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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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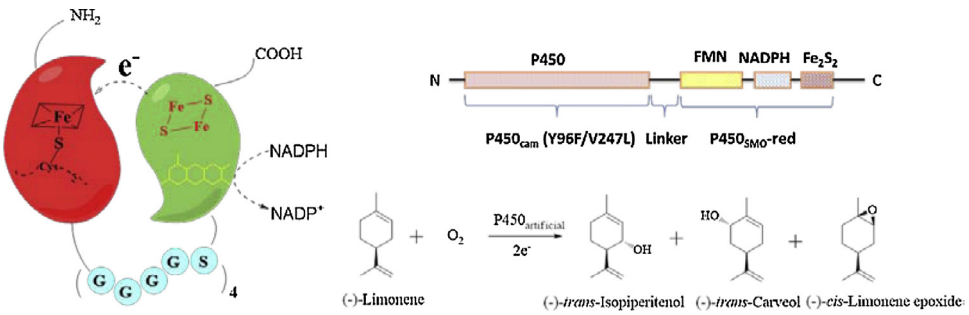


Scheme 1. Construction of an artificial P450 system: $P450_{cam}$ (Y96F/V247L)–$P450_{SMO\ red}$.

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

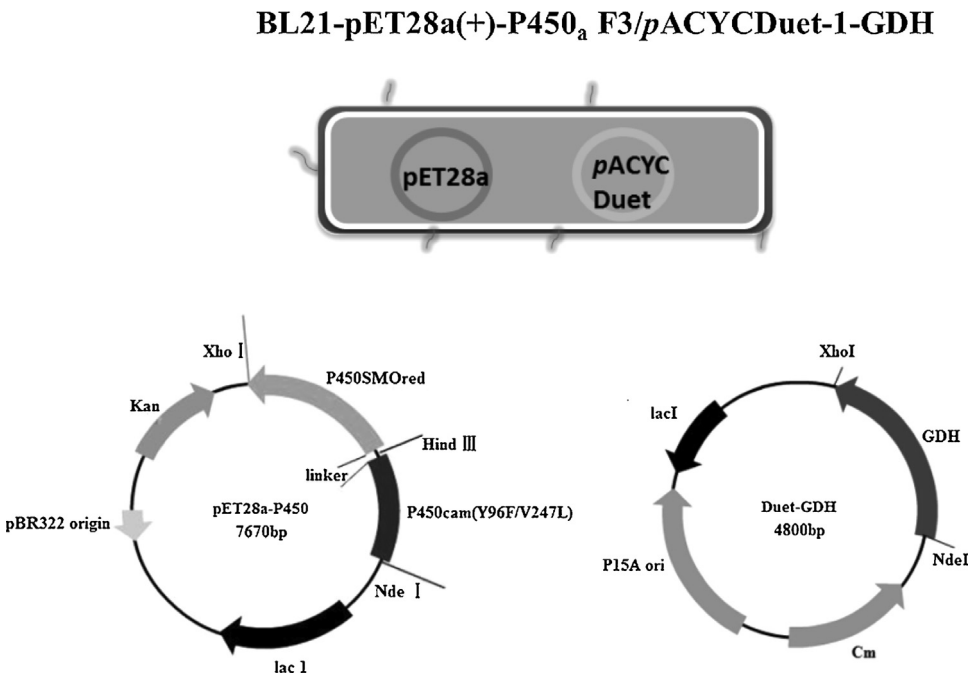
To determine the catalytic activity of the fused enzymes with different linkers, we performed a 1 mL reaction with the cell free extracts. The reaction mixtures containing 50 mM potassium phosphate buffer (pH 7.5), 1 μM $P450_a$ F1–F4, 100 μM (−)-limonene and 100 μM NADPH were incubated at 25 °C. After 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).



Scheme 2. The dual plasmid configurations for co-expression of $P450_a$ F3 and GDH in *E. coli*.

2.6. Monoterpene hydroxylation with BL21-pET28a-$P450_a$ F3/pACYCDuet-GDH

Biotransformation was performed in 10 mL reaction mixture with a 50 mL sealed conical flask. The reaction mixtures containing 50 mM potassium phosphate buffer (pH 7.5), 0.1 g recombinant wet cell/mL, 100 μM (−)-limonene (camphor or α-pinene), 100 μM $NADP^+$, 5 mM glucose were incubated at 25 °C. After incubation for a certain time, samples were withdrawn for GC–MS analysis.

