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Enhancing the catalytic performance of a CYP116B monooxygenase by transdomain combination mutagenesis

Ren-Jie Li,[a] Jian-He Xu,[a]* Qi Chen,[a] Jing Zhao,[b] Ai-Tao Li,[c] Hui-Lei Yu[a]*

Abstract: The cytochrome P450 monooxygenase discovered in *Labrenzia aggregata* ($P450_{LaMO}$) is a self-sufficient redox system with versatile oxygenation functions. However, its catalytic performance is severely hindered by a low reaction rate, poor electron coupling efficiency (CE) and fragile thermostability. Herein, a simple transdomain combination mutation strategy was proposed for engineering this multi-domain P450 enzyme with redox partners fused to the heme domain. After focused mutagenesis on the heme domain, a triple mutant H3 (N119C/V264A/V437G) was hit, that improved the turnover frequency (TOF) and CE of $P450_{LaMO}$ by about 7.8-fold and 3.0-fold, respectively. A redox domain-based mutant with higher cytochrome *c* reduction activity, MR1 (M612L/K774Y), mediated more efficient electron transfer, elevated the TOF by 4.9-fold, and the coupling efficiency by 4.2-fold. The beneficial effect was further enhanced by combining the mutation sites from different domains, resulting in a combinatorial mutant (N119C/V264A/V437G/M612L/N694D) with a 9.1-fold increase in coupling efficiency, 10-fold in TOF, as well as +3.8℃ in thermostability (T_{50}^{10}). Meanwhile, for series of tetrahydronaphthalene derivatives, this combinator showed higher hydroxylation activity. This work suggested that employing this combinatorial strategy targeting on both the redox and heme domains is efficient to improve holoenzyme activity, CE and stability of a CYP116B subfamily member from the low starting point.

Introduction

Cytochrome P450 monooxygenases are a superfamily of heme-containing catalysts in nature,[1] performing various reactions such as inert C-H bond hydroxylation,[2] *O*-dealkylation[3] and sulfoxidation.[4] Self-sufficient CYP116B subfamily members fuse the heme and redox domains into a single polypeptide chain, and the redox partners shuttle electrons from the reductant NADPH, through the specific FMN and Fe_2S_2 domains, to their acceptor, the heme domain.[5]

Until now, there have been a few typical members in the CYP116B subfamily, including $P450_{RhF}$ (CYP116B2),[6] CYP116B3,[7] CYP116B1,[8] CYP116B5,[9] CYP116B4,[10] $P450_{SMO}$,[11] $P450_{RpMO}$, $P450_{ArMO}$, $P450_{CtMO}$[12] as well as the latest reported CYP116B29, CYP116B46, CYP116B63, CYP116B64 and CYP116B65.[13] Moreover, various reaction types have been reported, such as aromatic hydroxylation, *O*-dealkylation, epoxidation and asymmetric sulfoxidation by $P450_{RhF}$; *N*-dealkylation by CYP116B1; benzene derivative dealkylation by CYP116B3; medium- and long-chain alkane (C_{14}, C_{16}, C_{24} and C_{36}) hydroxylation by CYP116B5. However, the poor activity and low efficiency of electron utilization of the members in this CYP116B subfamily, made it overshadowed compared to another self-sufficient redox family,[14] CYP102, such as the well-known $P450_{BM3}$. Therefore, it is highly desired to improve the catalytic properties and to release its oxyfunctionalization potential.

Protein engineering provides an efficient approach to improve P450 monooxygenases' catalytic efficiency. Many efforts have been made in terms of local segment mutagenesis, such as the random mutagenesis for heme domain of $P450_{LA1}$,[10b] which improved the efficiency of *anti*-Markvonikov addition of styrene and the domain-based engineering method for $P450_{BM3}$.[15] In particular, $P450_{BM3}$ mutants achieved the highest electron coupling efficiency for propane by introducing mutagenesis into the heme, FMN and FAD domains followed by mutation fusion. Moreover, high-throughput screening was focused on pseudosubstrate-based colorimetric assays. However, there are only very few high-throughput screening methods available for the CYP116B subfamily. For example, Liu et al.[3c] established a heme domain mutation library of CYP116B3 to enhance its dealkylation activity using a 7-ethoxycoumarin-based fluorescence method, which is limited to this specific type of substrate. Another report by Yan et al.[16] employed desorption electrospray ionization coupled with ion mobility mass spectrometry imaging (DiBT-IMMS) to screen for higher hydroxylation activity for diclofenac transformation within live bacterial colonies, but it seems that it is still far from generalization due to the high cost of special facilities for screening. Latest report by Hammer et al[10c] used a Purpald-based method to efficiently screen error prone randomly libraries to improve the productivity of CYP116B4, however, this method was aldehyde-specific could not be used to other reactions such as hydroxylation.

