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A functional role identified for conserved charged residues at the active site entrance of lipoxygenase with double specificity

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ABSTRACT

Plant lipoxygenases (LOXs) are a class of widespread dioxygenases catalyzing the hydroperoxidation of free polyunsaturated fatty acids, producing 9-hydroperoxides or 13-hydroperoxides from linoleic and α-linolenic acids, and are called 9-LOX or 13-LOX, respectively. Some LOXs produce both 9- and 13-hydroperoxides. The models proposed to explain the reaction mechanism specificity fail to explain the "double specificity" character of these LOXs. In this study, we used the olive LOX1 with double specificity to investigate the implication of the charged residues R265, R268, and K283 in the orientation of the substrate into the active site. These residues are present in a conserved pattern around the entrance of the active site. Our results show that these residues are involved in the penetration of the substrate into the active site: this positive patch could capture the carboxylate end of the substrate, and then guide it into the active site. Due to its position on α2 helix, the residue K283 could have a more important role, its interaction with the substrate facilitating the motions of residues constituting the "cork of lipoxygenases" or the α2 helix, by disrupting putative hydrogen and ionic bonds.

1. Introduction

Lipoxygenases (LOXs, EC 1.13.11.12) are a widespread class of enzymes catalyzing the hydroperoxidation of free polyunsaturated fatty acids containing a (1*Z*,4*Z*)-pentadienic system. They were found in plants, in animals [1,2], in some bacterial species [3] and in fungi [4,5]. They start a metabolic pathway leading to the production of compounds of biological importance in animals [2], such as leukotrienes, and in plants [6], such as jasmonic acid. LOXs catalyze a regiospecific and stereospecific insertion of molecular oxygen on the pentadienic system, in position $n+2$ or $n-2$, and occasionally in position n from the methylenic carbon [4,7–9].

In plants, linoleic and α-linolenic acids are the most common substrates. LOXs form 9-hydroperoxides or 13-hydroperoxides of the fatty acid used as substrate and thus they are called 9-LOX or 13-LOX, respectively [10]. LOXs with double specificity were also characterized: they are called 9/13-LOX or 13/9-LOX, depending on the product mainly formed [11].

The reaction mechanism of LOXs has been widely studied, and some models were proposed to explain the stereospecific and regiospecific insertion of molecular oxygen. The "Coffa–Brash" determinant was pointed to rule the stereospecificity of LOXs [12], but recent studies seem to show that it could not be involved in the reaction stereospecificity of several LOXs [13]. Two models were proposed to explain the regiospecificity of LOXs: according to Hornung et al. [14], the substrate entering into the active site by its methyl end would be characteristic of the 13-LOX family. When it enters by its carboxylate end, it would be characteristic of the 9-LOX family. In this model called "orientation-dependent", the entrance by the carboxylate end into the active site would be made possible by an interaction with a positively charged amino-acid at the bottom of the active site, mainly an arginine [14,15]. The orientation with carboxylate near the entrance could require the presence of a positively charged amino acid residue at the entrance of the active site. The second model was proposed by Hughes et al. [16]: the substrate always enters into the active site by its methyl end first, the carboxylate end oriented near the entrance. The regiospecificity of LOXs according to this model would be based on the depth the substrate can reach into the active site, positioning by this way either

Abbreviations: HPOD, hydroperoxyoctadecadienoic acid; HPOT, hydroperoxyoctadecatrienoic acid; IPTG, isopropyl-β-D-thiogalactopyranoside; LOX, lipoxygenase.

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the carbon 9 or 13 near the catalytic iron atom. Once again, the substrate could be stabilized by positively charged residues located at the entrance of active site.

An interaction between the carboxylate end of the substrate and a positively charged amino acid residue seems to be of great importance in the reaction mechanism of LOXs. The putative role of such residues was previously reported: Gan et al. [17] first proposed a positioning model of arachidonic acid obtained *in silico* from the structure of the soybean LOX1. A lysine present near the active site entrance would be involved in an ionic bond with the carboxylate end of the substrate. In the 8*R*-LOX of *Plexaura homomalla*, Neau et al. [18] have identified a "U-shaped" active site with two possible entrances. This LOX shows an arginine residue at each opening of the active site, R183 and R429. R429 is involved in an ionic bridge and is thus not involved in an interaction with the substrate, this role being probably attributable to R183. Finally, in the rabbit 12/15-LOX, Di Venere et al. [19] have shown that the residue R403 is involved in a network of ionic bonds with some residues of the α2 helix. Its replacement by a leucine has an impact on the tertiary structure of the protein. The catalytic efficiency of the enzyme mutant is far reduced with linoleic acid, whereas it is slightly modified with arachidonic acid. Other residues localized near the arginine 403 could participate in the orientation of substrate into the active site.

The previous studies show the importance of charged amino acid residues localized at the entrance of LOXs active site. The two models proposed to explain the reaction mechanism of LOXs are valuable when it comes to LOXs with single specificity. Nevertheless, they fail in explaining the double specificity of LOXs: the specificity as described by the two authors relies on the presence of some determinants in the active site. Thus, a given LOX cannot exhibit both regiospecificities. The charged amino acid residues at the entrance of the active site could play a key role in the positioning of substrate into the catalytic site.

