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Properties of a mini 9*R*-lipoxygenase from *Nostoc* sp. PCC 7120 and its mutant forms

Alexandra-Zoi Andreou [a], Marian Vanko [a,b], Lydia Bezakova [b], Ivo Feussner [a,*]

[a] Georg-August-University of Göttingen, Albrecht-von-Haller-Institute of Plant Sciences, Department of Plant Biochemistry, Justus-von-Liebig-Weg 11, D-37085 Göttingen, Germany
[b] Comenius University, Department of Cell and Molecular Biology of Drugs, Faculty of Pharmacy, Kalinčiakova 8, 832 32 Bratislava, Slovakia

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ABSTRACT

Lipoxygenases (LOXs) consist of a class of enzymes that catalyze the regio- and stereospecific dioxygenation of polyunsaturated fatty acids. Current reports propose that a conserved glycine residue in the active site of *R*-lipoxygenases and an alanine residue at the corresponding position in *S*-lipoxygenases play a crucial role in determining the stereochemistry of the product. Recently, a bifunctional lipoxygenase with a linoleate diol synthase activity from *Nostoc* sp. PCC7120 with *R* stereospecificity and the so far unique feature of carrying an alanine instead of the conserved glycine in the position of the sequence determinant for chiral specificity was identified. The recombinant carboxy-terminal domain was purified after expression in *Escherichia coli*. The ability of the enzyme to use linoleic acid esterified to a bulky phosphatidylcholine molecule as a substrate suggested a tail-fist binding orientation of the substrate. Site directed mutagenesis of the alanine to glycine did not cause alterations in the stereospecificity of the products, while mutation of the alanine to valine or isoleucine modified both regio- and enantioselectivity of the enzyme. Kinetic measurements revealed that substitution of Ala by Gly or Val did not significantly influence the reaction characteristics, while the A162I mutant showed a reduced v_{max}. Based on the mutagenesis data obtained, we suggest that the existing model for stereocontrol of the lipoxygenase reaction may be expanded to include enzymes that seem to have in general a smaller amino acid in *R* and a bulkier one in *S* lipoxygenases at the position that controls stereospecificity.

1. Introduction

Lipoxygenases (LOXs) are a family of structurally related non-heme iron containing dioxygenases (Brash, 1999). They catalyse the insertion of molecular oxygen into polyunsaturated fatty acids (PUFAs) that contain one or more 1,4-pentadiene moieties to give the corresponding hydroperoxides (Liavonchanka and Feussner, 2006). LOXs occur ubiquitously in plants and mammals, but have also recently been detected in coral, moss and a number of bacteria (Kühn et al., 2005; Lang and Feussner, 2007; Liavonchanka and Feussner, 2006). LOX products can be further metabolized to yield aldehydes and jasmonates in plants (Feussner and Wasternack, 2002) and lipoxins and leukotrienes in mammals (Samuelsson et al., 1987; Sigal et al., 1994). These signaling molecules play an important role in wound healing and defense processes in plants while in mammals they are involved in inflammation, asthma and heart disease. Very little is still known about the biological function of these enzymes in prokaryotes.

LOX classification in plants is done with respect to their positional specificity of fatty acid oxygenation against linoleic acid (LA). LA can be oxygenated either at carbon atom 9 (9-LOX) or at C-13 (13-LOX) of the hydrocarbon backbone, which leads to the formation of 9-hydroperoxy- and 13-hydroperoxy derivatives of LA (9- and 13-HPODE), respectively (Liavonchanka and Feussner, 2006). In mammals LOXs are similarly classified according to their positional specificity of arachidonic acid (AA) oxygenation, which can take place either at positions C-5 (5-LOX), C-8 (8-LOX), C-9 (9-LOX), C-11 (11-LOX), C-12 (12-LOX) or C-15 (15-LOX) (Schneider et al., 2007). Besides the high regiospecificity, the insertion of oxygen exhibits also high stereospecificity dependent on the primary sequence of the enzyme, which is predicted to determine the orientation and depth of substrate penetration into the active site (Feussner and Kühn, 2000; Schneider et al., 2007). While the regiospecificity of LOXs has been the focus of a number of studies, recent publications describe the importance of a conserved amino acid in the active site of the enzyme (Coffa et al., 2005). The so called "Coffa site" is reported to be a conserved alanine in *S*-specific LOXs and a glycine in all *R*-LOXs. Mutational studies converting the glycine to an alanine in enzymes of the latter category succeeded in partially switching the position of oxygenation and chirality of

Abbreviations: ALA, α-linolenic acid; ARA, arachidonic acid; CP-HPLC, chiral phase-HPLC; HODE, hydroxy octadecadienoic acid; HOTE, hydroxy octadecatrienoic acid; H(P)ETE, hydro(pero)xy eicosatetraenoic acid; H(P)ODE, hydro(pero)xy octadecadienoic acid; LA, linoleic acid; LOX, lipoxygenase; PUFA, polyunsaturated fatty acid; RP-HPLC, reversed phase-HPLC; SP-HPLC, straight phase-HPLC.

