepharose. The active fractions were pooled and aliquots exhibiting a similar arachidonic acid oxygenase activity were incubated for 5 min at room temperature in the standard 5-LOX assay. The oxygenation products were reduced with sodium borohydride, the samples were acidified, proteins were precipitated with 1 ml ice-cold methanol and after centrifugation aliquots of the supernatant were injected to RP-HPLC using a solvent system of methanol/water/acetic acid (80:20:0.1, v/v).

2.3. *Oxygenation of fatty acids by 5-LOX mutants with altered positional specificity*

Recently it was reported that the human 5-LOX could be converted to a 15-lipoxygenating enzyme species when sequence determinants of the positional specificity (F359, A424, N425 and A603) were mutated to more space-filling residues [9]. For this study we expressed the 15-lipoxygenating quadruple mutant F359W+A424I+N425M+A602I in *E. coli* and compared its reaction specificity with that of the wild-type enzyme using different polyenoic fatty acids as substrate. From Table 6 it can be seen that the C_{20} fatty acids containing $C_5=C_6$ and $C_8=C_9$ double bonds were good substrates for the wild-type enzyme. In contrast, C_{20} fatty acids lacking these double bonds as well as all C_{18} fatty acids were not oxygenated. The 15-lipoxygenating quadruple mutant oxygenated all polyenoic fatty acids containing $C_{11}=C_{12}$ and $C_{14}=C_{15}$ double bonds. Only the C_{20}:Δ5,8,11 substrate, which lacks the $C_{11}=C_{12}$ double bond was not oxygenated. These data suggest that for both the wild-type and mutant 5-LOX, the distance of the bisallylic methylene (site of hydrogen abstraction) from the ω end of the fatty acid may be of major importance.

The cucumber linoleate 13-LOX can be converted to a 9-lipoxygenating enzyme species by site-directed mutagenesis [20]. This alteration in positional specificity has been

Table 6
Oxygenation of different substrate fatty acids by 5-LOX mutants

substrate	5-LOX	F359W+A424I+N425M+A603I
C20:Δ5,8,11,14	100	100
C20:Δ5,8,11	132	0
C20:Δ 11,14,17	0	90
C20:Δ11,14	0	106
C18:Δ9,12	3	142
C18:Δ6,9,12	0	144
C18:Δ9,12,15	0	576

Wild-type human 5-LOX and its F359W+A424I+N425M+A603I mutant were expressed in *E. coli* and purified on ATP Sepharose. Aliquots of the enzyme preparation were incubated in the standard 5-LOX assay at room temperature for 5 min. After reduction with sodium borohydride and acidification 1 ml of ice-cold methanol was added and the protein precipitate was spun down. Aliquots of the clear supernatant were analysed by RP-HPLC (see Section 5). The amount of products formed by each mutant from arachidonic acid was set at 100 and the relative product formation with the other substrates is given.

suggested to be due to an inverse substrate orientation. It was hypothesised that the mutation carried out might demask a charged amino acid at the active site, which then may interact with the substrate's carboxylate [20]. To find out whether the human 5-LOX is also capable of burying a free carboxylate at its active site experiments with a dicarboxylic fatty acid (18-HOOC-C19:Δ5,8,11,14) were carried out. This substrate is a symmetric molecule (Table 2) and the distance of the bisallylic methylene C_7 from either carboxylate should be optimal for the human 5-LOX. If the enzyme is capable of burying a free carboxylate at its active site the dicarboxylic acid was expected to be a suitable substrate. However, we found that neither the wild-type enzyme nor the 15-lipoxygenating quadruple mutant was capable of oxygenating the dicarboxylic fatty acid.

