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TOPIC(s) : Enzyme discovery and engineering

Computational-aided engineering of a selective unspecific peroxygenase toward enantiodivergent beta-ionone hydroxylation

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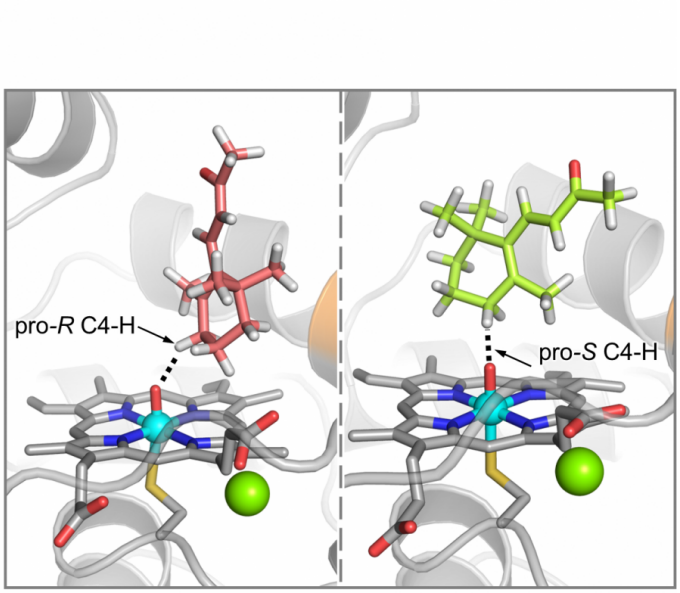
PURPOSE OF THE ABSTRACT
Unspecific peroxygenases (UPOs) are fungal, secreted, heme containing enzymes. They perform oxyfunctionalization reactions within a broad substrates scope utilizing H2O2 without additional reductive equivalents or electron transfer chains.[1] The development of these enzymes for industrial applications has been a focus of research over the last decade, with engineering efforts targeting heterologous expression, activity, stability, and improvements in chemo- and regioselectivity.[2, 3]However, the targeted engineering of enantioselectivity for specific substrates with poor starting enantioselectivity remained a missing integral piece until now. We pursued this endeavor using the terpene β-ionone as model substrate. Ionones are valuable substrates used in the fragrance industry and in the synthesis of carotenoids and Vitamin A.[4, 5]
The conversion of α- and β-ionone has already been shown with several UPOs, leading to a diverse range of hydroxylation and epoxidation products.[6] It also has been pursued using various P450s.[7-9] P450 engineering efforts led to a 280-fold increase in product formation rate toward α- and β-ionone hydroxylations. Enhancing the enantioselectivity, however, has proved challenging.[7] Enantioselective 4 hydroxy-β-ionone formation has been achieved solely through enzymatic kinetic resolution[10] and by recombinantly in T. ni cells expressed CYP2B6.[9]
We engineered MthUPO derived from Myceliophthora thermophila to enantioselectively access C4 hydroxylated stereoisomers of β-ionone.
In this study, a computational-aided engineering approach based on a combination of DFT model calculations and MD simulations has been applied. These simulations were used to characterize near-attack conformations of the selective hydroxylation which revealed relevant binding modes of the model substrate β-ionone (Figure 1). The identification of the relevant residues for substrate positioning facilitated the design of a small smart library to modify the active site pocket of MthUPO. In this way, we could direct the selectivity of the oxyfunctionalization toward enantioselective R/S C4 hydroxylation. Enzyme variants were expressed in Saccharomyces cerevisiae in a 96-well microtiter plate. The screening was performed by the previously developed Multiple Injection in a Single Experimental Run (MISER) GC-MS method [11, 12] focusing on activity increase. The MISER setup involves injecting 96 samples into the GC in a single experimental run, with product quantifications performed exclusively in the MS through different m/z ratios, eliminating the need for substrate/product separation. This setup enables an injection frequency of up to 30 s, allowing for GC analysis of one microtiter plate within 48 minutes. Rescreening of the best variants with a chiral GC-MS led to the determination of the enantioselectivities. After two rounds of iterative enzyme evolution, the activity increased up to 17-fold and the regioselectivity reached up to 99.6 % for the 4-hydroxy-β-ionone. Enantiodivergent variants were identified with enantiomeric ratios of 96.6:3.4 (R) and 0.3:99.7 (S), respectively (Figure 2). Finally, in silico analysis of the best performing, highly enantioselective variants revealed the molecular basis of the selectivity, which was achieved by only two (R-selectivity) and four (S-selectivity) mutations, respectively.

FIGURES



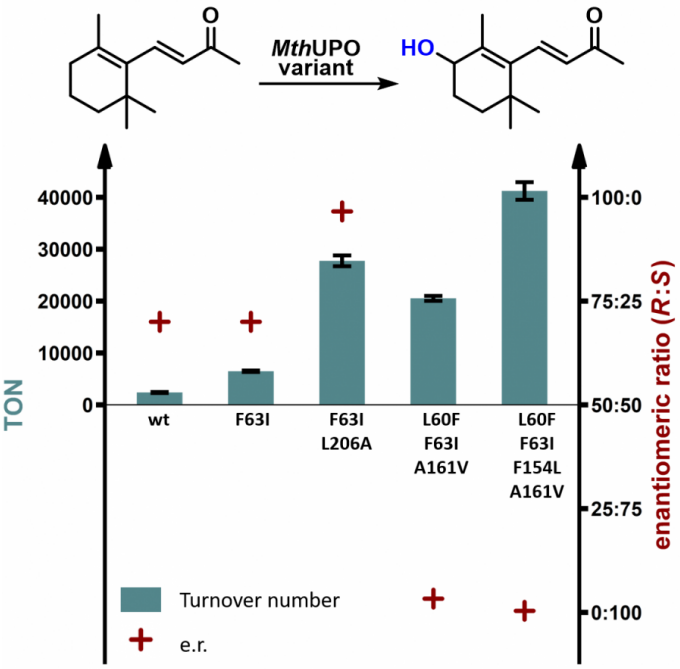


FIGURE 1

Analysis of beta-ionone near-attack conformations.

Restrained MD-simulations are performed to explore near-attack conformations for selective C4-hydroxylation and characterize relevant binding modes of the model substrate beta-ionone.

FIGURE 2

Turnover number and enantioselectivity of different engineered MthUPO variants.

Turnover data are mean +/- s.d. of measurements in triplicates. TON (teal bars) determined by GC-MS and enantiomeric excess (red cross) by chiral GC-MS.

KEYWORDS

unspecific peroxygenase | enantioselectivity | directed evolution | computational-guided protein engineering

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