* Corresponding author. Tel.: +49 551 395743; fax: +49 551 395749.
E-mail address: ifeussn@gwdg.de (I. Feussner).

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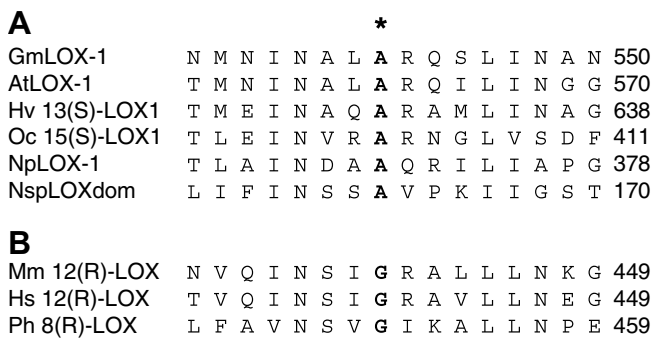

Fig. 1. Alignment of ΔNt-NspLOX with other *R* and *S* LOXs. Comparison of NspLOX (acc. no. NP_478445) from *Nostoc* sp. with: (A) the *S*-LOXs GmLOX1 from *Glycine max* (acc. no. S25064), AtLOX1 from *Arabidopsis thaliana* (acc. no. JQ2267), HvLOX1 from *Hordeum vulgare* (acc. no. U56406), Oc15LOX from *Oryctolagus cuniculus* (acc. no. M27214), NpLOX1 from *Nostoc punctiforme* (acc. no. ZP_00106490) and (B) the *R*-LOXs M12(*R*)-LOX from *Mus musculus* (acc. no. Y14334), Hs12(*R*)-LOX from human (acc. no. AF038461) and the LOX domain of Ph 8(*R*)-LOX from *Plexaura homomalla* (acc. no. AF003692). The "Coffa site", A in the case of *S*-LOXs and G in the case of *R*-LOXs is highlighted.

the product, thus converting a 13*S*- into a 9*R*-LOX enzyme (Coffa et al., 2005). The only reported exception among *S*-enzymes is the mouse platelet-type 12*S*-LOX which has a serine in this position (Coffa and Brash, 2004). Recently, a naturally occurring LOX with linoleate diol synthase activity from the filamentus cyanobacterium *Nostoc* sp. was reported (Lang et al., 2008). Sequence alignment of the protein revealed that an alanine aligned with the "Coffa site" and therefore suggested that the protein would be a 13*S*-LOX (Fig. 1). However, oxylipin measurements from *Nostoc* sp. PCC7120 as well as expression of the recombinant NspLOX carboxy-terminal portion (ΔNt-NspLOX) exhibited that the protein harbours a linoleate 9*R*-LOX activity. The aim of this study is to further characterize the enzyme and undertake mutagenesis experiments and examine whether substitution of this alanine with bulkier and smaller amino acids could alter the stereospecificity.

2. Results

2.1. ΔNt-NspLOX protein purification and kinetic determinations

In order to characterize the mini LOX domain of NspLOX (ΔNt-NspLOX) in more detail the recombinant ΔNt-NspLOX was expressed in BL21 (DE3) star cells and was initially purified using Ni affinity chromatography (Fig. 2A). Subsequently, the pooled fractions were further purified with gel filtration to a single 280-nm UV peak (Fig. 2B). At the end of the purification a unique protein band could be detected at electrophoretic homogeneity. The final protein yield was 10 mg/ml starting from 200 ml of bacterial expression culture. When the recombinant protein was incubated either with LA or α-linolenic acid (ALA), which are the fatty acids that have been previously been detected in *Nostoc* sp. (Lang et al., 2008), it converted the substrates to about 90% of (9*R*)-HPODE (Table 1 and Fig. 3A) or (9*R*)-HPOTE, respectively, and (*E*,*E*)-products were only minor products (1–2%). Thus this LOX produced only kinetically controlled products. The steady state kinetic parameters determined (Table 1) indicate that LA binds with higher affinity at the active site of ΔNt-NspLOX than ALA, while on the other hand the catalytic activity (v_{max}) of the enzyme was almost double in the case of ALA in comparison to LA.