The inefficient oxygenation of the dicarboxylic fatty acid may be due to energetic constraints associated with burying the free carboxylate in the hydrophobic environment of the substrate-binding pocket. If this conclusion is true monomethylation should render the substrate oxidisable. In fact, we found that the monomethyl ester of 18-HOOC-C19:Δ5,8,11,14) was oxygenated by 5- and 15-LOXs with a high reaction rate (Fig. 6, inset). The rabbit 15-LOX (Fig. 6) converted this substrate predominantly to the 15-hydro(pero)xy derivatives (free carboxylate carbon is numbered C1). The most informative ions in the negative ion mass spectra (explanation, relative abundance in parentheses) were as follows: *m/z* 349.7 (M^-, 2%), 317 ($M^- - MeOH$, 100%), 218.7 (α cleavage, 3%). In contrast, the wild-type 5-LOX (Fig. 6) catalysed a major 5-lipoxygenation (free carboxylate carbon is numbered C1) indicating that oxygen is introduced at a carbon atom which is far distant from the methylated carboxylate. Here again the following mass spectral data indicate the structure of the oxygenation product: *m/z* 349.4 (M^-, 3%), 330.9 ($M^- - H_2O$, 82%), 317.1 ($M^- - MeOH$, 100%), 114.7 (α cleavage, 16%). Since the methylated carboxylate is likely to slide into the active site because of energetic reasons one may conclude that a similar situation may be the case for free arachidonic acid. These data and the inability of the enzyme to oxygenate the free dicarboxylic acid may suggest that 5-lipoxygenation by the human 5-LOX involves a similar substrate alignment at the active site as 15-LOXs.

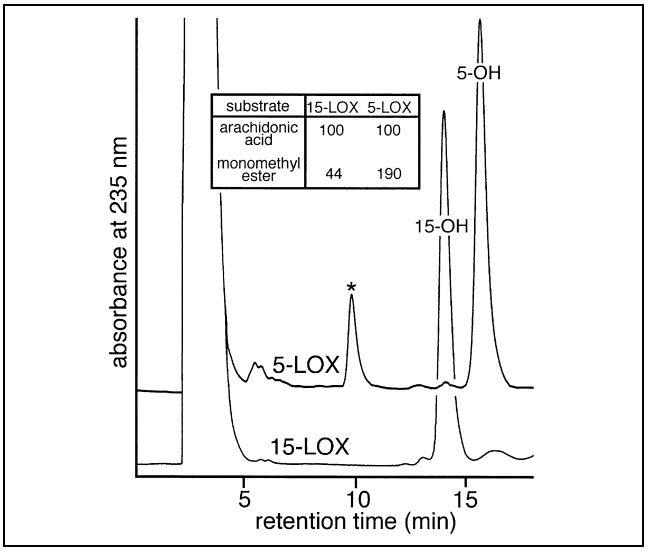


Fig. 6. Oxygenation products formed from the monomethyl ester of 18-HOOC-C_{19}:Δ5,8,11,14 by wild-type recombinant 5- and 15-LOXs. Wild-type human 5- and rabbit 15-LOXs were expressed in *E. coli*. Aliquots of the bacterial lysis supernatant exhibiting a comparable arachidonic acid oxygenase activity were incubated in the standard assay systems (optimal assay for each enzyme) for 5 min at room temperature. The oxygenation products were reduced with sodium borohydride, the proteins were precipitated with 1 ml ice-cold methanol and after centrifugation aliquots were injected to RP-HPLC using a solvent system of methanol/water/acetic acid (70:30:0.1, v/v). Inset: Comparison of the oxygenation rate of arachidonic acid and the dicarboxylic acid monomethyl ester. The rate of arachidonic acid oxygenation for each enzyme was set at 100%. The product marked with an asterisk was identified by MS as 5-hydroxylated free dicarboxylic acid. It was formed from the corresponding monomethyl ester by unspecific *E. coli* esterases, which were co-purified with the 5-LOX.

