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Polyunsaturated C_{18} fatty acids derivatized with Gly and Ile as an additional tool for studies of the catalytic evolution of fungal 8- and 9-dioxygenases

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ABSTRACT

The fungal linoleate diol synthase (LDS) family contains over twenty characterized 8-, 9-, and 10-dioxygenases (DOX), usually fused to catalytically competent cytochromes P450. Crystal structures are not available, but indirect evidence suggests that linoleic acid enters the active site of 8*R*-DOX-LDS headfirst and enters 9*S*-DOX-allene oxide synthase (AOS) with the ω-end (tail) first. Fatty acids derivatized with amino acids can conceivably be used to study oxidation in tail first position by enzymes, which bind natural fatty acids headfirst. The results might reveal catalytic similarities of homologous enzymes. 8*R*-DOX-5,8-LDS oxidize 18:2*n*-6-Ile and 18:2*n*-6-Gly in tail first position to 9*S*-hydroperoxy metabolites, albeit with less position and stereo specificity than 9*S*-DOX-AOS. The oxygenation mechanism of 9*S*-DOX-AOS with antarafacial hydrogen abstraction at C-11 and oxygen insertion at C-9 was also retained. Two homologues, 8*R*-DOX-7,8-LDS and 8*R*-DOX-AOS, oxidized 18:2*n*-6-Ile and 18:2*n*-6-Gly at C-9, suggesting a conserved feature of 8*R*-DOX domains. 9*R*-DOX-AOS, with 54% sequence identity to 9S-DOX-AOS, did not oxidize the derivatized C_{18} fatty acids. 9*Z*,12*Z*-16:2, two carbon shorter than 18:*n*-6 from the ω-end, was rapidly metabolized to an α-ketol, but *7Z*,10*Z*-16:2 was not a substrate. An unsaturated carbon chain from C-1 to C-8 was apparently more important than the configuration at the ω-end. 8*R*-DOX-LDS and 9*R*-DOX-AOS may thus bind 18:2*n*-6 in the same orientation. The oxidation of 18:2*n*-6 in straight or reverse head-to-tail positions illustrates evolutionary traits between 8- and *9*-DOX domains. Fatty acids derivatized with amino acids provide a complementary tool for the analysis of evolution of enzymes.

1. Introduction

Polyunsaturated fatty acids can be oxygenated by both dioxygenases (DOX) of the peroxidase-cyclooxygenase (COX) superfamily and by lipoxygenases (LOX) [1–3]. The peroxidase-COX superfamily contains three eukaryotic divisions: the animal COX, the plant α-DOX [4–6], and the fungal linoleate diol synthase (LDS) families [2]. DOX shares a common oxygenation mechanism [1,7–9]. With these enzymes, a conserved Tyr residue is oxidized to a radical, which abstracts hydrogen from an allylic or *bis*-allylic carbon or from an α-carbon. The carbon-centered radical reacts with molecular oxygen to form a peroxyl radical [1,7,10], which can be further transformed to prostaglandin endoperoxides or hydroperoxy fatty acids.

The 3D structures of fatty acids in the active sites have been determined in crystals of COX, α-DOX, and coral 8*R*-LOX. Ovine COX-1 binds 20:4*n*-6 and 18:2*n*-6 with the ω end in the hydrophobic active site ("tail first") and with the carboxyl group tethered to Arg120 close to the entrance [11–13]. In contrast, α-DOX of *Oryza sativa* oxidizes palmitic acid at the α-carbon with the carboxyl group deep in the active site ("headfirst"), stabilized by ionic interactions with His-311 and Arg-559, and with Tyr379 positioned close to the α-carbon [6] (Fig. 1A). In analogy, 8*R*-LOX of *Plexaura homomalla* binds the carboxyl group of 20:4*n*-6 by ionic interactions with Arg182 and Glu430 at the entrance and positions the ω end bound to hydrophobic residues in the substrate channel ("tail first") [14].