2.2. ΔNt-NspLOX activy with PC as substrate

The enzyme's activity against esterified polyenoic fatty acids was investigated to establish the substrate binding orientation.

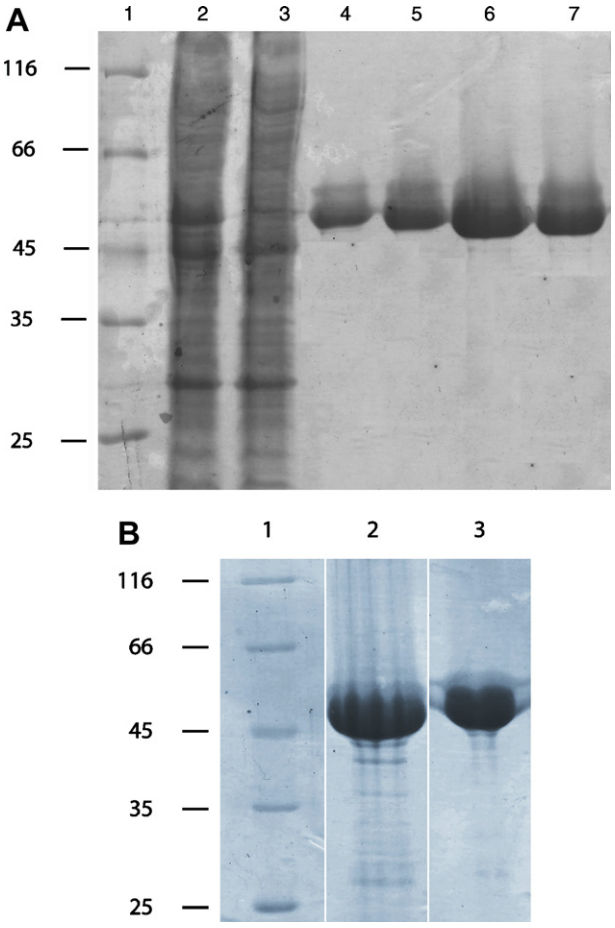

Fig. 2. SDS–PAGE analysis of the purification ΔNt-NspLOX. (A) His-trap purification: Lane 1, protein ladder; lane 2, BL21[DE3] star cell extracts; lane 3, column flow-through, lanes 4–7: elution fractions. (B) Lane 1, protein ladder; lane 2, purified enzyme, after Ni affinity chromatography; lane 3, purified enzyme after gel filtration. The purified ΔNt-NspLOX had a molecular weight of 50 kDa as shown by the single protein band. The experiment is representative for two independent experiments yielding similar results.

Table 1
Kinetic constants for purified ΔNt-NspLOX

Parameter	Linoleic acid	α-Linolenic acid
K_M	4.4 μM	26 μM
v_{max}	31.33 μM/mg protein min	53.91 μM/mg protein min

The values given represent the mean values of three independent experiments.

Oxygenation of the fatty acids of dilinoleoyl phosphatidylcholine (PC) can only be possible when a tail-first binding in the active site takes place. Purified recombinant protein was incubated with dilinoleoyl phosphatidylcholine (PC) in the presence of deoxycholate, the reduced products were transesterified and the HODE methyl esters were subsequently analysed by HPLC. Four products could be identified by SP-HPLC analysis: 9-(*Z*,*E*)-HODE-Me (41%) as the main product and additionally 13-(*Z*,*E*)-HODE-Me (29%), 9-(*E*,*E*)-HODE-Me (14%) and (D) 13-(*E*,*E*)-HODE-Me (15%) (Fig. 4) and as expected only 9-(*Z*,*E*)-HODE-Me was chiral (data not shown).