3. Discussion

The positional specificity of LOXs, which is the basis of the currently used enzyme nomenclature, is not an absolute enzyme property but depends on the alignment of the substrate at the active site. Modification of the substrate structure [9,11,12] and/or site-directed mutagenesis of critical amino acids [7,8,10] influence the substrate alignment and thus affect the specificity of the LOX reaction. For 12- and 15-lipoxygenation a topological model for substrate binding at the active site has been introduced (Scheme 1) suggesting that substrate fatty acids slide into the hydrophobic substrate-binding pocket with its methyl end ahead [21,22]. Although no direct structural data have been published as to the substrate alignment at the active site for any LOX isoform there is a plethora of experimental evidence suggesting that for 15-lipoxygenation arachidonic acid slides into the substrate-binding pocket with its methyl end ahead so that the bisallylic methylene C_{13} is located in close proximity to the enzyme's hydrogen acceptor. This model is supported by our results on the oxygenation of synthetic arachidonate derivatives by the wild-type rabbit 15-LOX and its 12-lipoxygenating mutants (I418A, F353L).

15-LOXs are capable of catalysing 5-lipoxygenation of substrates, which cannot be oxygenated at C_{15} because of their structural properties. In these cases the substrate may be aligned inversely at the active site and the energetic barrier associated with burying the carboxylate in the hydrophobic active site is reflected by a strongly reduced binding affinity [9,23]. The soybean LOX-1 converts 15*S*-HPETE inter alia to the corresponding 5*S*,15*S*-diHPETE but the K_M for this reaction was some 20 times higher

than for arachidonic acid oxygenation [23]. The rabbit 15-LOX introduces molecular dioxygen predominantly at C_5 of 15-HETE methyl ester and here again the K_M is much higher than that of arachidonic acid oxygenation [9]. The data obtained in this study indicate for the first time that under certain conditions 15-LOXs preferentially catalyse 5-lipoxygenation even though 15-lipoxygenation of the substrate is theoretically possible. As a mechanistic reason for this 5-lipoxygenation an inverse head-to-tail orientation of the substrate molecule was suggested (Scheme 2) and our results obtained with the 12-lipoxygenating 15-LOX mutant I418A (Table 5) support this hypothesis. To force the inverse substrate orientation the fatty acids must be modified simultaneously at both ends of the hydrocarbon chain. Introduction of a bulky or polar residue at the methyl terminus was not sufficient and neither was methylation of the carboxylate. However, if both types of modification were carried out simultaneously an inverse substrate alignment appeared to be favoured. With a defined substrate there may be a binding equilibrium between arachidonic acid-like orientation (methyl terminus slides into the binding pocket) and inverse substrate alignment (carboxylate group penetrates into the pocket). This equilibrium appears to be influenced by functional groups on either end of the fatty acid chain. Introduction of polar or bulky residues [–OH, C-$(CH_3)_3$] at the ω terminus is expected to shift the binding equilibrium towards an inverse substrate orientation. However, since the energy barrier associated with burying the polar carboxylate in the hydrophobic substrate-binding pocket is rather high, such an equilibrium shift may be prevented. When, in addition, the hydrophobicity of the carboxylate is augmented by methylation the energetic barrier is reduced and the binding equilibrium is shifted towards inverse substrate orientation. The increased share of 5-lipoxygenation may indicate this equilibrium shift. It is important to note that the pattern of oxygenation products may not quantitatively reflect the binding equilibrium. In other words, a lack of 5-lipoxygenation products does not exclude an inverse substrate binding. If, for instance, the inversely bound substrate is not oxygenated because of steric constraints (abortive substrate binding) an exclusive 15-lipoxygenation may be observed despite the fact that a certain percentage of the substrate is inversely aligned.

