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Identification of an amino acid determinant of pH regiospecificity in a seed lipoxygenase from *Momordica charantia* ☆

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ABSTRACT

Lipoxygenases (LOX) form a heterogeneous family of lipid peroxidizing enzymes, which catalyze specific dioxygenation of polyunsaturated fatty acids. According to their positional specificity of linoleic acid oxygenation plant LOX have been classified into linoleate 9- and linoleate 13-LOX and recent reports identified a critical valine at the active site of 9-LOX. In contrast, more bulky phenylalanine or histidine residues were found at this position in 13-LOX. We have recently cloned a LOX-isoform from *Momordica charantia* and multiple amino acid alignments indicated the existence of a glutamine (Gln599) at the position were 13-LOX usually carry histidine or phenylalanine residues. Analyzing the pH-dependence of the positional specificity of linoleic acid oxygenation we observed that at pH-values higher than 7.5 this enzyme constitutes a linoleate 13-LOX whereas at lower pH, 9-H(P)ODE was the major reaction product. Site-directed mutagenesis of glutamine 599 to histidine (Gln599His) converted the enzyme to a pure 13-LOX. These data confirm previous observation suggesting that reaction specificity of certain LOX-isoforms is not an absolute enzyme property but may be impacted by reaction conditions such as pH of the reaction mixture. We extended this concept by identifying glutamine 599 as sequence determinant for such pH-dependence of the reaction specificity. Although the biological relevance for this alteration switch remains to be investigated it is of particular interest that it occurs at near physiological conditions in the pH-range between 7 and 8.

1. Introduction

Lipoxgenases (linoleate:oxygen oxidoreductase; EC 1.13.11.12; LOX) are widely distributed in plant and animal kingdoms (Brash, 1999). They constitute a family of non-heme iron containing dioxygenases, which catalyze the regio- and stereoselective oxygenation of polyenoic fatty acids containing (1*Z*,4*Z*)-pentadiene systems (Liavonchanka and Feussner, 2006). In mammals, LOX are classified according to their positional specificity of arachidonic acid (AA) oxygenation (Kühn et al., 2005). Since AA is either not present in higher plants or is only a minor constituent of cellular lipids, plant LOX may be classified according to their positional specificity of linoleic acid (LA) oxygenation into 9- and 13-LOX (Feussner and Wasternack, 2002). A more comprehensive classification of plant LOX has been proposed based on the comparison of their primary structures (Shibata et al., 1994).

LOX are versatile enzymes catalyzing at least three different types of reactions: (i) Dioxygenation of lipid substrates (dioxygenase reaction), (ii) secondary conversion of hydroperoxy lipids (hydroperoxidase reaction), and (iii) formation of epoxy leukotrienes (leukotriene synthase reaction) (Feussner and Kühn, 2000). The mechanism of the dioxygenation reaction is rather complex and involves the formation of enzyme-bound radical intermediates (Kühn et al., 2005). The reaction is initiated by hydrogen abstraction from a bisallylic methylene (Hamberg and Samuelsson, 1967). The resulting carbon-centred radical intermediate is subsequently rearranged ([+2] or [−2] rearrangement) involving double bond conjugation (Fig. 1). For the time being there is no unifying

Abbreviations: AA, arachidonic acid; CP-HPLC, chiral phase-HPLC; RP-HPLC, reverse phase-HPLC; SP-HPLC, straight-phase-HPLC; hydroperoxy arachidonic acid, HPETE; 13-H(P)OD, (13,9*Z*,11*E*)-13-hydro(pero)xy-9,11-octadecadienoic acid; 9-H(P)OD, (9,10*E*,12*Z*)-9-hydro(pero)xy-10,12-octadecadienoic acid; LA, linoleic acid; LnA, α-linolenic acid; LOX, lipoxygenase(s).

☆ Sequence data: The nucleotide sequence reported in this paper has been submitted to the GenBank™ with Accession No. AM930395.