About 20 enzymes of the LDS family have been characterized, but

Abbreviations: AOS, allene oxide synthase; COX, cyclooxygenase; DOX, dioxygenase; EAS, epoxyalcohol synthase; LDS, linoleate diol synthase; LOX, lipoxygenase; TIC, total ion current; RP, reversed phase; TPP, triphenylphosphine; 8-H(P)ODE, 8-hydro(pero)xy-9*Z*,12*Z*-octadecadienoic acid; 18:2*n*-6-Gly, *N*-[9*Z*,12*Z*-octadecadienoyl]-*S*-glycine; 18:2*n*-6-Ile, *N*-[9*Z*,12*Z*-octadecadienoyl]-*S*-isoleucine; 18:3*n*-3-Gly, *N*-[9*Z*,12*Z*,15*Z*-octadecatrienoyl]-*S*-glycine; 18:3*n*-3-Ile, *N*-[9*Z*,12*Z*,15*Z*-octadecatrienoyl]-*S*-isoleucine; 8-H(P)OTrE, 8-hydro(pero)xy-9*Z*,12*Z*,15*Z*-octadecatrienoic acid; 9-H(P)ODE, 9-hydro(pero)xy-10*E*,12*Z*-octadecadienoic acid; 9-H(P)OTrE, 9-hydro(pero)xy-10*E*,12*Z*,15*Z*-octadecatrienoic acid; 10-H(P)ODE, 10-hydro(pero)xy-8*E*,12*Z*-octadecadienoic acid; 10-H(P)OTrE, 10-hydro(pero)xy-8*E*,12*Z*,15*Z*-octadecatrienoic acid; 13-H(P)ODE, 13-hydro(pero)xy-9*Z*,11*E*-octadecatrienoic acid; 13-H(P)OTrE, hydro(pero)xy-9*Z*,11*E*,15*Z*-octadecatrienoic acid; 16-H(P)OTrE, 16-hydro(pero)xy-9*Z*,12*Z*,14*E*-octadecatrienoic acid

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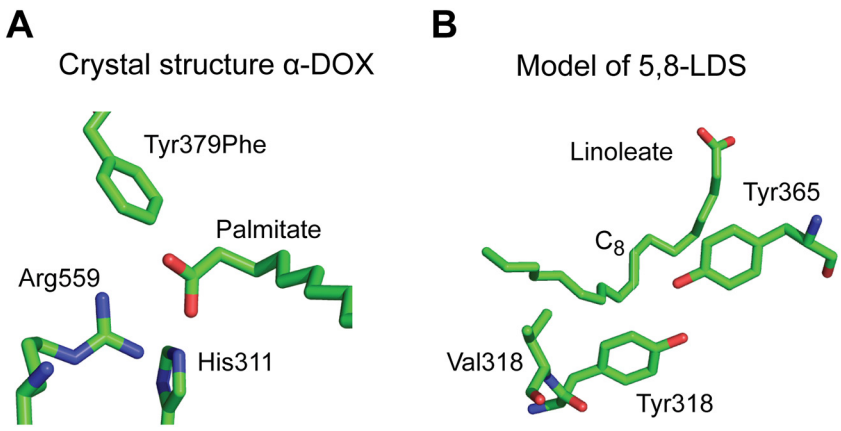

Fig. 1. Amino acids near the carboxyl group of palmitate in the crystal structure of α-DOX (*Oryza sativa*; PDB file 4KVL) and a model of amino acids of *At*-8*R*-DOX-5,8-LDS near C-8 of linoleate. A. Partial crystal structure of the Tyr379Phe mutant of α-DOX near the carboxyl group of palmitate. B. Swiss model of *At*-8*R*-DOX-5,8-LDS, based on the crystal structure of ovine COX-1 with linoleate (PDB file 1IGZ). Linoleate was positioned in the active site in reverse orientation compared to the orientation in COX-1 (see text). Val318 is important for substrate binding and Tyr365 for catalysis, but the Tyr318 residue in the vicinity is not required for catalysis.