In contrast to 15-LOXs for which many experimental data are available on the substrate alignment, much less is known for mammalian 5-LOXs. For the time being there are two alternative hypotheses, which rationalise the mechanistic basis for the positional specificity of 5-LOXs [13,14]. The volume theory suggested that all LOX isoforms align the substrates at the active site in a similar way irrespective of their positional specificity. The most important factor influencing the specificity of LOX isoforms may be the volume of the substrate-binding pocket. 15-LOXs have a small cavity and thus, polyenoic fatty acids are oxygenated close to the methyl terminus. In contrast, more space is available in the substrate-binding cleft of 5-LOXs. In this case the fatty acid substrates may slide farther into the cavity leading to an oxygenation close to the carboxylate. Sequence-based structural modelling suggested that the substrate-binding cavity of mammalian 5-LOXs is some 20% larger than that of 15-LOXs [5,14]. The major disadvantage of the conserved orientation hypothesis is that the stereochemistry of 5*S*-lipoxygenation is not straightforward. However, given the conformational flexibility of fatty acid derivatives it appears possible to position the pro-*S* hydrogen at C_7 close to the enzymes hydrogen acceptor. According to the orientation hypothesis [13,24] fatty acid substrates may slide into the active site with its carboxylate ahead for 5-lipoxygenation so that the bisallylic C_7 is located close to the enzyme's hydrogen acceptor. This model easily explains the stereochemistry of 5-lipoxygenation. However, the major drawback of this model is that an inverse substrate orientation may be prevented by an energy barrier associated with burying the polar carboxylate in the hydrophobic environment of the active site. It should be stressed that for the time being neither of the two hypotheses has been proven experimentally. Since direct structural investigation of 5-LOX/substrate interaction is not possible at the moment we approached the problem by a combination of targeted substrate modification and site-directed mutagenesis. Taken together our data suggest that 5-lipoxygenation by mammalian 15-LOXs involves an inverse head-to-tail substrate orientation. In contrast, 5-lipoxygenation catalysed by the human 5-LOX may follow the volume hypothesis and there are several lines of experimental evidence supporting this conclusion. (i) A gradual decrease in the active site volume induced by site-directed mutagenesis converted the human 5-LOX to a 15-lipoxygenating enzyme species (Fig. 5). (ii) The orientation hypothesis requires that 5-LOXs can bury a free carboxylate at the active site. If this is the case dicarboxylic fatty acids should be suitable substrates for these enzymes. However, we found that the dicarboxylic acid 18-HOOC-C_{19}:Δ5,8,11,14 was not oxygenated by the wild-type 5-LOX. (iii) The monomethyl ester of the dicarboxylic fatty acid was oxygenated with a high reaction rate and product analysis indicated major 5-lipoxygenation. If one assumes that the more hydrophobic methylcarboxylate slides into the substrate-binding pocket the initial hydrogen removal takes place at a bisallylic methylene which is far away from the methylated carboxylate. A similar situation may be the case for arachidonic acid. (iv) An inverse substrate orientation would be favoured if a positively charged amino acid was present at the bottom of the active site to interact with the substrate's carboxylate [20]. However, structural modelling did not provide any evidence for a charged group at the active site of the human 5-LOX [14]. If this is true, an inverse orientation would be hindered by an energy barrier, which should be reflected by a strongly reduced substrate affinity. However, the oxygenation kinetics (K_M and

V_{max}) of arachidonic acid by 5-LOXs are similar to those of the 15-LOX reaction [25].

Summarising our data one may conclude that substrate fatty acids may be oriented in either direction (straight or inverse orientation) at the active site of LOXs. The chemical structure of the substrate appears to be important for its orientation and different LOX isoforms behave differently. Although there are no direct structural data available on enzyme/substrate complexes of any LOX isoform structural modelling and our mutagenesis data suggest a conserved substrate alignment for mammalian 15- and 5-LOXs. For other LOX isoforms, in particular for plant enzymes [20], the situation may be different.