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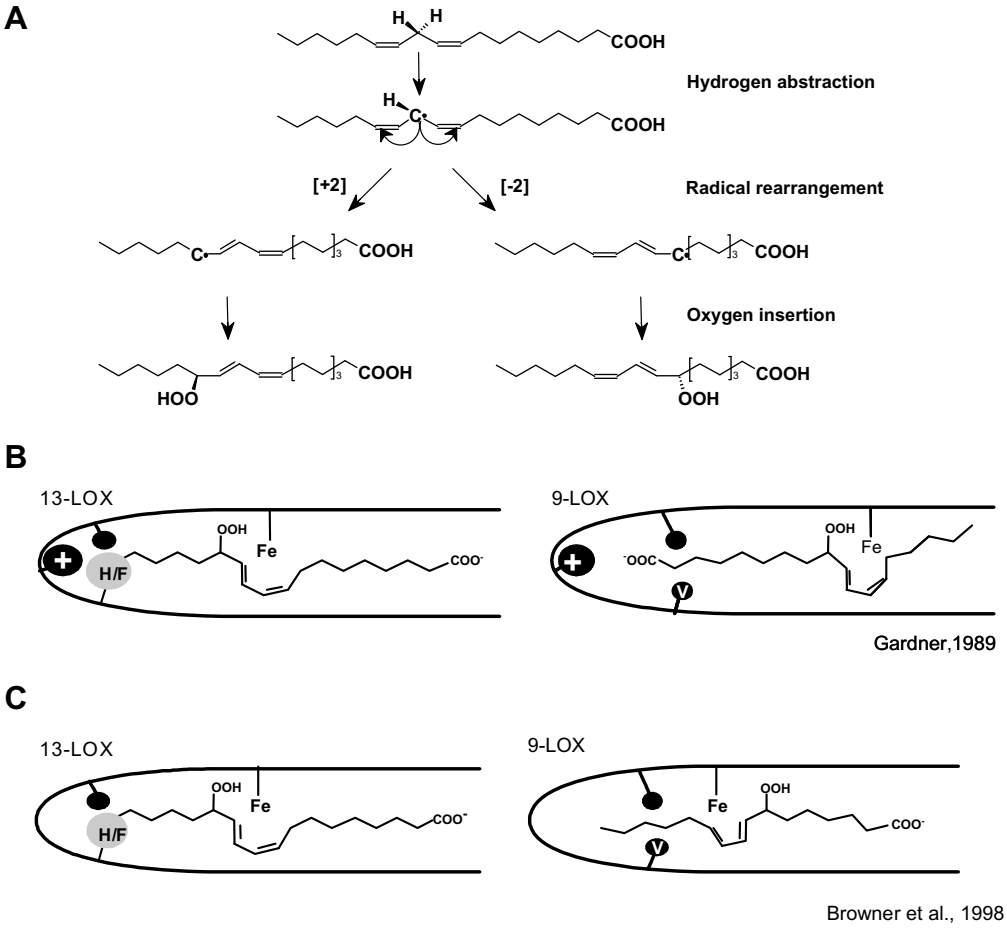

Fig. 1. LOX reaction mechanism and regio-specificity. Panel (A) specificity of LOX reaction with substrates containing one doubly allylic methylene. In this case, the specificity of the LOX reaction solely depends on the direction of the radical rearrangement. [+2] Radical rearrangement indicates that dioxygen is inserted at the second carbon atom in the direction of the methyl terminus of the substrate counted from the site of hydrogen removal. [–2] Indicates an inverse direction of radical rearrangement. Panels (B) and (C) Alternative explanations for the regio-specificity of LOX-isoforms: (B) model of inverse substrate orientation; and (C) space-related hypothesis of positional specificity.

concept explaining the structural basis for the reaction specificity of all LOX-isoforms. However, a number of critical amino acids have been identified at the active site of selected LOX-isoforms, which impact this enzyme property (Schneider et al., 2007). For mammalian LOX two alternative hypotheses have been developed to explain the reaction specificity. The space-related hypothesis (Browner et al., 1998) suggests that fatty acid substrate penetrate the active site with its methyl end ahead and that the volume of the substrate-binding pocket is important for the positional specificity. According to the orientation hypothesis substrate fatty acids may slide into the active site with its methyl end ahead leading to linoleate 13-lipoxygenation ([+2] radical rearrangement). However, when the substrate is forced into an inverse "head-to tail" orientation (Gardner, 1989) linoleate 9-lipoxygenation becomes plausible. For the cucumber lipid body 13-LOX (Hornung et al., 1999) a critical histidine residue was identified at the bottom of the substrate-binding pocket, which corresponds in amino acids alignment to a previously identified primary determinant for the positional specificity of the rabbit arachidonate 12/15-LOX (Sloane et al., 1991). Its replacement by less space filling residues altered the positional specificity from a linoleate 13-LOX to a 9-LOX by demasking a positively charged guanidino group. These volume-dependent charge alterations were suggested to favour "head-to tail" substrate orientation (Hornung et al., 1999) and thus, for this enzyme a combination of the two hypotheses may be applicable.

We recently cloned a LOX-isoform from *Mormordica charantia* seeds and found that it carries a glutamine at the position where other plant LOX harbour either histidine/phenylalanine (linoleate 13-LOX) or valine (linoleate 9-LOX) residues. Analyzing the reaction selectivity of this enzyme we found that it exhibits a variable positional specificity of linoleic acid oxygenation depending on the pH of the reaction mixture. Below pH 7.5; (9*S*,10*E*,12*Z*)-9-hydro-(pero)xy-10,12-octadecadienoic acid ((9*S*)-H(P)OD) was the major reaction product whereas at higher pH (13*S*,9*Z*,11*E*)-13-hydro-(pero)xy-9,11-octadecadienoic acid ((13*S*)-H(P)OD) was dominant. The structural basis for this variable reaction specificity was explored by site directed mutagenesis studies.