the first crystal structure is still pending [8]. The LDS family contains 8-, 9-, and 10-DOX usually fused to catalytically competent cytochromes P450 [15–17]. Electron paramagnetic resonance, site-directed mutagenesis, and modeling of 8*R*-DOX-LDS suggest that C-8 is positioned close to the catalytic Tyr residue (cf. Tyr365 in Fig. 1B) [7,8,18–20]. The aligned sequence identities between COX and 8*R*-DOX domains are unfortunately too low (about 25%) to allow modeling of the entire substrate channel.

LDS enzymes with 8*R*-DOX domains characteristically oxidize 18:2*n*-6 and 18:3*n*-3 efficiently and 20:2*n*-6 slowly, but neither 18:3*n*-6 nor 22:5*n*-6 [21]. The orientation of fatty acids in the 8*R*-DOX domains is difficult to predict from these observations. 9*R*-DOX of *Fusarium oxysporum* of the LDS family oxidizes 22:5*n*-6 almost as efficiently as 18:2*n*-6 [22], suggesting tail first orientation in the active site. In analogy, 13*S*-LOX-1 of *Glycine max* (*Gm*LOX-1) oxidizes 18:2*n*-6, long chain fatty acids (22:5*n*-6), and 18:2*n*-6 of phosphatidylcholine and lysophosphatidylcholine, which supports "tail first" binding to the active site [23–25]. These indirect methods are well suited to study enzymes with tail first orientation, but they are often less conclusive for enzymes with headfirst orientation.

It is likely that fatty acids in headfirst orientation tether the carboxyl group by ionic interactions with charged residues as illustrated by α-DOX (Fig. 1A). Fatty acids derivatized with amino acids will retain a carboxyl group; Gly conjugate will extend the carboxyl end with −H-N−CH_2−COOH. This will allow ionic interactions with the carboxyl group to charged residues; the amide group cannot be charged but the carbonyl may form hydrogen bonds and interact with the protein.

In a recent study, the Ile and Trp conjugates of 18:2*n*-6 and 18:3*n*-3 were found to be rapidly oxidized at C-9 by two DOX of *F. oxysporum*. *Fo*-9*R*-DOX and *Fo*-9*S*-DOX-allene oxide synthase (AOS) [26]. In analogy with the lipoxidation of lysophosphatidylcholine by *Gm*LOX-1 [25], these results suggested that fatty acids enter the substrate channel of *Fo*-9*R*-DOX and *Fo*-9*S*-DOX-AOS with their ω ends first (Fig. 2A). The oxidation of Gly, Ile, and Trp conjugates by 8*R*-DOX domains yielded unexpected results. 8*R*-DOX-7,8-LDS of *Magnaporthe oryzae* (*Mo*-8*R*-DOX-7,8-LDS) oxidized 18:2*n*-6 and the Gly conjugate rapidly to hydroperoxides at C-8 and to 7,8-diols [26]. The Ile and Trp conjugates of 18:2*n*-6 were not oxidized at C-8. Furthermore, three 8*R*-DOX-LDS homologues and 8*R*-DOX-AOS of *Coccidioides immitis* (*Ci*-8*R*-DOX-AOS) also oxidized the Gly conjugates at C-8, but not the Ile and Trp conjugates at this position [26]. This suggested that five homologous of 8*R*-DOX domains bind 18:2*n*-6 in headfirst orientation for oxidation at C-8 (Fig. 2B). Definitive proof will require the crystal structure of an 8*R*-DOX domain with 18:2*n*-6 in the active site.