4. Significance

LOXs constitute a heterogeneous family of lipid-peroxidising enzymes and the different mammalian LOX isoforms (15-LOXs, 12-LOXs, 8-LOXs and 5-LOXs) are categorised with respect to their positional specificity of arachidonic acid oxygenation. They have been implicated in physiological and pathophysiological events, such as cell differentiation, inflammation, carcinogenesis and atherosclerosis. For future investigations on the biological relevance of these enzymes genetically modified cells (transfectants) and organisms (transgenic animals and knockout mice) as well as isoform-specific inhibitors are needed. For the rational design of isoform-specific inhibitors detailed knowledge of the structural differences of the various isoforms and their catalytic peculiarities is required. It has been suggested before that substrate alignment at the active site is important for LOX specificity and for arachidonate 5-lipoxygenation two alternative hypotheses have been introduced. The space-based hypothesis suggests that the way of substrate binding is conserved among all LOX isoforms and that fatty acids may slide into the substrate-binding pocket with their ω end ahead. According to the orientation-based hypothesis fatty acid substrates may slide into the substrate-binding cleft with their carboxylate end ahead, and thus may be inversely aligned. We investigated the impact of targeted substrate modification and site-directed mutagenesis on substrate alignment and concluded that both hypotheses may be relevant. For 15-LOX-catalysed 5-lipoxygenation substrate fatty acids appear to be inversely aligned. In contrast, for 5-LOX-catalysed 5-lipoxygenation the space-related hypothesis may be more suitable to explain the experimental data. It should be stressed that final proof for either of the hypotheses can only be provided by direct structural investigations of enzyme/substrate complexes. However, such experiments are difficult to perform since they have to be carried out under strictly anaerobic conditions. Since such conditions can hardly be achieved on earth experiments in orbit may be helpful.

5. Materials and methods

5.1. Chemicals

The chemicals used were obtained from the following sources: arachidonic acid (5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid), HPLC standards of 5*S*-HETE, 12*S*-HETE, 15*S*-HETE and sodium borohydride from Serva (Heidelberg, Germany), ampicillin from Gibco (Eggenstein, Germany), isopropyl-β-D-thiogalactopyranoside (IPTG) from Sigma-Aldrich (Deisenhofen, 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). The *E. coli* strains HB 101 and M15 as well as the pQE-9 expression plasmid were purchased from Invitrogen (San Diego, CA, USA). Oligonucleotide synthesis was carried out by TiB-Molbiol (Berlin, Germany). All solvents were of HPLC grade and purchased from Baker (Deventer, The Netherlands). The polyenoic fatty acids used in this study were synthesised as reported before [26,27].

5.2. Preparation of the native rabbit 15-LOX

The native reticulocyte-type 15-LOX was prepared from the stroma-free haemolysis supernatant of a reticulocyte-rich blood cell suspension [28] by ammonium sulphate precipitation, hydrophobic interaction chromatography and anion exchange chromatography on a preparative Mono-Q column (Pharmacia, Uppsala, Sweden). The final enzyme preparation was electrophoretically pure ($<95\%$) and exhibited a linoleic acid turnover rate of about 30 s^{-1}.

5.3. Expression of the recombinant rabbit 15-LOX and enzyme purification

For convenient purification of the recombinant rabbit reticulocyte 15-LOX the enzyme was overexpressed as a His tag fusion protein in *E. coli*. To clone the cDNA into the expression plasmid a *Sal*I site was introduced at the starting methionine by PCR add-on mutation. The reverse PCR primer was placed in the cDNA coding region behind a unique *Sfi*I site. The PCR product was digested with *Sal*I and *Sfi*I and purified by gel electrophoresis. This *Sal*I/*Sfi*I fragment was ligated together with the *Sfi*I/*Hin*dIII restriction fragment of the LOX cDNA into the pQE-9 expression vector, which was previously cleaved with *Sal*I and *Hin*dIII to yield the 15-LOXpQE-9 expression plasmid. This procedure led to modification of the enzyme's N-terminus (Met-Arg-Gly-$(His)_6$-Gly-Ser-Val-Asp-LOX(Gly-Val-)), which did not influence the enzyme characteristics. The 15-LOXpQE-9 expression plasmid was used to transform *E. coli* (M15 cells) and cells were grown at 37°C in 10 ml LB medium containing 100 μg/ml ampicillin and 25 μg/ml kanamycin for 7 h. This culture was added to 400 ml of the same medium and grown at 37°C overnight to an approximate OD_{600} of 1. After addition of 1 mM IPTG, the culture was incubated at 30°C for 3 h and cells were pelleted by centrifugation. The expressed recombinant proteins were purified on a Ni-column using the Bug Buster Ni-NTA His bind kit from Novagen (Bad Soden, Germany). After cell lysis with the Bug Buster protein extraction reagent the lysis supernatant was incubated at 4°C with 0.5 ml of Ni-agarose equilibrated with 0.1