2.3. Expression and product analysis of wt and mutant ΔNt-NspLOX

In order to examine whether the amino acid residue aligning with the "Coffa site" is also in the case of ΔNt-NspLOX sufficient

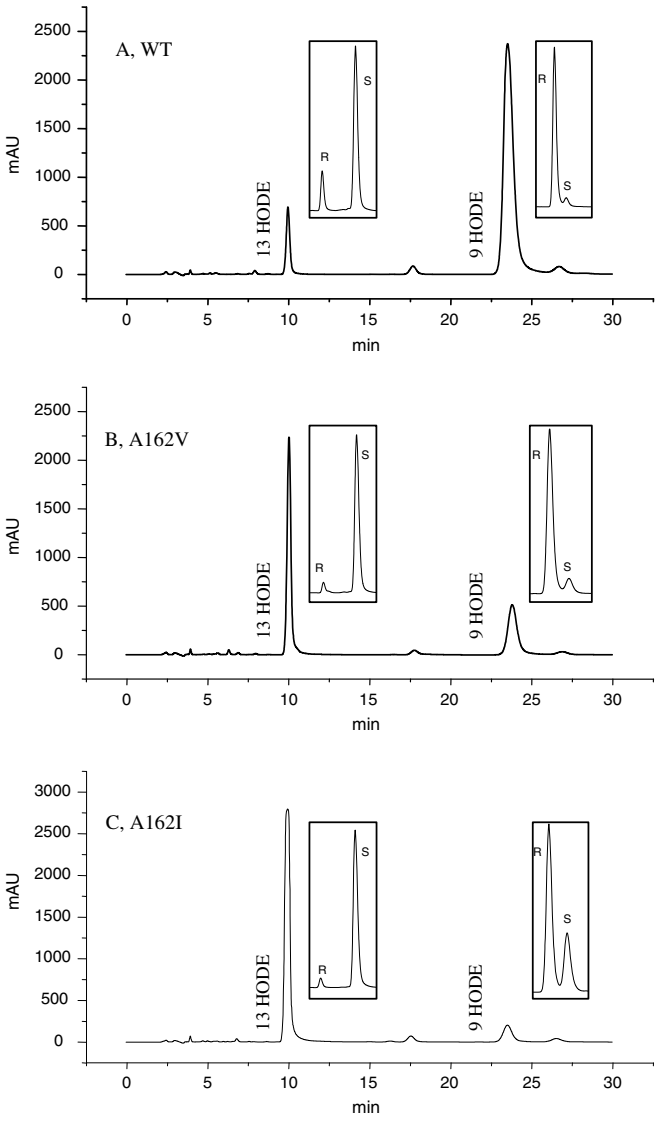

Fig. 3. Analysis of products formed by ΔNt-NspLOX wild type and "Coffa site" mutant enzymes. Enzyme preparations of wild type (wt), Ala → Val (A162V) and Ala → Ile (A162I) mutants of ΔNt-NspLOX were incubated with LA at pH 7.5 (here in their reduced form for SP-analysis). Insets show the analysis of *R* and *S* enantiomers by CP-HPLC analysis. The experiment is representative for two independent experiments yielding similar results.

to control the stereoselectivity of the reaction we performed site directed mutagenesis and converted the Ala residue at position 162 to Gly, Val and Ile. Expression of the wild type as well as the mutant versions of ΔNt-NspLOX was performed again in BL21 Star (DE3) cells.

To investigate the effect of Ala mutation (A162G mutant) on the stereospecificty of the LOX reaction, cell extracts of *Escherichia coli* cells were incubated with LA and the products were analysed by HPLC (Fig. 3). Wild type ΔNt-NspLOX produced primarily (9*R*)-HPODE (88%) from LA and the stereochemistry of the hydroperoxide was almost exclusively *R* (93%) (Table 2; Fig. 3A). (13*S*)-HPODE was only detected in small amounts as by-product. Therefore, the A162G mutant, the reported determinant amino acid conserved among *R*-lipoxygenases, appeared to have no effect on the reaction product in case of this specific LOX, since the mutant enzyme also converted LA primarily to (9*R*)-HPODE. According to the established model for regio- and stereospecificity of the LOX reaction, the change from the 9*R*- to the 13*S*-product imposed by the Gly