An intriguing observation was that Ile and Trp conjugates of C_{18} fatty acids appeared to be mainly oxidized at C-9 by several 8*R*-DOX domains [26], possibly by binding in tail first position. In addition, 8*R*-DOX domains could also oxidize the corresponding Gly conjugates at C-8 and C-9; apparently by hydrogen abstraction at C-8 and C-11, respectively. This suggested that fatty acids derivatized with amino acids might conceivably be used to study oxidation of fatty acids in tail first position of enzymes, which naturally bind fatty acids headfirst. The results might reveal catalytic similarities of homologous enzymes with different substrate orientation, although binding the substrates in the improper tail first orientation will be expected to reduce the catalytic turnover.

Based on this hypothesis, the first goal of the present study was to determine the oxidation of conjugated fatty acids at other positions than C-8 by 8*R*-DOX domains. 8*R*-DOX-5,8-LDS of *Aspergillus terreus* (*At*-8*R*-DOX-5,8-LDS) [27], 8*R*-DOX-7,8-LDS of *Gaeumannomyces graminis* (*Gg*-8*R*-DOX-7,8-LDS) [15,28], *Ci*-8*R*-DOX-AOS, and 8*S*-DOX-AOS of *Zymoseptoria tritici* (*Zt*-8*S*-DOX-AOS) [29] were chosen for these studies in an extension of the previous investigation [26]. The sequence similarities of their 8*R*-DOX domains is indicated by the phylogeny in Fig. 2C.

Fatty acids derivatized with amino acids are a novel tool to study enzymatic oxidation, but this method has shortcomings, which are also important to document. The second goal was to illustrate these limitations with 9*R*-DOX-AOS of *A. niger* (*An*-9*R*-DOX-AOS) [30]. The 9-DOX domains of *An*-9*R*-DOX-AOS [30] and *Fo*-9*S*-DOX-AOS [16] can be aligned with 54% sequence identity (Fig. 1C). *An*-9*R*-DOX-AOS could therefore be expected to bind fatty acids tail first in analogy with *Fo*-9*S*-DOX-AOS, however, it was found that this was not the case. The fatty acids derivatized with amino acids were not transformed and provided little other information, but the classical method of oxidation of substrate analogs (16:2*n*-4 and 16:2*n*-6) confirmed headfirst binding to the active site of *An*-9*R*-DOX-AOS.

2. Materials and methods

2.1. Materials

18:2*n*-6 (99%), 18:3*n*-3 (99%), *S*-isoleucine, *S*-glycine, ethyl chloroformate, triphenylphosphine (TPP), *Gm*LOX-1 (Lipoxidase), and triethylamine were from Sigma. 16:2*n*-6, 16:2*n*-4 and 20:2*n*-6 were from Larodan (Solna, Sweden). [$^{13}C_{18}$]18:2*n*-6 (98% ^{13}C; 95%) was from Cambridge Isotope Laboratories (Andover, MA, USA). 9*S*- and 9*R*-hydroperoxy-10*E*,12*Z*,15*Z*-octadecatrienoic acid (9-HPOTrE) and 9*S*-hydroperoxy-10*E*,12*Z*-octadecadienoic acid (9-HPODE) were obtained by biosynthesis (9*S*-LOX of tomato [31] and recombinant 9*R*-LOX of *Anabaena* sp. PCC 7120 [32]). The amino acid conjugates of fatty acids were prepared as described [26,33]. The same method was used to prepare conjugates of 9*S*- and 9*R*-HOTrE. [^{2}H,11*S*]18:2*n*-6 (99% ^{2}H), [^{2}H,11*R*]18:2*n*-6 (25% ^{2}H), and 9*S*-hydroxy-10*R*(11*R*)epoxy-12*Z*-octadecenoic acid from Larodan were derivatized with Gly in the same way. The ester derivative of the hydroxy group of 9-HOTrE conjugates was regenerated by alkaline hydrolysis (0.5 M KOH in methanol with 10% water under Ar; 70 °C, 1 h). Rac HPOTrE-Ile, rac HPODE-Ile, and rac