M phosphate buffer, pH 7.4 for 1 h in an overhead shaker. The agarose was transferred to a polypropylene column, washed twice with 4 ml washing buffer (10 mM imidazole, 500 mM NaCl, 20 mM Tris, pH 7.9) and the recombinant enzyme species were eluted with 2 ml elution buffer (200 mM imidazole, 500 mM NaCl, 20 mM Tris, pH 7.9).

To express the human 5-LOX as a non-fusion protein (the His tag 5-LOX was catalytically inactive) its cDNA was subcloned into the expression plasmid pKK 233-2 [9] and bacteria (HB 101) were transformed with the recombinant plasmid. Bacteria were grown overnight in 10 ml LB medium containing ampicillin (0.1 mg/l). 1 ml of this pre-culture was added to 200 ml LB medium (0.1 mg/l ampicillin) and the bacteria were grown at 37°C to an optical density at 600 nm of 1.0. Expression of the recombinant 5-LOX species (wild-type or mutant) was then induced with 1 mM IPTG and the culture was incubated for an additional 12 h at 30°C. Cells were spun down, washed with phosphate-buffered saline, resuspended in 2 ml of 50 mM TRA/HCl buffer, pH 7.3 containing 2 mM EDTA and 10 mM mercaptoethanol. Cells were lysed by sonication (three times for 20 s with a Labsonic U-tip sonifier), cell debris was removed by centrifugation and the lysis supernatant was used as the enzyme source. In selected experiments the recombinant enzyme species (wild-type and mutants) were purified by affinity chromatography on ATP Sepharose [9].

Our purification strategy for the two recombinant enzyme species and their mutants did not lead to electrophoretically homogeneous enzyme preparations, but all attempts of further purification were accompanied by severe losses in enzyme activity. In order to avoid misinterpretations of the experimental data most of the experiments were repeated with crude enzyme preparations (bacterial lysis supernatant). In neither case did we observe significant differences in the positional specificity of crude and purified enzyme preparations.

5.4. Site-directed mutagenesis

Site-directed mutagenesis of the LOX isoforms (I418A, F353L for the rabbit 15-LOX; F359W+A424I+N425M+A603I for the human 5-LOX) was carried out by the PCR-mediated overlap extension technique using mismatching synthetic oligonucleotides. The cDNA constructs were sequenced completely to verify their primary structures. At least 10 LOX-positive clones were assayed for their LOX activities and recombinants exhibiting the highest activity were used for the experiments.

5.4.1. Assay system

The oxygenation kinetics of the different fatty acid derivatives were assayed spectrophotometrically measuring the increase in absorbance at 235 nm. The assay mixture, which consisted of 0.1 M phosphate buffer, pH 7.4 containing various concentrations of substrate fatty acid, was sonicated in an ultrasonic bath before the reaction was started by addition of enzyme. Measurements were carried out at room temperature.

For analysis of the oxygenation products formed by the recombinant LOX species aliquots of partially purified enzyme preparations were incubated with various substrates (0.1 mM final substrate concentration) at room temperature for 15 min. For the 15-LOX and its mutants the assay mixture was 1 ml of sodium/potassium phosphate buffer, pH 7.4 containing 0.1 mM substrate fatty acid. The hydroperoxy fatty acids formed were reduced to the corresponding hydroxy derivatives with sodium borohydride. After acidification to pH 3 an equal volume of methanol was added, protein precipitate was spun down and aliquots were injected to RP- or SP-HPLC analysis. To analyse the product pattern of the wild-type and mutant 5-LOX aliquots of the bacterial lysis supernatant or of the purified enzyme preparation were incubated in 1 ml Tris–HCl buffer, pH 7.4 containing 0.1 mM arachidonic acid, 0.4 mM $CaCl_2$, 40 μg/ml dipalmitoyl phosphatidylcholine and 0.1 mM ATP. After 15 min of incubation the hydroperoxy compounds formed were reduced with sodium borohydride to the corresponding hydroxy derivatives, the mixture was acidified to pH 3 and 1 ml of ice-cold methanol was added. The protein precipitate was spun down and aliquots of the clear supernatant were injected to RP-HPLC quantification of the LOX products.