2. Results

2.1. Cloning of *M. charantia* seed LOX

To clone an unknown LOX-isoform from *M. charantia* an EST-library from developing seeds was analyzed. Database searches and nucleotide alignments revealed a cDNA of 1673 bp with significant similarity to other plant LOX. The deduced amino acid sequence exhibited highest degree of similarity to the lipid body LOX from cucumber (Accession No. X92890). To create a full-length cDNA clone, 5′-RACE was performed and the RACE-fragment was fused with the EST. The complete cDNA had a length of 2930 bp. The open reading frame named MoLOX1 (Accession No. AM930395)

had a size of 2724 bp encoding a protein of 881 amino acids with a theoretical molecular weight of 99.5 kDa. The sequence showed the highest degree of similarity to cucumber lipid body linoleate 13-LOX (82%). Additional sequence analysis revealed that MoLOX1 does not contain a putative chloroplast transit peptide and that the putative iron ligands (three histidines, one asparagine and the C-terminal isoleucine) are well conserved (Liavonchanka and Feussner, 2006). Inspection of active site residues critical for the regiospecificity indicated that this enzyme harbours a glutamine at the position 599 where phenylalanine or histidine are found in other plant 13-LOX. In linoleate 9-LOX a valine residue is located at this position (Table 1). Moreover the enzyme harbours an alanine at the position 584 suggesting an *S*-LOX (Coffa and Brash, 2004).

Table 1
Alignment of amino acid residues possibly determining the positional specificity of plant LOX according to Liavonchanka and Feussner (2006)

Enzyme	Accession No.	Position of amino acid residues	Amino acid residues
13-LOX:			
Cucumber lipid body LOX	X92890	596/597	Thr/His
Soybean seed LOX-1	P08170	556/557	Thr/Phe
Potato LOX-H1	X96405	614/615	Ser/Phe
Arabidopsis LOX-4	CAC19364	642/643	Cys/Phe
Momordica charantia	AM930395	598/599	Thr/Gln
9-LOX:			
Potato tuber LOX	P37831	575/576	Thr/Val
Tobacco elicitor-Induced LOX	X84040	580/581	Thr/Val
Barley grain LOX-A	L35931	574/575	Thr/Val
Arabidopsis LOX-1	Q06327	576/577	Thr/Val

2.2. Enzymatic characterization of M. charantia seed LOX

For enzymatic characterization MoLOX1 was expressed as his-tagged fusion protein in *E. coli*. Under standard assay conditions (0.9 mM fatty acid substrate, room temperature, 30 min incubation period, pH 8.0) AA was almost exclusively converted to (15*S*)-H(P)ETE (hydroperoxy arachidonic acid) and (13*S*)-H(P)OD was identified as major product of LA oxygenation (Fig. 2). These data identified MoLOX1 as linoleate 13-LOX and arachidonate 15-LOX. The enzyme exhibits a broad substrate specificity metabolizing various C18 and C20 fatty acids. Substrate preference was determined by incubating the enzyme for 30 min at pH 8.0 with an equimolar mixture of LA, AA and α-linolenic acid (LnA). Afterwards the reaction was stopped and product formation was quantified. Under these conditions AA oxygenation was twofold higher than the formation of C18-derived products.

