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Monoterpene hydroxylation with an artificial self-sufficient P450 utilizing a $P450_{SMO}$ reductase domain for the electron transfer

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ABSTRACT

Cytochrome $P450_{SMO}$ from *Rhodococcus* sp. ECU0066 is a natural self-sufficient P450 monooxygenase, consisting of a heme domain, a flavin-reductase domain containing FMN and NADPH binding sites, and a $[Fe_2S_2]$ ferredoxin domain. $P450_{cam}$ catalyzes the hydroxylation of camphor to 5-exo-hydroxycamphor. The variant $P450_{cam}$ (Y96F/V247L) was reported for the oxidation of monoterpene by protein engineering. In this work, we constructed an artificial self-sufficient P450-type monoterpene hydroxylase by connecting the $P450_{SMO}$ reductase domain and the $P450_{cam}$ (Y96F/V247L) domain together with a linker region $(G_4S)_4$. The resultant chimeric P450 enzyme could catalyze the hydroxylation of (−)-limonene and α-pinene as well as camphor, which were all inactive for the natural fusion protein $P450_{SMO}$. Co-expression of the fused P450 with a glucose dehydrogenase (GDH) improved the (−)-limonene conversion as sufficient NADPH was regenerated in the system with glucose as a cosubstrate. This work illustrated that $P450_{SMO}$ reductase might act as an electron donor partner of P450s and might be used for fusion with heterogeneous P450 domains to elucidate the catalytic function of other unknown P450s.

1. Introduction

P450s are hemoproteins encoded by a super-family of genes. They can convert a broad variety of substrates and catalyze a series of interesting chemical reactions. It is worth mentioning that P450s can participate in hydroxylation of terpenes, which are essential intermediates for the spices industry [1]. The cytochrome P450 reactions must be associated with electron donor partner protein(s) for two electrons transferred from nicotinamide adenine dinucleotide phosphate [NAD(P)H] to the heme domain of a P450. For example, adrenal mitochondrial P450 systems obtain electrons from NADPH *via* adrenodoxin reductase and adrenodoxin; and the liver microsomal P450s obtain electrons from NADPH *via* a FAD and FMN-containing P450 reductase [2]. The inherent requirement for the individual protein partner(s) usually made P450s difficult for significant discovery and application [3,4]. Meanwhile, electrons generated by the redox partner, which are intended to fuel the P450 oxidation process, are not always transferred effectively. Even the reducing equivalents are successfully transferred to the heme domain, they can be employed in the formation of water or hydrogen peroxide and are therefore useless for substrate oxidation [5,6].

$P450_{BM3}$, which is naturally fused to a eukaryotic-like reductase, represents an effective solution to these limitations. The fusion nature of this enzyme greatly improves the electron-transfer efficiency and the oxidation activity for fatty acids [7]. Based upon the self-sufficiency of this naturally fused enzyme, a number of engineered proteins of diverse eukaryotic P450s bearing a reductase domain from $P450_{BM3}$ have been generated with *in vitro* activities [8–10]. This provides ready access to the great catalytic versatility of the membrane-bound eukaryotic P450s. Recently, it has been reported that the reductase domain of $P450_{RhF}$ from *Rhodococcus* sp. NCIMB 9784 can be fused to some Class I P450s or Class II eukaryotic microsomal P450s ($P450_{cam}$, $P450_{bzo}$, $P450_{balk}$, $P450_{PikC}$, $P450_{XplA}$, CYP73A5) for the generation of self-sufficient enzymes in whole cell biotransformation or in cell free assays [11–15]. $P450_{SMO}$, a new member of CYP116 family, has been discovered in our laboratory, showing significant sulfoxidation activity toward several sulfides [16,17]. $P450_{SMO}$, belonging to Class IV P450 family, contains a heme domain, FMN and NADPH binding motifs and a $[Fe_2S_2]$ ferredoxin-like center. The composition of $P450_{SMO}$ reductase domain has the similarity to the electron transfer partner of

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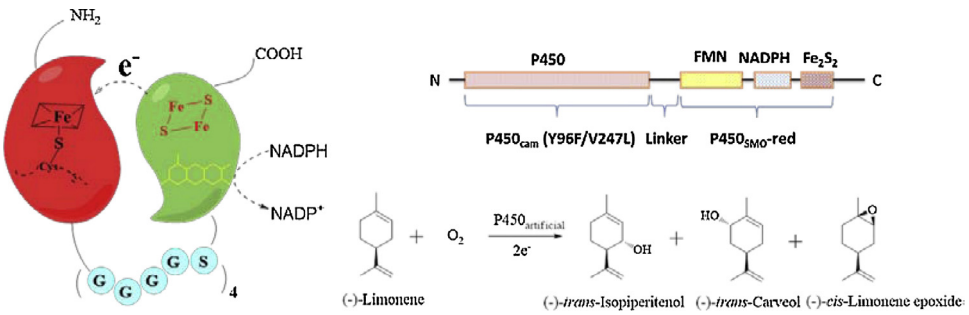

Scheme 1. Construction of an artificial P450 system: $P450_{cam}$ (Y96F/V247L)–$P450_{SMO\ red}$.

Class I P450. So we speculated whether the $P450_{SMO}$ reductase domain could act as an effective redox partner for generating efficient P450 chimeras.

