Hydrocarbon biosynthesis by Atypical P450 Enzymes

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ABSTRACT

Fatty acid peroxygenases have been underscored in hydrocarbon biosynthesis due to their capacity to perform C-C scission en route, producing olefins, a central building block for the production of sustainable plastics, polymers, and fuels. These biocatalysts possess non-canonical and complex mechanisms, which involve redox partners, co-factors, hydrogen abstraction, controlled electrons and protons delivery that culminate in bifurcated chemoselectivity into hydroxylation or decarboxylation. Herein, we revealed different structural complexes of iso-functionally clustered decarboxylases bound to either saturated or unsaturated substrates, along with structure-guided protein engineering, molecular dynamics simulations and unsupervised machine learning. This mechanism is underpinned in concerted molecular arrangements involving the distal binding pocket, known as hydrophobic cradle, and substrate to orient the Cβ atom towards the catalytic heme-iron. We also demonstrated that the lack of the aromatic residue from the Phe-His-Arg triad, positioned at the heme proximal site, preserved the chemoselectivity for alkenes, underlining a distinct standpoint regarding the structural determinants for β-regiochemistry. Taken together, these findings untangle key molecular factors governing the tunable biocatalytic selectivity of P450 peroxygenases that are central for the sustainable production of olefins from oleic acid, the most abundant and relevant renewable fatty acids in nature.

Keywords: Peroxygenase P450. Hydrocarbon. Unsaturated fatty acid. Drop-in biofuels.

1 INTRODUCTION

Olefins (alkenes) are key intermediates and commodity chemicals in the industrial production of polymers, plastics, surfactants, plasticizers, and fuels. In organic synthesis, olefins play central roles in constructing C–C, C–O, and C–N bonds en route to active pharmaceutical ingredients, biological probes, and advanced functional materials. Given the critical position of olefins derived from petroleum extraction, significant efforts have been directed into the development of strategies aiming at synthesizing these compounds from renewable sources [1,2,3,4].

Over the last decade, a distinctive class of enzymes termed fatty acid decarboxylases have attracted great attention because of their ability to produce olefins, acting on the removal of the fatty acyl carboxyl group. These biocatalysts possess non-canonical and elaborate mechanisms, which involve hydrogen abstraction, controlled electrons and protons delivery, and bifurcated chemoselectivity, representing an eminent field for investigation [1,2,3,4,5,6,7,8,9,10].

Here, we solved the crystal complex of the oleic acid bound to a CYP152 decarboxylase, unveiling structural adaptations in the catalytic pocket involved in the modulation of (chemo)specificity. In this regard, the decarboxylation of oleic acid is favored by the rotation of the distal phenylalanine, which composes the hydrophobic cradle motif and allows the elongation of the catalytic pocket for substrate accommodation.

MATERIAL & METHODS

Heterologous expression and purification of OleTs and mutants.

Recombinant-pET-28a vector were co-transformed into E. coli BL21(DE3) with pG-TF2 plasmid (Takara Bio, Kusatsu, JPN), which encodes GroEL, GroES, and Tig chaperones. The expression and purification were performed as described by Rade et al. Briefly, the transformed strain was grown in 500 mL of a selective (chloramphenicol and kanamycin) Terrific Broth medium (TB) supplemented with 125 mg/L thiamine hydrochloride and 500 µL trace metals solution (containing 50 mM FeCl3, 20 mM CaCl2, 10 mM MnCl2, 10 mM ZnCl2, 2 mM CoCl2, 2 mM CuCl2, 2 mM NiSO4) at 37 °C. When the optical density (OD600nm) of the culture reached 0.6, the growth temperature was reduced to 20 °C, and 200 µM isopropyl-β-D-thiogalactopyranoside (IPTG) (Invitrogen), 5 µM of FeCl3, 100 µM of δ-aminolevulinic acid (ALA), and 10 µg/mL tetracycline were added. The culture was grown at 20 °C overnight. Cells were harvested by centrifugation at 7.000 x g (10 min at 4 °C) and subsequently the cell pellets were resuspended in buffer A [100 mM potassium phosphate (pH 7.5), 300 mM NaCl, 10 mM imidazole, and 5 % (v/v) glycerol] and disrupted by sonication in a Vibracell VCX 500 device (Sonics and Materials, Newtown, USA), by performing 2 min of sonication (40% amplitude and no pause), followed by 5 minutes of stirring at 4 °C. This procedure was repeated 5 times. The cell lysate was centrifugated at 12.000 x g for 25 min and the clear supernatant was loaded onto a 5 mL His-Trap chelating HP column (GE Healthcare Biosciences), pre-equilibrated with buffer A. The target protein was eluted with a gradient of imidazole (5 to 300 mM)

in buffer A. The eluted sample was dialyzed overnight against 100 mM phosphate buffer (pH 7.5) containing 50 mM NaCl and 5% (v/v) glycerol. Dialyzed protein was loaded onto a fast flow Q-sepharose column (Sigma-Aldrich Co., St Louis, USA) and eluted by a NaCl gradient (50 mM to 1 M). The heterologous expression and enzyme purification steps were evaluated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 12%) and spectrophotometric analysis of typical Soret bands. Steady-state Turnover- Fatty acid substrate conversion

Activity assays were carried out with different fatty acid substrates (C10-C20 and C18:1).