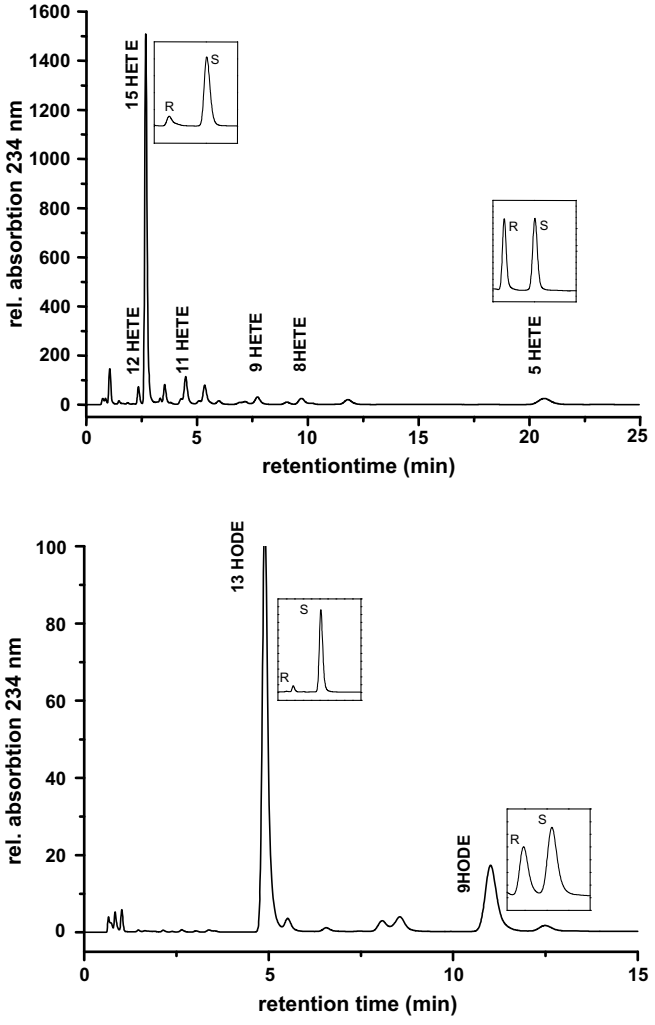

Fig. 2. HPLC analysis of hydroxy fatty acids formed from AA and LA or AA by MoLOX1. MoLOX was incubated with 0.9 mM LA or AA at room temperature for 30 min. After reduction of lipids with sodium borohydride, the reaction mixture was acidified and the lipids were extracted. Oxygenated fatty acid derivatives were isolated by RP-HPLC, and positional isomers were analyzed by SP-HPLC. Ratios of *S* and *R* were determined by CP-HPLC (Insets). In the upper panel the right inset shows the *S*/*R*-ratio for 5-H(P)ETE being representative for all others except the one of 15-H(P)ETE which is shown as left inset.

Next, we recorded the pH-profile of linoleic acid oxygenation and explored the impact of pH-alterations on the reaction specificity. From Fig. 3A it can be seen that the global activity optimum was around pH 8. In addition, there were two putative local pH optima below pH 7.5 but owing the large errors ranges the activity differences were not significant. It should be stressed at this point that except from the pH all other reaction parameters were kept constant. When the reaction specificity of linoleic acid oxygenation was analyzed over a broad pH-range, we found that at pH < 7.4 MoLOX1 oxygenated LA preferentially at carbon 9 of the fatty acid chain. In contrast, above this pH 13-H(P)OD was the major oxygenation product (Fig. 3A). Thus, there was a clear pH-dependence in the positional specificity of linoleic acid oxygenation and this alteration was observed in the near physiological range. Chiral HPLC of the major positional isomers indicated that 13-H(P)OD was preferentially formed as *S* enantiomer (<85%) and that the *S*/*R*-ratio did hardly vary over the entire pH-range (Fig. 3B). In contrast, the enantiomers composition of 9-H(P)OD was pH-dependent. Below pH 7.5 a strong preponderance of the *S* enantiomer was observed. However, at higher pH-values the relative share of (9*R*)-H(P)OD became increasingly abundant reaching a 1:1 ratio at pH 9 (Fig. 3C). Taken together these results indicate that MoLOX1 constitutes a seed LOX that exhibits a variable reaction specificity depending on the pH of the reaction mixture. Similar enzymes have been characterized before in peas, soybean and corn seed as well as in potato tubers (Egmond et al., 1972; Gardner, 1989; Hughes et al., 2001a,b).

2.3. Site-directed mutagenesis studies affecting regioselectivity

As indicated in Table 1 Gln599 of MoLOX1 aligns with amino acids of other LOX, which have previously been identified as sequence determinants for the positional specificity. To explore whether this amino acid may be involved in pH-dependent alterations of the positional specificity we mutated this residue to phenylalanine or histidine, which are normally found at this position in linoleic 13-LOX. The positional specificity of the resulting mutants was compared with that of the wild-type enzyme over a pH-range