2.7. The dissociation constants (K_d) of $P450_a$ F3 with (−)-limonene, camphor and α-pinene

The dissociation constants (K_d) of $P450_a$ F3 were determined at 30 °C by titration of 1 mL purified $P450_a$ F3 (1 μM) in 50 mM phosphate buffer (pH 7.5) with substrates (−)-limonene, camphor and α-pinene in ethanol. The spectroscopic changes (350–500 nm) associated to the sequential addition of substrate (in the range of 0.1–35 μM) were recorded. The difference in absorbance between the wavelength maximum (418 nm) and minimum (390 nm) was plotted *versus* the substrate concentration into a Michaelis–Menten equation to estimate K_d [21].

2.8. Analytical methods

The analysis of products was performed by GC–MS (Shimadzu GC–MS QP2010 Plus) with an intercap-5 column (30 m length; 0.25 mm diameter). The injection and detection temperatures were 250 °C and 230 °C, respectively. The column was subjected to an initial temperature of 50 °C for 3 min, with the temperature then being raised to 250 °C at a rate of 10 °C/min for 10 min. The MS spectrum of product was the same as the literature [22]. (−)-Isopiperitenol gave a 12.05 min peak and was quantified by a selective ion monitoring mode generating standard curves. (−)-Carveol and (−)-limonene epoxide showed the peaks at 12.30 and 10.95 min, respectively.

3. Results and discussion

3.1. Construction of fusion enzymes $P450_a$ F1–F4 with different linkers

The successful construction of a recombinant fusion protein requires two indispensable elements: the component proteins and the linker [23]. Here based on the desired monoterpene hydroxylation of the fusion protein, we selected $P450_{cam}$ (Y96F/V247L) and $P450_{SMO\ red}$ directly. On the other side, the selection of a suitable linker to join the protein domains together is very crucial in this work. We designed four different linkers for connecting $P450_{cam}$ (Y96F/V247L) and $P450_{SMO\ red}$ domains with different length and secondary structure. Linkers F1 [$(G_4S)_3$] and F3 [$(G_4S)_4$] contain flexible residues glycine and serine so that the adjacent protein domains can move freely to close one another and maintain necessary inter-domain interactions. Meanwhile we constructed the relatively rigid linkers F2 [RLAST] and F4 [$(RLAST)_2$] for a comparison. Fig. 1 shows the SDS-PAGE of the protein expression. The production of active P450 protein and the activity of the reductase domain were on the same level for different fused enzymes (Table 1).

As shown in Table 1, (−)-limonene biotransformations were performed using $P450_a$ with different linkers (F1–F4). The reactions

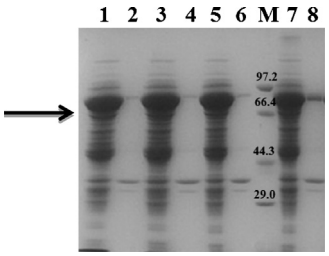


Fig. 1. SDS-PAGE analysis of the fused enzymes. Lanes 1, 3, 5 and 7 were the supernatant of $P450_a$ F1–F4, respectively. Lanes 2, 4, 6 and 8 were the precipitant of the fused enzymes.

Table 1
Activity assay of the fused enzymes.

	F1 [$(G_4S)_3$]	F2 [RLAST]	F3 [$(G_4S)_4$]	F4 [$(RLAST)_2$]
[P450] (nmol/$g_{wet\ cell}$)[a]	32.6 ± 0.3	40.4 ± 1.5	54.6 ± 0.8	39.4 ± 1.1
Reductase activity (kU/$g_{wet\ cell}$)[b]	9.76 ± 0.1	11.0 ± 0.2	10.0 ± 0.5	11.4 ± 0.1
[(−)-Isopiperitenol] (μM)[c]	2.1 ± 0.1	n.d.[d]	5.2 ± 0.3	n.d.

[a] Ferrous CO *versus* ferrous difference spectrum was used for the quantitation of the P450 (see Fig. S1).
[b] The activity of reductase domain was measured by NADPH-cytochrome *c* reduction assay. One unit of enzyme activity was defined as the amount of enzyme catalyzing the reduction of 1 nmol of cytochrome *c* per min.
[c] The concentration of (−)-isopiperitenol was measured by GC–MS. The TIC and mass spectra for the products were shown in Fig. S2.
[d] n.d.: no product was detected.