For this, a 500 µL reaction was prepared by mixing 500 µM fatty acid substrate, 2 µM enzyme and 600 µM H2O2 in the best buffer for each enzyme (previously characterized). Hydrogen peroxide was added in six steps of 1 µL of hydrogen peroxide (10 mM stock) every 10 min on positive control over the course of 1 hr. For the negative control, the addition of buffer was performed instead of the H2O2. Reactions were carried out at the best temperature (previously characterized) and 700 rpm. The reaction was immediately stopped with 5 µL of 50% HCl. Then, the internal standards 1-tetradecene and heptadecanoic acid (final concentration of 0.98 mg/mL) were added to the reaction mixtures. The products were extracted with 500 µL chloroform by homogenization in a tube revolver rotator (20 min) followed of centrifugation at 5000 rpm for 5 min. 400 µL of the organic phase was transferred to a 2 mL glass vial for the derivatization step, in which 25 µL of HCl (2 M in methanol) and 75 µL of a second internal standard solution (1-hexadecene and methyl pentadecanoate at 50 µg/mL final concentration in methanol) were added. The sample was incubated at 50 °C for 30 min and then quenched with 40 µL NaOH solution (1 M in methanol). 500 µL of pure water was added for phase separation, and, after centrifugation at 5000 rpm for 5 min, the organic phase was analyzed by gas chromatography.

Analytical analyses for fatty acid and products quantification

The reaction products were determined by gas chromatography analysis (7890A, Agilent Technologies, Santa Clara, USA). The following conditions were used: RTx-5MS (30 m x 0,25 mm x 0,25 µm), detector temperature of 290 °C, injector temperature of 230 °C, split ratio 1:10, and oven temperature: 30 °C for 2 min, increase to 220 °C at a rate of 10 °C/min, increase to 240 °C at a rate of 2 °C/min and increase to 350 °C at 50 °C/min. To the identification of the peaks, the comparison of the retention time with authentic standards of fatty acids, alkenes, and hydroxylate products was performed. Quantification of the compounds was carried out by the addition of internal standards at each sample (1-tetradecene for alkene quantification and heptadecanoic acid for fatty acids and hydroxylates quantification), and the exact quantification of the internal standards added was performed by using calibration curves for each internal standard (1-hexadecene and methyl pentadecanoate).

2 RESULTS & DISCUSSION

The repertoire of P450 fatty acid decarboxylases with elucidated structures is scarce, relying on only two enzymes complexed with either palmitic (OleTPRN) or arachidic acid (OleTJE), which is an extremely rare biological substrate. As abovementioned, OleTCL contains a peculiar performance towards the production of alkenes from unsaturated substrate; and therefore, structural studies aiming at obtaining such decarboxylase complexed with oleic acid would provide instrumental acumen on the molecular aspects that enables such biocatalytic reaction. Hence, the oleic acid-bound OleTCL structure was herein elucidated revealing that the substrate accommodation throughout the catalytic pocket as well as its configuration are expressively altered compared to the saturated substrate (Fig. 1). Remarkably, this phenomenon is majorly generated by a ~110° rotation of the residue F19 that leads to the rearrangement of the residues L48 and L49 (Fig. 1). Interestingly, in terms of catalytic performance, OleTCL-L26F mutant presented around 30% reduction in total oleic acid conversion (Fig. 3C), indicating that a higher rigidity of F19 partially impairs the productive binding of unsaturated substrates. On the other hand, the consumption of saturated fatty acids, especially C12, was considerably enhanced, suggesting that bulkier residues contribute to increasing the hydrophobicity of the hydrophobic cradle, thereby shortening the catalytic pocket and creating a more accommodating space to bind shorter substrates. This result is aligned with what we observed for OleTKM, which displays high efficiency in metabolizing and producing alkenes from C12 and C10 substrates due to its shorter catalytic pocket. Therefore, these results allude that the F19 mobility can modulate the substrate preference and, especially, its pivotal for oleic acid decarboxylation by OleTCL. However, the rigidity imposed by Phe-Phe interactions is not a conserved feature associated with decreased activity on unsaturated among CYP152s, since OleTPRN has such Phe-Phe interaction[1] and yet it can efficiently decarboxylate oleic acid into olefins.