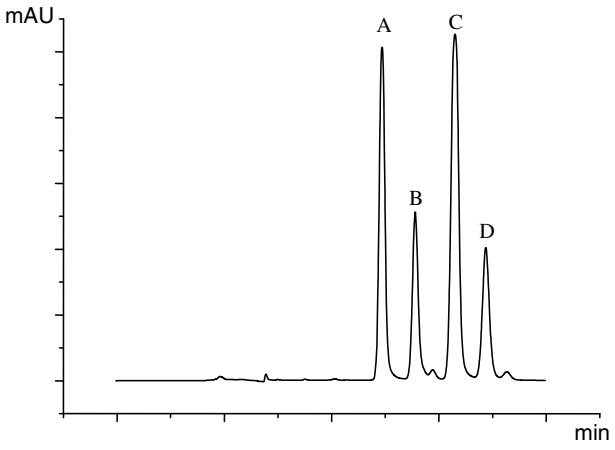

Fig. 4. Analysis of products formed by wt ΔNt-NspLOX incubated with PC. Enzyme preparations of ΔNt-NspLOX were incubated with 1,2-dilinoleyl-*sn*-glycero-3-phosphocholine at pH 7,5. SP-HPLC (here in their reduced form) of 13-(*Z*,*E*)-HODE-Me (peak A), 13-(*E*,*E*)-HODE-Me (peak B), 9-(*Z*,*E*)-HODE-Me (peak C) and 9-(*E*,*E*)-HODE-Me (peak D).

Table 2
Kinetic constants for purified ΔNt-NspLOX mutants for LA

Enzyme	K_M	v_{max}
Ala → Gly	8.9 μM	48.05 μM/mg protein min
Ala → Val	5.0 μM	35.03 μM/mg protein min
Ala → Ile	5.6 μM	0.27 μM/mg protein min

The values given represent the mean values of three independent experiments.

to Ala exchange is linked to the size difference between the two amino acids (Coffa and Brash, 2004). We therefore proceeded in exchanging the Ala residue of ΔNt-NspLOX into a bulkier amino acid, such as valine. Indeed the A162V mutant was active and converted LA primarily to 13-HPODE (64%) and to a lesser extent to 9-HPODE (36%). Chiral analysis by CP-HPLC showed that the main product was as predicted of predominantly *S* configuration (94%) while the secondary product consisted mainly of the *R* enantiomer (88%). To investigate whether the substitution of the Ala amino acid residue by an even bulkier amino acid would further convert the 9*R*-LOX to a 13*S*-enzyme, we replaced Ala by Ile. Analysis of the reaction products by HPLC showed indeed that this mutant produced almost exclusively 13-HPODE (90%) in an *S* configuration (96%; Table 2).

2.4. Purification and kinetic determination of ΔNt-NspLOX mutants

The mutant forms of ΔNt-NspLOX were purified by His-Trap affinity chromatography at a final concentration of 1–2 mg/ml to electrophoretic purity of >95% as judged by SDS-PAGE stained with Coomassie Brilliant Blue. The purified proteins were used in order to determine the steady state kinetic parameters of the mutants and obtain more information on the mechanism that underlies the observed change of the stereospecificity of the LOX reaction

Table 3
Fatty acid hydroperoxides formed from the reaction of ΔNt-NspLOX wild type and mutant enzymes with LA at pH 7.5

Enzyme	9 HODE (*R* configuration) %	13 HODE (*S* configuration)
Wild type	88 (93)	12 (84)
Ala → Gly	87 (94)	13 (85)
Ala → Val	36 (88)	64 (94)
Ala → Ile	10 (72)	90 (96)

The values given are mean values from three measurements.

upon mutation of the Ala residue aligning with the "Coffa site". Using LA as substrate the mutants had very similar K_M to the wt suggesting that the affinity of the enzyme for the fatty acid remains unaffected. On the other hand, v_{max} calculations revealed that replacement of Ala by Gly or Val did not greatly influence the reaction rate. On the other hand, Ala to Ile mutation resulted in a mutant with a 100-fold smaller rate of LA oxygenation in comparison with the mutant (Table 3).

3. Discussion

The aim of this study was to characterize the minimal LOX domain of a bifunctional LOX from *Nostoc* sp. with respect to its catalytic properties. Therefore the carboxyterminal portion of the protein was purified to homogeneity after expression in *E. coli* and mutagenesis experiments were performed in order to examine whether substitution of this alanine with bulkier and smaller amino acids could alter the stereospecificity of the enzyme. The steady state kinetic parameters of the enzyme using the LA and ALA, the PUFAs found endogenously in *Nostoc* sp. (Lang et al., 2008), as substrate were determined. The obtained K_m for ΔNt-NspLOX is smaller in comparison to the one measured for plant LOXs from banana leaf (Kuo et al., 2006), tomato (Todd et al., 1990) and the bacterial LOX from *Thermoactinomyces vulgaris* (Iny et al., 1993) and similar to the one for soybean LOX-1 (Tomchick et al., 2001) and the manganese lipoxygenase from the take-all fungus *Gäumannomyces graminis* (Su and Oliw, 1998). The difference observed in the v_{max} of the reaction when LA and ALA were, respectively, added as substrate also correlates with the increased conversion of the latter to its product under substrate excess conditions, which has previously been reported (Lang et al., 2008).