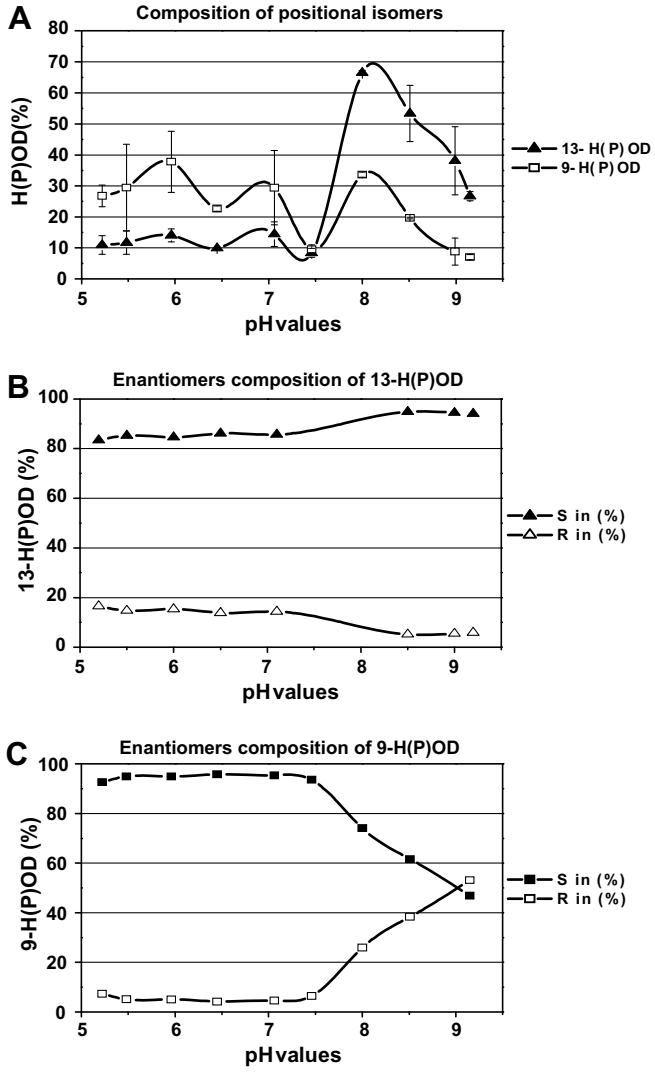

Fig. 3. pH-dependence of the reaction specificity of linoleic acid oxygenation catalyzed by recombinant MoLOX. MoLOX was incubated with 0.9 mM of LA at room temperature for 30 min at variable pH-values. The reaction was terminated by addition of a molar excess of sodium borohydride, which reduces the hydroperoxy fatty acids formed to the corresponding alcohols. After acidification, total lipids were extracted and the oxygenated fatty acid derivatives were purified by preparative RP-HPLC. The composition of the positional isomers was subsequently analyzed by SP-HPLC (panel A, solid triangles – 13-H(P)OD, empty squares – 9-H(P)OD). The enantiomers composition of both, 13-H(P)OD (panel B, solid triangles *S*-isomer, empty triangles – *R*-isomer) and 9-H(P)OD (panel C, solid squares – *S*-isomer, empty squares – *R*-isomer) was analyzed by chiral phase-HPLC as indicated in Section 4. The figure represents the mean value and error range of two experiments. The error bars represent repetitions of the experiments with a single enzyme preparation. Maximal product formation (pH 8.0) was set to 100%. This relative level corresponded to the formation of about 10 μM H(P)OD per incubation sample.

from 5 to 9.5. As indicated in Fig. 4A the wild-type enzyme was a linoleate 13-LOX above pH 7.5 but below this value it functioned as a linoleate 9-LOX. The Q599F-mutant lost its 13-LOX activity at higher pH-values (Fig. 4B). In fact, at pH-values above 7.5 a rather unspecific product pattern (1:1 ratio of 13-H(P)ODE and 9-H(P)ODE) was observed with this enzyme mutant. The unspecific oxygenation reaction was also indicated by the *R*/*S*-ratio (4:6) of the major reaction products. In contrast, the Q599H-mutation converted the enzyme to a linoleate 13-LOX lacking any pH sensitivity (Fig. 4C). The pH optimum remained unaffected, and the major product isomers were in the *S*-configuration.

3. Discussion

The structural basis for the high reaction specificity of most LOX-isoforms has been studied for many years (Veldink and Vliegenthart, 1991; Kühn et al., 2005; Schneider et al., 2007), but still many aspects remain unclear. For various LOX-isoforms sequence determinants for the reaction specificity have been characterized but there is no unifying concept for all LOX variants. In principle, the LOX reaction consists of four elementary reactions (hydrogen abstraction, radical rearrangement, oxygen insertion, peroxide reduction) and during LOX-catalyzed fatty acid oxygenation the stereochemistry of the elementary reactions is tightly controlled. A major element for the positional specificity of initial hydrogen abstraction is the distance of the bisallylic methylene from the reactive non-heme iron centre. The stereochemistry of oxygen insertion is far more complex and various control elements have recently been reviewed (Kühn et al., 2005; Schneider et al., 2007). (i) Shielding: This concept suggests that fatty acid substrates are bound at the active site of LOX in such a way that only one of reactive carbon atoms of substrate molecules may be accessible for hydrogen abstraction and oxygen insertion. Reactivity of other potential candidates is impaired by interaction with the enzyme molecule. (ii) Oxygen channelling: Atmospheric dioxygen may selectively be transported from the reaction buffer to the site of oxygen insertion via preformed oxygen access channels and thus, the position of oxygen insertion is predetermined by the protein structure. Mutagenesis studies and molecular dynamic simulations on the arachidonic acid 15-LOX from soybeans (Knapp and Klinman, 2003) and rabbit reticulocytes (Saam et al., 2007) supported the concept of targeted intra-enzyme oxygen movement. It should, however, been stressed that for the rabbit enzyme the oxygen access channel does not constitute a continuously open channel but rather a chain of cavities which are temporarily interconnected by movement of amino acid site chains (Saam et al., 2007). (iii) Peroxy radical trapping: This concept is based on the idea that oxygen addition at the carbon-centred radical forming the oxygen centred peroxy radical is a reversible reaction and occurs randomly during LOX catalysis. Formation of a distinct product depends on selective reduction of a certain peroxyl radical to the corresponding hydroperoxide. This mechanism requires favourable positioning of an electron donor. (iv) Substrate twisting: In non-enzymatic lipid peroxidation the carbon-centred pentadienyl radical adopts a planar configuration, which allows oxygenation at multiple positions and chirality. At the active site of an enzyme the pentadienyl radical may be twisted, the radical electron is delocalized and oxygenation takes place at a preferred position and with selective chirality.

