Creating lipoxygenases with new positional specificities by site-directed mutagenesis

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Abstract

In order to analyse the amino acid determinants which alter the positional specificity of plant lipoxygenases (LOXs), multiple LOX sequence alignments and structural modelling of the enzyme-substrate interactions were carried out. These alignments suggested three amino acid residues as the primary determinants of positional specificity. Here we show the generation of two plant LOXs with new positional specificities, a γ-linolenate 6-LOX and an arachidonate 11-LOX, by altering only one of these determinants within the active site of two plant LOXs. In the past, site-directed-mutagenesis studies have mainly been carried out with mammalian lipoxygenases (LOXs) [1]. In these experiments two regions have been identified in the primary structure containing sequence determinants for positional specificity. Amino acids aligning with the Sloane determinants [2] are highly conserved among plant LOXs. In contrast, there is amino acid heterogeneity among plant LOXs at the position that aligns with P353 of the rabbit reticulocyte 15-LOX (Borgräber determinants) [3].

For a more comprehensive understanding of the mechanistic reasons for the positional specificity of plant LOXs, structural modelling of the active site of plant LOXs was carried out using the X-ray co-ordinates of soya bean LOXs-1. In wild-type plant LOXs there is a phenylalanine, a cysteine or a histidine located at the position, which aligns with the Sloane determinants of mammalian LOXs [4]. These amino acids may shield the positive charge of an arginine and thus the substrate may only penetrate the active site with its methyl end since there is no counterpart to neutralize the charge of the carboxy group [4]. Mutagenesis studies of one of these Sloane determinants with the lipid-body LOX indicated for the first time the possibility of converting a plant LOX catalysing a [+2] radical rearrangement into a LOX catalysing a [−2] rearrangement [4]. A single point mutation (H608V) converted the wild-type linoleate 13-LOX into a 9-lipoxygengating mutant species.

To investigate the role of other sequence determinants for the positional specificity of plant LOXs we point-mutated the Borgräber determinant of the cucumber lipid-body LOX (V542F). Introduction of the space-filling phenylalanine in addition to the already existing bulky H608 leads to an enzyme species that exhibited a thus-far unknown positional specificity (Figure 1, left-hand panel). This mutant converted γ-linolenic acid mainly into (6S,7E,9Z,12Z)-6-hydroperoxy-7,9,12-octadecatrienoic acid. In contrast, the wild-type enzyme produced mainly the corresponding (13S)-hydroperoxy derivative. Here again, the direction of radical rearrangement was inverted by the mutation.

This information may be helpful to design stable LOX species exhibiting a desired positional...
specificity for biotechnological application. Moreover, it may be possible to optimize the biochemical properties of LOXs already being used for such purposes. The most physiologically relevant mammalian LOXs are the 5-LOXs, which catalyse the initial steps in leukotriene biosynthesis. Leukotrienes are important mediators of hyperergic and inflammation disease [5] and inhibitors are already available as anti-asthmatic drugs. Although mammalian 5-LOXs have been purified from natural and recombinant sources, the purified enzymes are unstable and difficult to handle [6]. Moreover, they require a set of essential co-factors such as Ca$^{2+}$, ATP and phospholipid vesicles for maximal activity. To circumvent these problems several plant 5-LOXs have been tested as models for the physiologically more relevant mammalian enzymes. Among plant 5-LOXs at least, only the potato tuber enzyme converts arachidonic acid with acceptable yields [7]. It does not require essential cofactors and it is stable over months in suspension without a significant loss in activity when it is stored at $-20\,^\circ\mathrm{C}$ [7]. Unfortunately, the positional specificity of this enzyme, as with other plant LOXs [8], is not as stringent as that of the human enzyme. It forms a mixture of 11-, 8- and 5-HETE (hydroxyeicosatetraenoic acid) in a ratio of 1:1:2 (results not shown [1]). Since the recombinant enzyme is available [9], it should be possible to alter the positional specificity by site-directed mutagenesis to make the product pattern more specific. As one example of our approach we created the V576F mutant of this enzyme (Borngräber determinant) and obtained a more specific product pattern of arachidonic acid oxygenation (Figure 1, right-hand panel). Moreover, this mutation did not affect other biochemical parameters of the enzyme.

References

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