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Expanding the substrate scope and reactivity of cytochrome P450 OleT

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ABSTRACT

The efficient hydrogen peroxide-dependent hydroxylation and epoxidation of hydrocarbons is catalysed by a P450 fatty acid decarboxylase (OleT) active-site variant. The introduction of an acidic functionality in the protein framework circumvents the necessity for a carboxylate that is typically provided by the substrate for efficient H_2O_2 heterolysis. Spectroscopic and turnover studies show that the mutation eliminates the binding and metabolism of prototypical fatty acid substrates, but permits the oxidation of a broad range of inert hydrocarbon substrates.

1. Introduction

The use of cytochrome P450 enzymes (CYPs) for the controlled and selective insertion of oxygen into chemically inert C–H bonds provides an attractive alternative to more conventional synthetic routes for generating higher value-added products [1–3]. The transformations catalysed by P450s offer numerous advantages to synthetic transition metal complexes for C–H functionalization, including highly regio- and stereo-selective conversions, and robust activity under mild temperature and environmentally benign aqueous solvent conditions. The catalytic proficiency of CYPs largely owes to the highly oxidizing nature of the principle intermediate formed at the heme-iron active-site, an iron(IV)-oxo pi-cation radical intermediate known as Compound I that is formally two redox equivalents higher than the ferric resting state of the enzyme [4]. In most P450s, this potent oxidant is generated from the binding and subsequent reduction of atmospheric O_2 (reviewed in Refs. [5–7]). The activation of atmospheric dioxygen involves the orchestrated delivery of reducing equivalents from NAD(P)H through an associated redox chain, and finely coordinated active-site proton delivery. The dual requirement for accessory enzymes and an expensive pyridine nucleotide co-substrate imposes significant limitations on the successful deployment of P450 enzymes for large-scale biotechnological applications. As a result, a number of more cost-effective strategies for electron delivery have been pursued, including alternative chemical [8], photo- [9,10] and electrochemical reduction methods [11], and cofactor recycling [12], among others.

A highly sought alternative is provided by eliminating the necessity for electron transfer processes altogether, and initiating P450 catalysis through a “shunt” pathway in which hydrogen peroxide (H_2O_2) is used to generate Compound I. However, the efficiency of “peroxygenase” turnover by O_2 activating P450s is typically meagre, due to inefficient O–O heterolysis and subsequent enzyme inactivation. Nonetheless, some success has been achieved by using directed evolution methods to enhance enzymatic stability [13]. By comparison, the CYP152 family of P450s are efficiently tuned for the utilization of H_2O_2 as a co-substrate for fatty acid (FA) hydroxylations with variable regiospecificities [14–19]. A structurally similar CYP152 [20] (termed OleT), catalyzes a highly atypical C–C lyase reaction to afford 1-alkenes [21,22] and carbon dioxide [23]. In CYP152 enzymes, the substrate carboxylate, which is anchored in place via electrostatic interactions with a conserved active-site arginine (Arg245 in OleT) is obligatory for efficient catalysis. While providing a strategy for O–O bond cleavage, this imparts significant limitations on the range of substrates that can be metabolized. To circumvent this requirement, Watanabe, Shoji, and co-workers have exquisitely demonstrated that non-native chain length fatty acids, as short as acetic acid, can activate CYP152 enzymes and permit the hydroxylation of wide range of secondary substrates that lack a terminal carboxylate [24–27]. In a structurally unrelated class of H_2O_2 dependent thiolate-ligated heme fungal enzymes termed aromatic or unspecific peroxygenases which include the archetypal *Aae*-APO [28–31],

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a similar stratagem is used. X-ray crystallographic studies of *Aae*-APO [28] reveal that an active-site glutamate instead provides the carboxylate necessary for efficient peroxide utilization. Although robust catalysts, limitations in the heterologous expression of these highly glycosylated enzymes in genetically tractable hosts may hamper their more widespread biotechnological use.

Inspired by the internal solution provided by *Aae*-APO, we reasoned that introduction of an appropriately placed active-site carboxylate side chain may permit OleT to utilize H_2O_2 for the metabolism of a broad range of hydrocarbon substrates. It would also provide a means to evaluate whether the OleT Compound I species, which has been directly shown to initiate the decarboxylation reaction sequence, is capable of more traditional oxygen insertion reactions. In this work, we show that a suitably placed distal carboxylate residue enables the efficient utilization of H_2O_2 by OleT, and dramatically alters the substrate preference of the enzyme. The engineered OleT variant is shown to catalyze epoxidation and hydroxylations with several non-fatty acid substrates.

2. Materials and methods

2.1. Heterologous expression and purification of WT OleT and P246D OleT

Wild-type (WT) cytochrome P450 OleT from Jeotgalicoccus sp. ATCC 8456 was overexpressed, purified and quantified as previously described [23]. Introduction of the P246D mutation was achieved using the following primer and its reverse complement:

5′-GACTTGATGAACACCTTTCGCGATCTGATCGCCATCAATCGCTTC.

Successful mutagenesis was verified via gene sequencing at EtonBio Inc, and purification was identical to the wild-type enzyme.