We previously reported the cloning and characterization of an olive LOX with double specificity, producing both 9*S*-hydroperoxide and 13*R*-hydroperoxide from linoleic and α-linolenic acids, and we identified the entrance of its active site [20,21]. From a model of olive LOX1 and sequence alignments with several plant and animal LOXs, we identified conserved positively charged amino-acids surrounding the active site entrance. We used site-directed mutagenesis to investigate the role of these residues in the reaction mechanism of LOXs with double specificity. In views of the results, we discussed the involvement of the positively charged amino acid residues in an interaction with the carboxylate end of substrates for their penetration into the active site of olive LOX1.

2. Materials and methods

2.1. Materials, bacterial strains

The oligonucleotides used in site-directed mutagenesis were synthesized by Invitrogen (Cergy Pontoise, France). Cobalt–affinity resin (Talon® Superflow Metal Affinity) was purchased from Clontech (Heidelberg, Germany). All other chemicals were commercial products of analytical grade or molecular biological grade.

Escherichia coli M15 containing the pREP4 plasmid was transformed with the product of site-directed mutagenesis, and used as expression strain.

2.2. Sequence alignments and homology modeling

The alignment of LOX primary sequences was performed using the ClustalW program [22] in order to identify conserved residues. A model of olive LOX1 was constructed by homology modeling using the server I-TASSER [23,24]. Template used was soybean LOX3 (PDB: 1NO3, resolution 2.15 Å) with sequence identity of 64%. Identification of potential cavities inside the protein corresponding to the putative active site, was performed using the software PyMOL [25] and the web-based tool CASTp [26]. The generated model was then superimposed with the soybean LOX3 co-crystallized with a ligand 13(*S*)-hydroperoxy-9(*Z*), 11(*E*)-octadecadienoic acid (13*S*-HPOD) (PDB: 1IK3) using PyMOL, and the ligand appeared lying in the predicted active site. The mutagenesis tool incorporated in PyMOL was used to model the residues substitution in the model described above.

2.3. Site-directed mutagenesis and expression

The construct named pQE-30-LOX containing the full length wild type olive LOX1 cDNA inserted in the plasmid pQE-30 (Qiagen) [20,21] was used as template. Site-directed mutagenesis experiments were performed using the Quick Change™ site-directed mutagenesis kit (Stratagene) according to the manufacturer's protocol with primers containing the following mutations: R265Q (5′-GATATTTACATTCCA**CAA**GATGAACGTTTTGGA-3′/5′-TCCAAAACGTTCATC**TTG**TGGAATGTAAATATC-3′), R268Q (5′-GATATTTACATTCCAAGAGATGAA**CAA**TTTGGACACTTGAAG-3′/5′CTTCAAGTGTCCAAA**TTG**TTCATCTCTTGGAATGTAAATATC-3′), K283Q (5′-CTTGCTTATGCACTA**CAA**TCCGTCGTTCAGTTTC-3′/5′-GAAACTGAACGACGGA**TTG**TAGTGCATAAGCAAG-3′), R265Q/R268Q (5′-GATATTTACATTCCA**CAA**GATGAA**CAA**TTTGGACACTTGAAG-3′/5′-CTTCAAGTGTCCAAA**TTG**TTCATC**TTG**TGGAATGTAAATATC-3′) and R265Q/R268Q/K283Q built from the plasmid containing the mutation R265Q/R268Q with the K283Q primer.

2.4. Expression and purification of the recombinant wild type and mutant olive LOX1

Expression of recombinant proteins was performed as described previously [20], with some modifications: cells were grown to an OD_{600} of about 1 before induction [27], and protein expression was induced by 1 mM IPTG in presence of 250 μM ammonium iron(III) citrate [27]. Proteins were purified by a one-step purification procedure using an Äkta Purifier FPLC system (GE Healthcare) at 4 °C. The soluble protein fraction obtained by bacterial lysis was collected and charged on a cobalt resin (Talon® Superflow Metal Affinity resin, Clontech) with a bed volume of 1mL. Resin was equilibrated with 50 mM sodium phosphate buffer at pH 7 containing 300 mM NaCl. His-tagged recombinant olive LOX1 was eluted with 100 mM imidazole in the buffer previously described.

2.5. Protein analysis

Protein concentration for the pure protein fraction was determined spectrophotometrically with the theoretical OD_{280} extinction coefficient ϵ calculated from the tyrosine and tryptophan content [28] of the deduced amino-acid sequence. SDS-PAGE was performed in 12% Tris–glycine gel with 2% SDS under reducing conditions, and protein were stained with EZBlue Staining Reagent (Sigma) or transferred onto nitrocellulose (Bio-rad) and revealed with a monoclonal peroxidase-conjugated anti-His antibody (Sigma).

2.6. Enzymatic assay and kinetic studies

LOX activity was determined by continuously monitoring the formation of conjugated dienes at 234 nm [29] as described previously [20] in a sodium phosphate buffer 50mM pH 6, and calculated