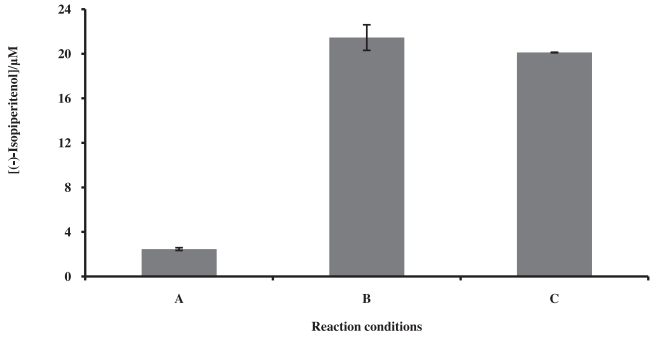


Fig. 2. Hydroxylation of (−)-limonene with or without GDH. Condition A: biotransformation with whole cells of BL21-pET28a(+)-P450$_a$ F3, plus 100 μM NADPH; condition B: biotransformation with whole cells of the co-expression system BL21-pET28a(+)-P450$_a$ F3/*p*ACYCDuet-GDH, plus 100 μM $NADP^+$; condition C: *in vitro* biotransformation with the cell free extract of BL21-pET28a(+)-P450$_a$ F3, plus GDH (20 U) and 100 μM $NADP^+$.

were carried out using the same amount of P450s (1 μM). No product was detected for the negative control, among the fused enzymes, P450$_a$ F2 and F4 gave no hydroxyl product after 12 h. The fused enzyme F1 and F3 both mediated the conversion of limonene to isopiperitenol, and that the activity of F3 appeared to be higher. Perhaps the appropriate length of the flexible linker F3 [$(G_4S)_4$] could provide the joined domains the chance for necessary movement or interaction. While, the α-helix linkers may act as a rigid spacer between protein domains and make the independent structures that did not interact with the adjacent protein domains. So the following experiments were all based on P450$_a$ F3.

3.2. Co-expression of GDH with the fused enzyme P450$_a$ F3

As stated above, P450s require a cofactor to supply electrons for the substrates oxygenation. However, the most common source of reducing equivalents, NAD(P)H, has proven to be a major barrier hampering the industrial implementation of P450s [24–26]. So we introduced the cofactor regeneration pathway into the *E. coli* cell containing the P450$_a$ F3. Comparing the different co-expression systems, the dual plasmid co-expression system was chosen, which showed higher activity of GDH, as well as higher protein expression level of P450 (Table S4). As sufficient NADPH was regenerated, the concentration of (−)-isopiperitenol was significantly improved by approximately 10 folds upon the introduction of GDH co-expressing system (Fig. 2). It suggested that NADPH was limiting in the absence of the GDH regeneration system.

3.3. Biotransformation of (−)-limonene by BL21-pET28a-P450$_a$ F3/pACYCDuet-GDH

(−)-Limonene is the major component of peel oil from oranges and lemons, so it is volatile like other terpenes. Thus high-yield biotransformations should be performed with a closed system. On the other hand, the hydroxylation reaction catalyzed by P450s needs oxygen as the oxidant, thus the availability of oxygen in the closed flask might have a significant influence on the hydroxylation. So we optimized the reaction system in flasks with different loading volume, indicating that the loading of 10 mL cell suspension in a 50-mL closed conical flask was better (Fig. S3). Here, the reaction was conducted using 0.1 g wet cell/mL buffer, 100 μM (−)-limonene, 100 μM $NADP^+$, and 5 mM glucose. Under the optimum condition, we got the reaction progress curve of the (−)-limonene

Table 2
The K_d values and product distribution of P450$_a$ F3 with different substrates.

Substrate	K_d^0 (μM)[a]	K_d (μM)[b]	Product distribution (%)[c]		
(−)-Limonene	8.6 ± 2.6	9.6 ± 1.4	46	38	16
Camphor	5.2 ± 1.0	4.1 ± 0.8	89		11
α-Pinene	6.5 ± 0.6	3.6 ± 0.9	54		46

[a] The dissociation constants of P450$_{cam}$ (Y96F/V247L) toward (−)-limonene, camphor and α-pinene were shown in Fig. S4 (A).
[b] The dissociation constants of purified P450$_a$ F3 toward (−)-limonene, camphor and α-pinene were shown in Fig. S4 (B).
[c] Values were obtained from GC–MS peak area integrations. The oxidation products were analyzed by GC–MS. Fig. S5 gives the mass spectra of the products.
[d] Not detected.