2.2. Optical spectroscopy

A Hewlett-Packard 8453 spectrophotometer was used for optical spectroscopic studies. For substrate titration experiments of WT OleT with palmitic acid, an 800 μL of a mixture containing 10 μM of WT OleT in 200 mM KPi (pH 7.5) was titrated with small aliquots of 10 mM palmitic acid stock dissolved in DMSO with a 10 μL Hamilton syringe. The final amount of DMSO never exceeded 5% (v/v). The substrate induced absorbance changes at 392 nm and 417 nm was fitted with the following modified quadratic function (Morrison equation) in Origin software. A_{max} is the maximum absorbance change at ligand saturation, S is the fatty acid concentration, E_t is the concentration of peroxide-treated WT OleT, and K_d is the dissociation constant.

$$A_{obs} = \left(\frac{A_{max}}{2E_t}\right)(S + E_t + K_d) - \left(\left((S + E_t + K_d)^2 - (4SE_t)\right)^{0.5}\right)$$

In the case of P246D OleT, a prolonged 30 min incubation of a 25 fold molar excess of palmitic acid with the enzyme (4 μM) failed to produce significant changes in the optical spectrum.

Carbonmonoxy (CO) difference spectra were collected in a sealed cuvette containing 10 μM P246D OleT and 1 μM of methyl viologen. The solution was gently bubbled with CO at 4 °C for 20 min. Subsequently, 25 μL of 10 mM sodium dithionite (prepared in N_2 buffer) was added using a Hamilton syringe. The ferric enzyme was used as a baseline.

2.3. Electron paramagnetic resonance spectroscopy

Samples contained 195 μM of P246D OleT. For substrate binding studies, 410 μM of palmitic acid (prepared as a 10 mM stock in ethanol) was added and allowed to incubate on ice for 30 min before flash freezing in liquid N_2. X-band EPR spectra were recorded using a Bruker EMXplus spectrometer equipped with an Oxford Instruments ESR900 liquid helium continuous flow cryostat under the following conditions: temperature 10 K, modulation amplitude 10 G, microwave power 2 mW. A total of 5 scans were collected for each sample.

2.4. Product analysis of WT OleT and P246D OleT

A 2 mL reaction mixture containing 5 μM of WT OleT or P246D OleT, 10 μL of neat small molecules (cyclohexane, nonane, or styrene), 500 μM of eicosanoic acid (from a 10 mM stock dissolved in ethanol), or 500 μM of palmitic acid (dissolved similarly), in 200 mM KPi (pH 7.5), 200 mM NaCl and 10% glycerol (v/v) was allowed to stir at room temperature for 15 min prior to the addition of 5 mM of H_2O_2 oxidant at the rate of 2 mL/h for an hour. The reaction was terminated by the addition of equal volume of chloroform followed by the internal standard. The mixture was vortexed and centrifuged to separate the organic phase, and 5 μL of the organic phase was immediately loaded onto an FID-GC or GC-MS instrument equipped with a DB-5MS column for analysis. Oven conditions for the various metabolites follow. Styrene: 80 °C for 3 min, 10 °C/min to 250 °C, hold for 2 min. Cyclohexane and nonane: 60 °C for 3 min, 10 °C/min to 180 °C, 20 °C/min to 280 °C, hold for 3 min. C_{16} and C_{20} fatty acids: 170 °C for 3 min, 10 °C/min to 220 °C, 5 °C/min to 320 °C, hold for 3 min. Myristic acid was used as an internal standard for the quantification of fatty alcohols produced from eicosanoic acid and palmitic acid. 1-hexadecene was used as a standard for alkene products. Phenol was used as a reference for cyclohexane derived products. Hexane was used as a reference quantitation of nonane products. Cyclohexanol was used as a reference for styrene metabolites.

3. Results and discussion

3.1. Design, spectroscopic characterization, and fatty acid binding properties of the OleT P246D variant

Based on a comparison of the crystal structures of *Aae*-APO [28] and eicosanoic acid bound OleT [21] (Fig. 1A) the position of the nearest carboxylate oxygen is fairly invariant, ranging from 5.1 to 5.3 Å respectively. A modeled structure of the proline to aspartate OleT variant (P246D), shown in Fig. 1B, positions the introduced carboxylate within an effective range to interact with Arg245 (3.5–4 Å) and facilitate the heterolysis of H_2O_2 (4 Å from the heme iron). The P246D OleT variant was generated, expressed in *E. coli* in a soluble form and purified using techniques previously described by our laboratory [23]. Retention of the thiolate axial ligand was verified by a prominent carbonmonoxy difference spectrum at 445 nm (Fig. 2A inset) and by electron paramagnetic resonance (EPR). Intriguingly, the addition of a ten-fold molar excess of palmitic acid (C_{16}) caused no appreciable change in the optical spectrum of P246D (Fig. 2A, gray trace). This is in sharp contrast to the wild-type (WT) enzyme. Although dissociation constants for medium to long chain length saturated fatty acids have been previously reported for OleT [21], Munro and colleagues have noted significant variations in binding parameters with different preparations of the enzyme. For direct comparison, the WT enzyme was purified using similar conditions used for P246D. The optical changes associated with the binding of palmitic acid to WT are shown in Fig. 2C. A significant low-to high-spin conversion (~60% at saturation) is clearly visible, resulting in changes in the Soret λ_{max} from 417 to 392 nm. A dissociation constant (K_D) for palmitic acid was determined by fitting the optical changes with a quadratic Morrison binding expression, resulting in a $K_D = 700 \pm 50$ nM

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