5.4.2. Analytics

RP-HPLC was performed with a Shimadzu LC-6A liquid chromatograph connected to a Hewlett Packard diode array detector 1040A. Separation of the fatty acid derivatives was performed on a Nucleosil C-18 column (Macherey-Nagel, Düren, Germany; 250×4 mm, 5 μm particle size) and a guard column (30×4 mm, 5 mm particle size, same vendor). For analysis of monohydroxylated fatty acids solvent systems consisting of methanol/water/acetic acid (80:20:0.1 or 70:30:0.1, v/v) and a flow rate of 1 ml/min were used. The absorbance at 235 nm was recorded. SP-HPLC was performed on a Nucleosil 100-7 column (Macherey-Nagel, Düren, Germany; 250×4 mm, 5 μm particle size) with the solvent system hexane/2-propanol/acetic acid and a flow rate of 1 ml/min. The 2-propanol content varied between 1 and 5% depending on the chemical structure of the substrate. For quantification of the chromatograms a calibration curve (five-point measurement at different concentrations) was established.

For GC/MS analysis the hydroxy fatty acids were prepared by RP- and/or SP-HPLC, methylated with diazomethane and their hydroxy groups were silylated with bis(trimethylsilyl)trifluoroacetamide (Sigma Chemicals, Deisenhofen, Germany) in dry pyridine. GC/MS was carried out on a Shimadzu GC-MS QP-2000 system equipped with a fused silica column SPB 1 (10 m×0.25 mm, coating thickness 0.25 μm). An injector temperature of 270°C, an ion source temperature of 180°C and an electron energy of 70 eV were adjusted. The derivatised fatty acids were eluted with the following temperature programme: from 130°C at a rate of 50°C/min to 200°C, then at a rate of 5°C/min to 260°C followed by an isothermal post-run of 10 min.

Negative ion mass spectrometry of the underivatised HPLC-pure hydroxy fatty acids was performed with an LCQ ion trap mass spectrometer (Thermoquest, Engelsbach, Germany) using electrospray ionisation at 4 kV. The hydroxy fatty acids were dissolved in a 1:1 mixture of 50 mM ammonium bicarbonate buffer pH 8.0 and methanol. Aliquots were injected with a syringe pump (3 μl/min) into the injection capillary, which was heated to 200°C. Molecular mass ions were detected in the range of *m/z* 100–500. Fragmentation of the selected molecular ions was carried out with a voltage ranging between 10 and 40 V.

5.4.3. Miscellaneous methods

Protein concentration of the LOX preparations was determined with the Bio-Rad kit. For more informative mass spectra the methylated trimethylsilyl derivatives of the polyenoic fatty acids

(10 μg dissolved in 1 ml of ethanol) were hydrogenated using 5 mg of palladium asbestos as the catalyst. Hydrogen gas was bubbled through this mixture for 2 min at room temperature. The solution was filtered to remove the catalyst, the solvent was evaporated and the residue was reconstituted in 20 μl of dodecane. Aliquots were injected to GC/MS as described above. It should be noted that hydrogenation was carried out before silylating the OH groups.

Acknowledgements

The authors wish to thank Mrs C. Gerth for technical assistance and Dr K. Janek for carrying out electrospray mass spectrometry. Financial support for this study was provided by the Deutsche Forschungsgemeinschaft (Ku 961/7-1) and by the European Community (BMH4-CT98-3191). Parts of this work were carried out during a sabbatical of I.I. at the Humboldt University.

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