Since positional specificity is an important enzymatic parameter, which should be determined for all LOX-isoforms, we first characterized this enzyme property for the novel MoLOX1 under standard conditions (Fig. 2). Next, we recorded the pH-profile of linoleic acid oxygenation and found that pH-alterations impacted the positional specificity of linoleate oxygenation (Fig. 3). Interestingly, these changes occurred in a narrow pH-range at near physiological conditions. To explore the mechanistic basis of this phenomenon we performed site-directed mutagenesis studies and found that Gln599His and Gln599Phe mutants exhibited reduced pH sensitivity (Fig. 4). In fact, the Gln599His mutant oxygenated LA mainly to 13-H(P)ODE independent of the pH of the reaction mixture, which contrasts the pH-dependent alterations of the wild-type enzyme. The mechanistic basis for these effects remains to be investigated. In principle, pH changes impact the acid/base properties of both, fatty acid substrates and amino acid site chains. However, previous studies suggested that in the water bulk phase the vast majority of fatty acid molecules are present as

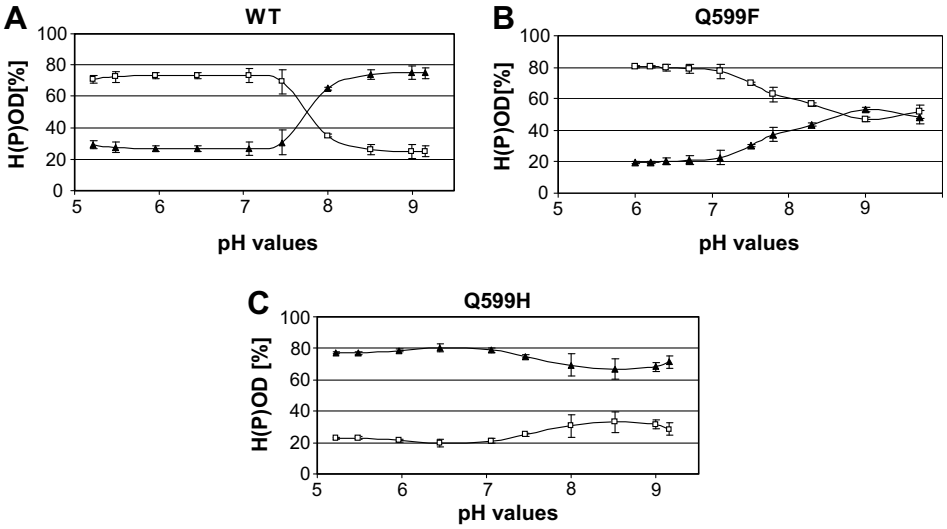

Fig. 4. Reaction specificity of various MoLOX1 mutants using LA as substrate. Wildtype and mutant MoLOX1 species expressed in *E. coli* were incubated with 0.9 mM of LA at room temperature for 30 min at variable pH-values. After reduction of the primary reaction products with sodium borohydride, the reaction mixture was acidified and the lipids were extracted. Oxygenated fatty acid derivatives were isolated by RP-HPLC and the mixture of positional isomers was analyzed by SP-HPLC (13-H(P)OD, black triangles; 9-H(P)OD, white squares). The sum of 9- and 13- H(P)OD formed was set to 100% for each LOX species. (A) wild-type, (B) Q599F, and (C) Q599H. Panel (A) represents two independent experiments, all other panels three of them. Repetitions were performed with aliquots from a single enzyme preparation.

uncharged molecules owing to the high pKa of the fatty acid carboxylate in aqueous solutions (Kanicky and Shah, 2002, 2003). These data suggest that the degree of dissociation of the fatty acids in the reaction mixture (water bulk phase) is only slightly altered between pH 7 and 9. Thus, alterations in the acid/base properties of the amino acid site chains appear to be more important. It should, however, been stressed that the physico-chemical state of the fatty acid molecules in the water bulk phase may not necessarily mirror the situation at the active site. If basic residues are accessible at the active site they may act as proton acceptors to force dissociation of the carboxylate regardless of the situation in the water bulk phase. On the other hand, when acidic residues are present the substrate's carboxylate may be protonated and potential salt bridges, which might contribute to substrate-binding, are broken. It should, however, been stressed that it is rather difficult to quantify the association/dissociation behaviour of a fatty acid molecule inside the active site of an enzyme and thus, the discussion on the physico-chemical state of the substrate molecules is rather speculative.

If pH-alterations do not impact the physico-chemical state of the substrate, pH-dependent changes in the structure of the active site are more likely the reasons for the alterations in reaction specificity. Gln599 carries a non-dissociable side chain and thus, this amino acid itself may not be sensitive for pH-alterations. However, our studies clearly indicated that mutation of Gln599 to a more bulky amino acid induced reduction of pH sensitivity. To explain these data one might suggest that Gln599 modifies the interaction of an acid/base sensitive residue at the active site with the substrate's carboxylate. When we introduced a more bulky site chain (phenylalanine, histidine) at position 599 this acid/base residue may be shielded so that the interaction with the fatty acid is weakened. To identify such an acid/base sensitive amino acid we searched the immediate surrounding of Gln599 in the 3D-model of MoLOX1, which was structured on the basis of the X-ray coordinates for the soybean-LOX1, and identified as candidate amino acid Gln537. This amino acid residue was mutated into leucine, isoleucine, asparagine or glutamic acid. However, all mutants turned out to be inactive (data not shown). In addition to these spatial effects local charge alterations need to be considered for the Gln599His mutant. In contrast to phenylalanine, which is insensitive for pH-alterations, histidine is an amino acid that changes its degree of dissociation depending on pH. At alkaline pH-values the imidazole site chain delivers a proton losing its positive charge. This alteration may favour a "head-to head" substrate orientation (Fig. 1B), in which the fatty acid enters the active site with its methyl end ahead, which is consistent with our experimental data.