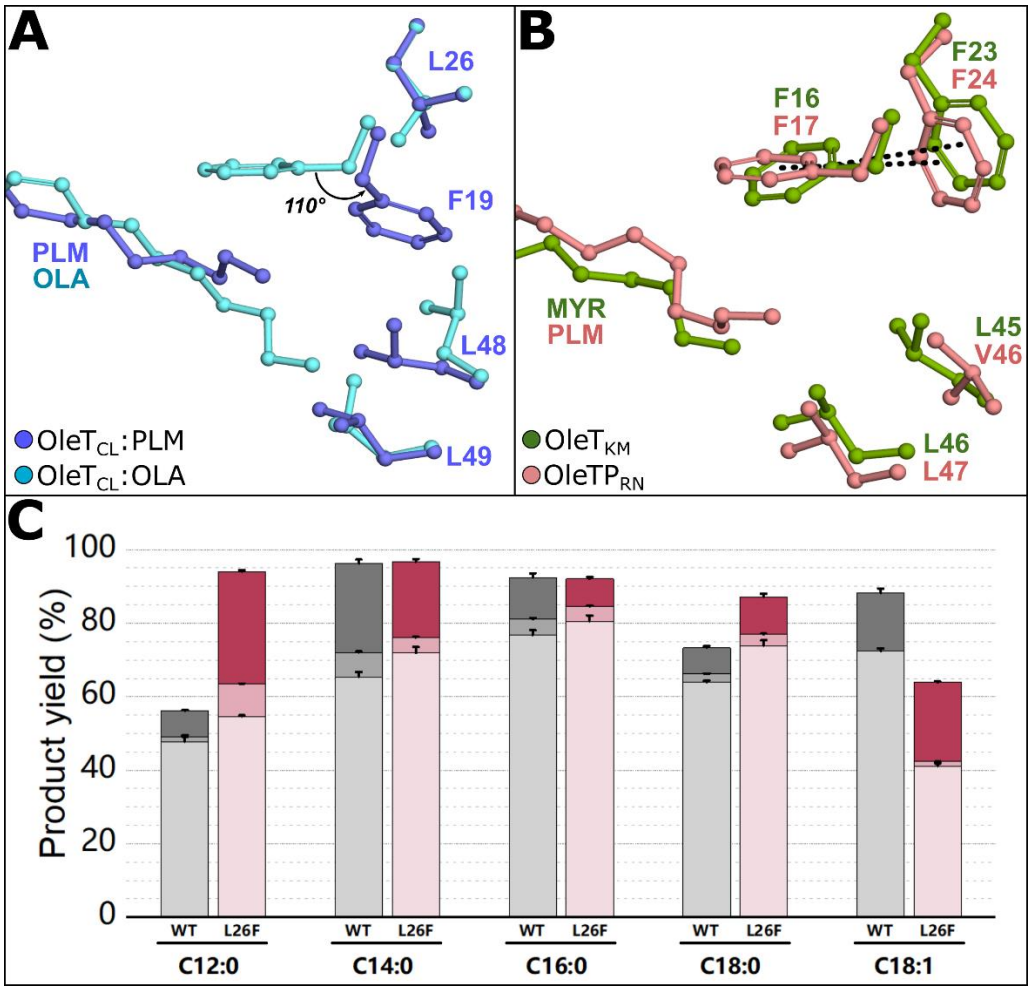

Figure 1 Rotation of Phe19 at OleTCL binding pocket distal, leading to rearrangement of L48 and L49 to hold palmitic and oleic acid inside the binding pocket. (B) Representation of the rigid phenylalanine of both OleTKM (F16) and OleTPRN (F17) that might not be subjected to this side chain flexibility due to an interaction with F23 and F24, respectively. (C) Turnover assays conducted with OleTCL wildtype (WT) and L26F mutant with different FA substrates. Lighter, medium and darker bar color stands for alkene, α-hydroxy- and β-hydroxy-fatty acid, respectively. Experimental conditions: 2 µM enzyme, 500 µM total substrate, 600 µM H2O2, 45°C in 100 mM sodium-phosphate buffer (pH 7.5) per 1 h.

3 CONCLUSION

This study shed light on the role of structural adaptations, accompanied by coordinated arrangements, in enabling the decarboxylation of an abundant and relevant unsaturated substrate by CYP152 enzymes. This contributes to addressing the urgent need to reduce fossil resource utilization, given the importance of olefins in petrochemical and bioenergy industries.

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