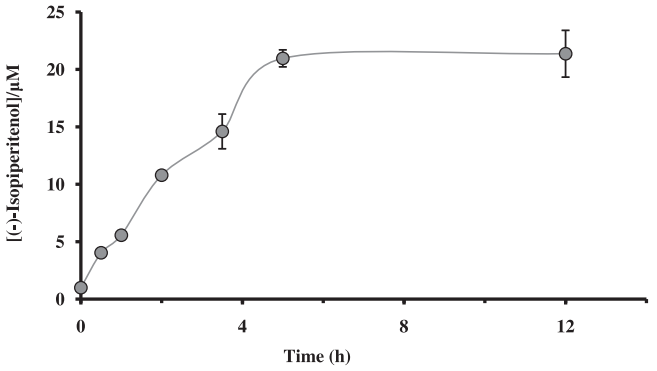


Fig. 3. Biotransformation of (−)-limonene by BL21-pET28a-P450$_a$ F3/*p*ACYCDuet-GDH.

biotransformation (Fig. 3) where the concentration of desired product (−)-isopiperitenol was quantitatively measured by GC–MS.

3.4. Bioconversion of other substrates by BL21-pET28a-P450$_a$ F3/pACYCDuet-GDH

The catalytic activity of the artificial self-sufficient P450 toward other terpenes was checked in the 10 mL reaction system. As shown in Table 2, the fused enzyme P450$_a$ F3 exhibited the activity toward the hydroxylation of these substrates. The K_d values of the purified enzyme P450$_a$ F3 with different substrates were also measured, which are similar to the mutant P450$_{cam}$ (Y96F/V247L). This indicates that the attachment of the heterologous reductase domain has no significant impact on the substrate binding with P450$_{cam}$ (Y96F/V247L) domain.

4. Conclusions

In this report, we successfully constructed an artificial self-sufficient P450 system (P450$_a$), *i.e.*, P450$_{cam}$ (Y96F/V247L)–P450$_{SMO\ red}$, utilizing the P450$_{SMO}$ reductase domain for the electron transfer. The fused enzyme could effectively hydroxylate (−)-limonene to (−)-isopiperitenol, (−)-carveol and (−)-limonene epoxide, which are important intermediates for spices and pharmaceutical industry. Two major factors, the secondary structure and the length of the linker between the P450$_{cam}$ (Y96F/V247L) domain and the P450$_{SMO}$ reductase domain, have been varied to optimize the fusion system. The results have shown that the linker modification may alter electron transport or influence the extent to which correctly folded fusion protein is formed. In our study, the flexible loop linker, $(G_4S)_4$, could enable the two joined domains to move and interact, which is crucial for the electron transfer. Analysis of the P450 concentration and the activity of the reductase domain revealed that the fused enzyme P450$_{cam}$ (Y96F/V247L)–P450$_{SMO\ red}$ F3 retains the original characteristics of the two domains. Meanwhile, the K_d values of the fused enzyme with different substrates are similar to the wild type, indicating that the fused heterologous reductase domain has no significant influence on the substrate binding with P450$_{cam}$ (Y96F/V247L) domain. Considering providing sufficient cofactor NADPH, we constructed the co-expression system of GDH with P450$_a$ F3, which improved the hydroxylation by 10-fold due to the enzymatic regeneration of NADPH from $NADP^+$ at the cost of glucose.

P450-catalyzed reactions depend on the redox partner for generation of active ferryloxo species which is a key for these enzymes to carry out oxidation reactions. The Class I or biosynthetic P450s lack such a universal reductase partner and the discovery of their natural partner may be very laborious. The chimeric technology we presented here might be a solution to this problem. On the other hand, because the P450s and their reductase domains are originated heterogeneously, the fused enzyme showed a relatively low NADPH coupling efficiency (less than 10% toward (−)-limonene, compared to 62% coupling efficiency of P450$_{cam}$-PdR-Pdx system). Co-expression of a glucose dehydrogenase in the recombinant *E. coli* cell containing P450 gene could solve the above problem and supply sufficient NADPH for the hydroxylation reaction. For further study, we need to pay a close attention to the interactions between the two heterogeneous domains and the improvement of their coupling efficiency.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molcatb.2015.02.006.

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