Cytochrome $P450_{cam}$ (PdR, Pdx) catalyzes the oxidation of the bicyclic compound D-(+)-camphor to 5-exo-hydroxycamphor, the first step in the camphor metabolism pathway of the soil bacterium *Pseudomonas putida*. The Y96F/V247L mutant of $P450_{cam}$ could catalyze the hydroxylation of (−)-limonene in one step, producing (−)-*trans*-isopiperitenol, (−)-*trans*-carveol and (−)-*cis*-limonene epoxide [18]. In the present work, we therefore constructed a self-sufficient chimera $P450_{cam}$ (Y96F/V247L)–$P450_{SMO\ red}$, by fusing the $P450_{SMO}$ reductase domain with the $P450_{cam}$ mutant domain together (see Scheme 1). By optimizing the linker sequence between the two domains and co-expressing with glucose dehydrogenase (GDH, used for NADPH regeneration), the whole cell could be used for monoterpene hydroxylation, indicating that the $P450_{SMO}$ reductase might be used as the electron transfer partner for heterologous P450s. Furthermore, the biosynthetic P450s (Class I) lacking such a universal reductase may be engineered similarly into diverse self-sufficient P450s for either functional characterization or potential application.

2. Experimental

2.1. Engineering the chimeric $P450_{cam}$ (Y96F/V247L)–$P450_{SMO\ red}$ F1–F4

The genes of $P450_{cam}$ and $P450_{SMO}$ were cloned from *P. putida* PpGl (ATCC17453) and *Rhodococcus* sp. ECU0066, respectively. Site-directed mutagenesis of $P450_{cam}$ (Y96F/V247L) gene was carried out with the Quick Change® Site-directed Mutagenesis Kit. The primers for the mutagenesis were shown in Table S1 (Supporting Information).

As shown in Table S1, $P450_{cam}$ (Y96F/V247L) and $P450_{SMO}$ reductase were amplified with primers A, C1/D1, C2/D2, C3/D3, C4/D4, B to construct four different linkers. The amplified P450 gene has *Nde*I site at 5′-ends and *Hin*dIII at the 3′-ends. Meanwhile, the $P450_{SMO\ red}$ gene has *Hin*dIII site at 5′-ends and *Xho*I at the 3′-ends. Then the two genes were ligated into pET28a(+) and fused together. The fused enzymes $P450_{cam}$ (Y96F/V247L)–$P450_{SMO\ red}$ F1–F4 have the His-tag at the *N*-terminal for subsequent purification. The fused enzymes are abbreviated as $P450_a$ F1–F4 in this article.

2.2. The expression of fusion enzymes

Recombinants containing pET28a(+)-$P450_a$ F1–F4 were grown in 100 mL Luria-Bertani (LB) medium with 100 μg/mL of kanamycin at 37 °C. After the optical density OD_{600} had reached 0.5, 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and 0.2 mM δ-amino levulinic acid (δ-ALA) were added, and then the cultivation was continued with reciprocal shaking for 18 h at 25 °C. The cells were collected, suspended in 50 mM potassium phosphate buffer (pH 7.5), and disrupted by sonification. After centrifugation, the soluble fractions were subjected to spectrophotometric analysis or reductase domain activity assay.

2.3. Spectrophotometric analysis and reductase activity assay of $P450_a$ F1–F4

To quantitate the content of active cytochrome P450 in the recombinant *E. coli*, the soluble fraction of $P450_a$ F1–F4 were used to measure the CO-reduced P450 absorption with a UV-vis spectrophotometer [19]. The reductase activity was determined by measuring the increase in absorbance at 550 nm due to the reduction of cytochrome *c* [20].

2.4. Bioconversion of (−)-limonene by the fused enzymes with different linkers

To determine the catalytic activity of the fused enzymes with different linkers, we performed a 1 mL reaction with the cell free extracts. The reaction mixtures containing 50 mM potassium phosphate buffer (pH 7.5), 1 μM $P450_a$ F1–F4, 100 μM (−)-limonene and 100 μM NADPH were incubated at 25 °C. After incubation for 12 h, the reaction mixture was extracted with 900 μL ethyl acetate by vigorous shaking. The organic layer was separated by centrifugation, dried over anhydrous sodium sulfate, and then subjected to GC–MS analysis.

The negative control was conducted under the same condition using the same amount $P450_{cam}$ (Y96F/V247L) and the $P450_{SMO}$ reductase, which were not fused together. The positive control was conducted by $P450_{cam}$ system under the same condition, which contained $P450_{cam}$ (Y96F/V247L), putidaredoxin reductase (PdR) and putidaredoxin (Pdx).

2.5. Plasmid construction for co-expression of $P450_{cam}$ (Y96F/V247L)–$P450_{SMO\ red}$ F3 and GDH genes

Plasmids and the primers used for co-expression in this study are listed in Tables S2 and S3. For construction of the co-expression system, the glucose dehydrogenase (GDH) gene was amplified with primers 1 and 2 using genome DNA from *Bacillus megaterium*. The resulting 786 bp fragment was digested with *Nde*I and *Xho*I and then ligated into *p*ACYCDuet-1 which was digested with the same restriction enzymes, generating the plasmid *p*ACYCDuet-1-GDH. Successful ligation into *p*ACYCDuet-1 was confirmed by restriction analysis.

Co-expression of $P450_a$ F3 and GDH genes was performed in a two-plasmid system with different origins for replication, $P450_a$ and GDH genes were respectively cloned into pET28a(+) and *p*ACYCDuet-1. The same *E. coli* strain transformed with both pET28a(+)-$P450_a$ and *p*ACYCDuet-1-GDH was named as BL21-pET28a-$P450_a$ F3/*p*ACYCDuet-GDH (see Scheme 2).