The results presented here also clarify the manner of fatty acid binding in the active site of ΔNt-NspLOX. It has been proposed that in the case of plant lipoxygenases substrate binding is one of the parameters that determine the regiospecificity of the catalysed oxygen insertion (Liavonchanka and Feussner, 2006). Enzymes that insert oxygen at position 9 in the case of LA and 5 in the case of ARA are thought to bind the fatty acid in "head first" orientation, with the carboxyl-end positioned deep in the active site pocket (Prigge et al., 1996). Taking into account the regiospecificity of ΔNt-NspLOX it was especially interesting to establish if the enzyme is able to insert molecular oxygen in the fatty acid moieties of PC, which would only be possible in case of a "straight" substrate alignment, as has previously been shown in the case of (15*S*)-LOX (Coffa and Brash, 2004). Under high substrate concentration we were able to detect the multiple reaction products as well. The enzyme was able to convert the esterified LA to hydroperoxides, with 9-(*Z*,*E*)-HPOD as the main product (Fig. 4), but the specificity of the reaction is decreased. This suggests that the substrate binding is suboptimal and that free fatty acids are the preferred substrate.

The stereospecificity of the LOX reaction has been reported to be controlled by a single amino acid residue ("Coffa site"), which is a highly conserved Ala in *S*-LOXs and a the smaller Gly in all *R*-LOXs described so far (Coffa and Brash, 2004). However, in a recent study a LOX of cyanobacterial origin which exhibits *R*-LOX activity in spite of harbouring an Ala in the "Coffa site", was identified (Lang et al., 2008). In this study, site-directed mutagenesis was employed in order to examine whether the existing theory describing stereocontrol of LOX products could be expanded from a Gly vs. Ala classification into a broader model of a small vs. a more bulky amino acid depending possibly on the size of the active site pocket of the enzyme. In agreement with this assumption in our mutagenesis experiments we found that changing Ala into a smaller Gly did not influence the stereospecificity of the enzyme, while substituting Ala with a bulkier valine succeeded in converting a 9*R*-LOX to an enzyme producing 13*S*-hydroperoxide as major product. Further substitution of the Ala by the even more space-filling Ile confirmed this tension and achieved in complete conversion of the enzyme to a 13*S*-LOX (Table 2). Previous studies have shown the same tendency upon substitution of the Gly present in the active site of the 8*R*-LOX domain of the peroxidase LOX fusion protein from the coral *Plexaura homomalla* by an Ala (Coffa and Brash, 2004). Interestingly, in the case of *S*-LOXs which harbour an Ala in the active site, such as human 15-LOX2, replacement of Ala with a bulkier amino acid such as Val or Thr resulted in an inactive enzyme (Coffa et al., 2005).

Kinetic characterization of the mutant enzymes revealed that substitution of the "Coffa site" Ala by other amino acids did not influence affinity of the enzyme for the substrate, as can be seen by the unaffected K_M. The rate of the reaction also appears unaffected in the case of the Ala to Gly and Ala to Val mutation, but is drastically decreased in the case of the Ala to Ile mutation. Interestingly, this decrease is not apparent in the amount of substrate converted to the hydroperoxide by crude bacterial extracts expressing the various proteins (Fig. 3). This observable discrepancy is possibly due to the increased protein amount and reaction time in the latter experiment.

Whether changes in stereospecificity are strictly connected with changes in the regiospecificity is still a matter of discussion, since in the case of the murine 12*R*-LOX mutation of the "Coffa site" from a Gly into an Ala did not significantly change the regiospecificity but the stereospecificity (Meruvu et al., 2005). In another study mutation of the "Coffa site" lead to conversion of an 8*S*- into a 12*R*-LOX, respectively, and an 8*R*- to a 12*S*-lipoxygenating enzyme (Coffa and Brash, 2004). Our results are in accordance with the latter, since conversion of the *R*-LOX ΔNt-NspLOX to an *S* producing enzyme by mutation of the Ala into an Ile was also accompanied by a change in the position of oxygenation, namely from C-9 of LA to