As indicated above a key problem for the understanding of the reaction specificity of LOX is the alignment of fatty acid substrates and reaction intermediates at the active site. Unfortunately, for the time being there is little direct experimental information on this interaction. In the absence of any structural data the first concepts for the regiocontrol of the LOX reaction were developed on the basis of the product structure and our knowledge of the stereochemistry of hydrogen abstraction (Egmond et al., 1972; Gardner, 1989; Veldink et al., 1972). Later on, when X-ray coordinates and mutagenesis data became available these models were refined and modified (Kühn et al., 2005; Schneider et al., 2007). Unfortunately, the current models are solely based on static experimental data and do not consider the high degree of motional flexibility of both, fatty acid substrates and LOX. Recent molecular dynamics simulations (Saam et al., 2007), small angle X-ray scattering measurements (Hammel et al., 2004; Daniese et al., 2005) and fluorescence life time studies (Mei et al., 2008) have visualized the conformational flexibility but exact quantification is still pending. However, we now know that movement of amino acid side chains induces frequent opening and closure of preformed cavities in the 3D-structure of LOX and that there are considerable differences in the motional flexibility of LOX-isoforms. For interpretation of future experiments dynamic feature of protein–substrate interaction should increasingly been considered.

4. Experimental

4.1. Materials

The chemicals used were from the following sources: Standards of chiral and racemic hydroxy fatty acids from Chayman Chem. (Ann Arbor, MI, USA) methanol, hexane, 2-propanol (all HPLC

grade) from Baker (Griesheim, Germany). Restriction enzymes were purchased from MBI Fermentas (St. Leon-Rot, Germany).

4.2. Plant growth

M. charantia was grown from seeds in a greenhouse under 16 h of artificially supplemented illumination at a temperature ranging between 22 °C and 30 °C. For all experiments developing seeds from green fruits were used.

4.3. RNA and cDNA isolation and protein expression

For RNA isolation 20 g developing seeds were shelled, ground in liquid nitrogen, 200 ml of extraction buffer I (100 mM Tris–HCl, pH 7.5, 25 mM EDTA, 2% (w/v) laurylsarcosyl, 4 M guanidinium thiocyanate, 5% (w/v) PVPP, 1% (v/v) β-mercaptoethanol) was added, further homogenized with an Ultraturrax and the homogenate was shaken for 10 min. After centrifugation at 3000*g* for 15 min the floating solid lipid phase and the pellet were discarded and the remaining liquid phase was extracted with an equal volume of PCI (phenol:chloroform:isoamyl alcohol, 20:20:1). After centrifugation at 3000*g* for 10 min the hydrophilic phase was re-extracted with an equal volume of chloroform and the centrifugation step performed as before. The hydrophilic phase was loaded on a CsCl cushion (5 M CsCl) of 8 ml and centrifuged at 18 °C and 100,000*g* for 18 h. The RNA precipitate was dried, resuspended and extracted for 15 min in a mixture consisting of 7.5 ml extraction buffer II (100 mM Tris–HCl, pH 8.8, 100 mM NaCl, 5 mM EDTA, 2% (w/v) SDS) and 10 ml PCI. Centrifugation and washing of the hydrophilic phase with chloroform followed and the RNA was precipitated from the hydrophilic phase by an equal volume of 5 M LiCl overnight at 4 °C. After centrifugation for 30 min at 12,000*g* at 4 °C the precipitate was washed with 70% ethanol, dried and dissolved in 1 ml water. From this total RNA fraction poly(A)$^+$ RNA was enriched using the Poly-Attract-Kit (Promega, Mannheim, Germany) according to the provided manual and used for all further experiments.

Five microgram poly(A)$^+$ RNA were used for the construction of a cDNA-library in Lambda ZAPII (Stratagene, Amsterdam, The Netherlands). The resulting library consisted of cDNA inserts cloned directionally (5′–3′) in the *EcoR*I and *Xho*I sites of pBluescript SK-vector in Lambda ZAP II and were stored as DMSO stocks at −80 °C until used for expressed sequence tag (EST) isolation.

