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

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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.

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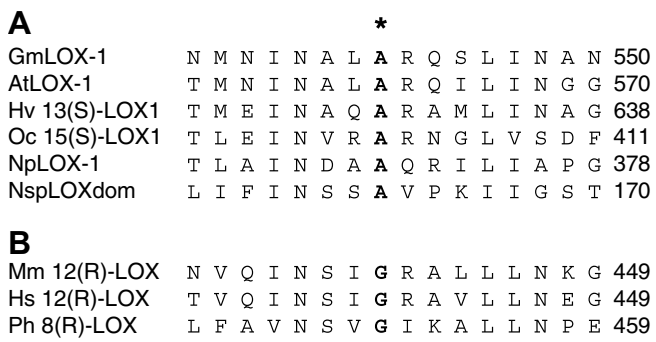

Fig. 1. Alignment of ΔNt-NspLOX with other *R* and *S* LOXs. Comparison of NspLOX (acc. no. NP_478445) from *Nostoc* sp. with: (A) the *S*-LOXs GmLOX1 from *Glycine max* (acc. no. S25064), AtLOX1 from *Arabidopsis thaliana* (acc. no. JQ2267), HvLOX1 from *Hordeum vulgare* (acc. no. U56406), Oc15LOX from *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.

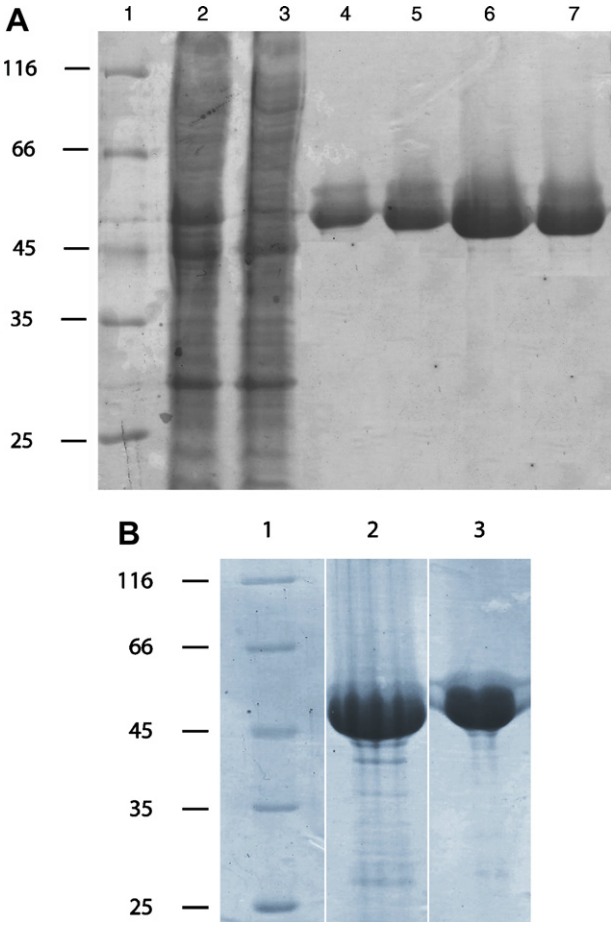

Fig. 2. SDS–PAGE analysis of the purification ΔNt-NspLOX. (A) His-trap purification: Lane 1, protein ladder; lane 2, BL21[DE3] star cell extracts; lane 3, column flow-through, lanes 4–7: elution fractions. (B) Lane 1, protein ladder; lane 2, purified enzyme, after Ni affinity chromatography; lane 3, 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