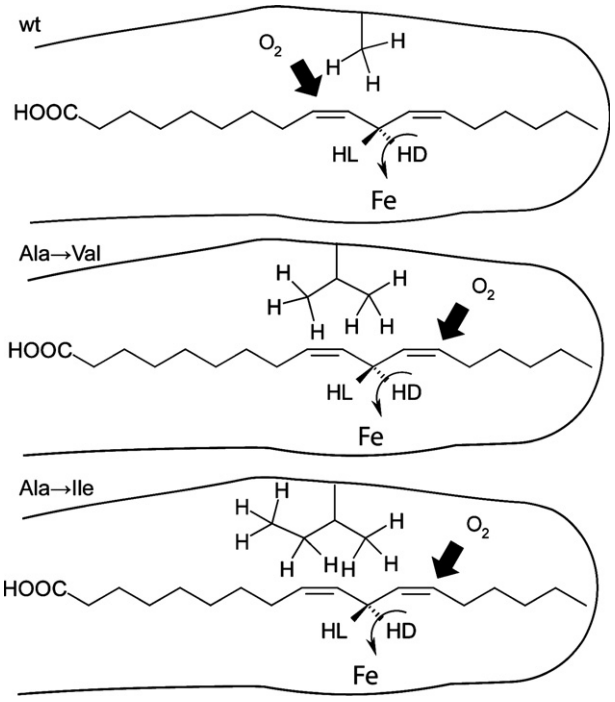

Fig. 5. A model for the control of stereospecificity in the active site of ΔNt-NspLOX. In wt ΔNt-NspLOX LA may have a tail-first orientation in the active site and the reaction begins with an abstraction of D-hydrogen. Oxygen attack takes place on the other side of the molecule. The Ala residue in the "Coffa site" allows oxygen insertion at the C-9 of the fatty acid. In the Ala → Val and Ala → Ile mutants the substrate binding takes place in the same orientation, but presence of the bulky amino acids shields the C-9 from oxygen attack and result in C-13 oxygenation.

C-13. This suggests that also in the case of ΔNt-NspLOX the substrate orientation remains unaffected by the performed mutations and the insertion of a bulkier amino acid (Val and Ile) possibly shields the C-9 from the oxygen attack (Fig. 5).

However, the low sequence similarity of ΔNt-NspLOX with LOXs which have elucidated structures such as LOX-1 and LOX-3 from soybean does not allow modelling of the protein and therefore does not allow prediction of the location of the oxygen channel. Moreover ΔNt-NspLOX has a large deletion in the protein sequence, which possibly renders the active site pocket of the protein different then the majority of LOXs (Lang et al., 2008). It can be assumed that in the case of ΔNt-NspLOX the space in the active site pocket of ΔNt-NspLOX is larger, since the presence of an Ala in the position of the "Coffa site" does not inhibit oxygenation at the C-9. Additionally the replacement of Ala by significantly more space filling amino acids such as Val or an Ile can be accommodated and still yields catalytically active enzymes.

In summary, what can be implied by our results and the comparison with previous studies on the stereospecificity of LOXs is that the residue aligning with a conserved Ala in the case of *S*-LOXs and a conserved Gly in *R*-LOXs is crucial in determining the stereospecificity of the product. However, the presence of a Gly vs. an Ala in the primary structure of a LOX is not sufficient to determine whether the enzyme is *R* or *S* specific, but the morphology and size of the active site also plays a determining role.

Note: While this manuscript was under revision another paper describing a similar set of experiments was published (Zheng et al., 2008).

4. Experimental

4.1. Site-directed mutagenesis

Construction of mutants of the ΔNt-NspLOX domain of NspLOX were performed using the QuikChange site-directed mutagenesis kit (Stratagene). The overlapping mismatching primers used were NspLOXA162GF 5′-TTATCAACTCATCAGGTGTTCCAAAGATTATC-3′; NspLOXA162GR 5′-GATAATCTTTGGAACACCTGATGAGTTGATAA-3′, NspLOXA162VF 5′-TTATCAACTCATCAGTTGTTCCAAAGATTATC-3′, NspLOXA162VR 5′-GATAATCTTTGGAACAACTGATGAGTTGATAA-3′, NspLOXA162IF 5′-TTATCAACTCATCAATTGTTCCAAAGATTATC-3′ and NspLOXA162R 5′-GATAATCTTTGGAACAATTGATGAGTTGATAA-3′. The His-tagged ΔNt-NspLOX domain in the pET15b expression vector was used as template (Lang et al., 2008). The desired mutations were confirmed by sequencing.