EST-sequencing of 1600 clones was performed using this cDNA-library and an EST-clone with a putative LOX-sequence was identified. To obtain a full-length cDNA clone a 5′-end RACE was performed using the Marathon cDNA amplification kit (Clontech, Heidelberg, Germany) and the LOX-specific primer A: 5′-CAC TTG AAA CAC AGA CTC TCC AGC G-3′. The PCR reaction was carried out according to the supplier's instruction. The RACE-fragment was clone in pGEM-T Easy (Promega, Mannheim, Germany) and sequenced. To isolate the full length cDNA clone by PCR, specific primers of the expected open reading frame containing suitable recognition sites for specific restriction endonucleases were used for amplification: sense primer B: 5′-CGG GAT CCA TGT TTG GGA TTG GGA AGA G-3′, antisense primer C: 5′-GTG GGT CGA CTT ATA TGG AGA TAC TGT TGG G-3′. The PCR reaction was carried out with the Expand High Fidelity System (Roche Diagnostics, Mannheim, Germany) using an amplification program of 2 min denaturation at 94 °C, followed by 10 cycles of 30 s at 94 °C, 30 s at 52 °C, 3 min at 72 °C, followed by 18 cycles of 30 s at 94 °C, 30 s at 52 °C, 3 min at 72 °C (time increment 5 s) and terminated by 5 min extension at 72 °C. The resulting fragment was cloned into pGEM-T Easy and sequenced. For expression in *E. coli* the open reading frame was cloned into pQE30 (Qiagen, Hilden, Germany) as a *BamH*I/*Sal*I-fragment to yield pQE-MoLOX and the plasmid was transformed in *E. coli* strain SG13009. Expression was performed by growing cells at 37 °C to an OD_{600} of 0.6, chilling the cells on ice, inducing the cells with 1 mM IPTG and cultivating them at 10 °C for 50–60 h. Cells were harvested by centrifugation at 4 °C at 3000*g*, cells from 100 ml culture were resuspended in 10 ml lysis buffer (50 mM Tris–HCl, pH 7.5, 500 mM NaCl, 10% (v/v) glycerol, 0.1% (v/v) Tween 20) and lysed by sonification. The lysate was incubated with fatty acids for 30 min at RT, the resulting hydroperoxides were reduced with sodium borohydride, acidified with glacial acetic acid to pH 3 and lipids were extracted as described (Bligh and Dyer, 1959). Analysis of the fatty acids was performed on a HPLC.

4.4. Site-directed mutagenesis

Site-directed mutagenesis of pQE-MoLOX was carried out by using the QuikChange site-directed mutagenesis kit (Stratagene, Amsterdam, The Netherlands). The oligonucleotides used for mutagenesis contained apart from the appropriate base changes additional conservative base exchanges which created new restriction sites for the identification of mutants. The following oligonucleotides were used: MoLOX-Q599Ha 5′-AAC GCG GGT GGT CTT ATT GAA AGT ACT CAC TTT CCG GCA AAG TAT GCT-3′; MoLOX-Q599Hb 5′-AGC ATA CTT TGC CGG AAA GTG AGT ACT TTC AAT AAG ACC ACC CGC GTT-3′; MoLOX-Q599Fa 5′-AAC GCG GGT GGT CTT ATT GAA AGT ACT TTC TTT CCG GCA AAG TAT GCT-3′; MoLOX-Q599Fb 5′-AGC ATA CTT TGC CGG AAA GAA AGT ACT TTC AAT AAG ACC ACC CGC GTT-3′. In addition all mutations were sequenced and at least three different bacterial colonies were expressed and used for analysis of enzymatic parameters.

4.5. Analytics

HPLC analysis was carried out on an Agilent (Waldbronn, Germany) 1100 HPLC system coupled to a diode array detector. RP-HPLC of the free fatty acid derivatives was carried out on a Nucleosil C-18 column (Macherey-Nagel, Düren, FRG; 250 × 2 mm, 5 μm particle size) with a solvent system of methanol/water/acetic acid (90/10/0.1; by vol.) and a flow rate of 0.18 ml/min. The absorbances at 234 nm (conjugated diene system of the hydroxy fatty acids) and 210 nm (polyenoic fatty acids) were recorded simultaneously. Oxygenated linoleic acid containing triacylglycerides were separated on a Nucleosil C-18 column (Macherey-Nagel, Düren, FRG; 250 × 4 mm, 5 μm particle size) using a flow rate of 1 ml/min and a binary gradient system; solvent A: methanol/water/acetic acid (90/10/0.1; by vol.); solvent B: methanol/acetic acid (100/0.1, by vol.) and the following gradient program: 10 min at 100% solvent A, then in 20 min with a linear increase of solvent B to 100% solvent B followed by an isocratic postrun of 50 min at 100% B. The absorbance at 234 nm was recorded. Straight phase-HPLC (SP-HPLC) of hydroxy fatty acid isomers was carried out on a Phenomenex Luna Silica column (Aschaffenburg, Germany; 50 × 4.6 mm, 3 μm particle size) with a solvent system of *n*-hexane/2-propanol/acetic acid (100/1/0.1, by vol.) and a flow rate of 1 ml/min. The enantiomer composition of the hydroxy fatty acids was analyzed by chiral phase-HPLC on a Chiralcel OD-H column (Diacel Chem. Industries, distributed by Merck, Darmstadt, Germany; 150 × 2.1 mm, 5 μm particle size) with a solvent system of hexane/2-propanol/acetic acid (100/5/0.1, by vol.) and a flow rate of 0.1 ml/min.

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