4.2. Overexpression and protein purification

Expression of the protein was performed in *E. coli* BL21 Star (DE3) cells. The cultures were grown at 37 °C until OD_{600} 0.6, induced with 0.1 mM IPTG and further incubated for 48 h at 16 °C. For purification of ΔNt-NspLOX frozen cell pellets were lysed using BPer (Pierce) according to manufacturer's instructions. Cell debris was removed by centrifugation at 27,000*g* for 20 min at 4 °C and supernatant was applied to a 1 ml HisTrap FF column (GE Healthcare) previously equilibrated with 50 mM Tris pH 7.5. The column was then washed with 50 mM Tris pH 7.5, 300 mM NaCl, 10 mM imidazole and protein was eluted with 50 mM Tris pH 7:5, 300 mM NaCl, 200 mM imidazole. ΔNt-NspLOX was further purified using the Superdex 200 HR (GE Healthcare).

4.3. LOX activity assay

For activity assays the pellets of 30 ml expression cultures of *E. coli* cells were resuspended in 5 ml lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 10% (w/w) glycerol, 0.1% (v/v) Tween20). The cells were disrupted by 2 pulses of 20 s of sonification on ice. 250 μg of LA was added to a 900 μl aliquot of the crude extract and reactions allowed to proceed for 30 min at room temperature. Hydroperoxides formed were reduced to their corresponding hydroxides with 900 μl of 50 mM $SnCl_2$, dissolved in methanol. After acidification to pH 3.0 with glacial acetic acid, fatty acids were extracted as described (Bligh and Dyer, 1959).

After removing the organic solvent, the residue was reconstituted in 80 μl of methanol/water/acetic acid (85:15:0.1, v/v) and subjected to HPLC analysis. HPLC analysis was performed with an Agilent 1100 HPLC system (Waldbronn) coupled to a diode array detector. Hydroxy fatty acids were separated from fatty acids by reversed phase HPLC (RP-HPLC; EC250/4 Nucleosil 120-5 C18, Macherey-Nagel) eluted with a solvent system of: solvent A, methanol/water/acetic acid (85:15:0.1, v/v) and solvent B, methanol/acetic acid (100:0.1, v/v) at a flow rate of 0.18 ml/min. Straight phase HPLC (SP-HPLC) of hydroxy fatty acid isomers was carried out on a Zorbax SIL column (250/4.6, 5 μm particle size, Agilent) eluted with a solvent system of hexane/2-propanol/acetic acid (100:1:0.1, v/v) at a flow rate of 0.2 ml/min. CP-HPLC of the hydroxy fatty acids was carried out on a Chiralcel OD-H column (2.1 × 150 mm, 5-μm particle size, Daicel, distributed by VWR) with a solvent system of *n*-hexane/2-propanol/acetic acid (100:5:0.1, v/v) and a flow rate of 0.1 ml/min. The absorbance at 234 nm (conjugated diene system of the hydroxy fatty acids) was recorded simultaneously during all chromatographic steps.

The activity of ΔNt-NspLOX using PC as substrate was measured by incubating 0.7 nmol purified protein with 0.6 mg 1,2 Dilinoleyl-*sn*-Glycero-3-Phosphocholine (Avanti Polar Lipids) in 5 ml buffer (50 mM Tris pH 7.5, 4 mM sodium deoxycholate) under constant stirring for 30 min at room temperature. Reduction of the hydroperoxides was performed as described above. The lipid bound fatty acids were the converted to the corresponding methyl esters by adding 500 μl of 1% sodium methoxide solution to the dried after chloroform evaporation samples and shaking for 20 min at room temperature. 500 μl 6 M NaCl was added to the reaction and the methyl esters were then extracted twice with 750 μl hexane. The solvent was removed by evaporation under streaming nitrogen and the sample was dissolved in 80 μl of methanol/water/acetic acid (85:15:0.1, v/v/v). Methyl esters were separated by reversed phase-HPLC (RP-HPLC) with a solvent system of methanol/water/acetic acid (75:25:0.1 v/v) at a flow rate of 0.18 ml/min. Straight phase-HPLC (SP-HPLC) analysis was carried out with a solvent system hexane: 2-propanol: trifluoroacetic acid (100:1:0.02, v/v/v) at a flow rate of 0.1 ml/min.

4.4. Steady-state kinetic measurements

K_M and v_{max} were determined by monitoring the formation of the conjugated double bond of the product at 234 nm ($\varepsilon = 2.5 \times 10^4\ M^{-1}\ cm^{-1}$) with CARY 100 Bio (Varian). The assay samples were 1 ml in volume and substrate concentrations were 3–100 μM (Borate buffer, pH 7.5). Linear parts of the curves were used for Dixon plot.

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