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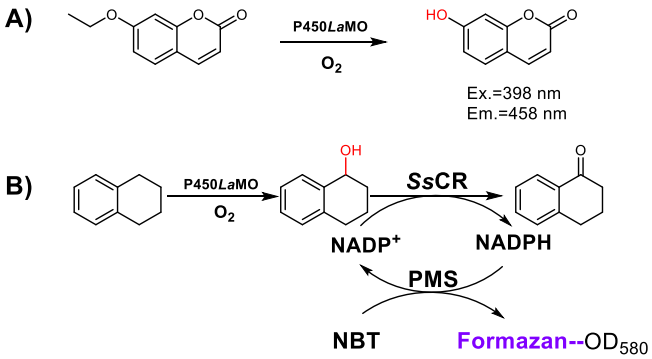

NBT: nitro blue tetrazolium; **PMS:** phenazine methosulfate
***Ss*CR:** *Scheffersomyces stipitis* carbonyl reductase

Scheme 1. High-throughput screening methods constructed for the engineering of P450$_{LaMO}$. A) The high-throughput screening of redox domain libraries was based on 7-ethoxycoumarin-based fluorescence method. B) The high-throughput screening of the heme domain libraries was based on the NBT-PMS colorimetric assay.

The P450$_{LaMO}$ discovered from *Labrenzia aggregate*, as reported in our previous work,[10a] showed hydroxylation activity for the core structure of tetralin substrates, albeit with a low reaction rate (0.018 min^{-1}, Table 1) and an extremely low electron coupling efficiency (0.7%, Table 1). In the catalytic cycle of P450s, obtaining a better electron coupling efficiency seems to be more important towards utilizing this catalyst in bioconversions. There are two aspects leading to the low coupling efficiency:[17] a) an unnatural substrate is difficult to form an effective intermediate within the heme domain, resulting in the formation of H_2O_2 and other oxygen species; b) the redox domain cannot efficiently accept the electrons from NADPH and transfer the electrons to the heme domain. Therefore, to improve the electron coupling efficiency and holoenzyme's activity of P450$_{LaMO}$, we used a transdomain combination mutation in this work. Redox domain-based mutants with higher electron transfer efficiency were screened by a high-throughput screening method using a 7-ethoxycoumarin-based fluorescence substrate (Scheme 1A). On the other hand, higher electron utilizing heme-domain based mutants, forming more amounts of hydroxylation product, were selected by an NBT-PMS (nitro blue tetrazolium-phenazine methosulfate) assay that is correlated to changes in the concentration of NADPH produced by the dehydrogenation of sequential product alcohol.[21](Scheme 1B) We expected that both the coupling efficiency and the activity of this CYP116B member could be promoted by this transdomain combination mutation strategy (TDCM) with the aid of appropriate high-throughput screening methods for the screening of different domain-based mutants. We also tried to find some key clues to the factors limiting both the electron utilization and the electron transfer efficiency using this simple combination strategy.

Results and Discussion

Mutagenesis in the Redox Domain of P450$_{LaMO}$

It was reported that the redox domain plays an irreplaceable role in the catalytic cycle of P450s, especially important for accelerating the catalytic efficiency of the heme domain.[18] In this work, introduction of mutations into the redox domain, through random mutagenesis using 7-ethoxycoumarin as the initial screening substrate, resulted in four single-site mutants (R1~R4) with significant improvements in cytochrome *c* reduction activity (Table 1: R1, 319 U/mg, 41-fold; R3, 139 U/mg, 18-fold; R4, 80 U/mg, 10.4-fold), which are also helpful for increasing the turnover frequency (R1, 0.028 min^{-1}, 1.6-fold; R3, 0.029 min^{-1}, 1.6-fold; R4, 0.031 min^{-1}, 1.7-fold) and improving the coupling efficiency (R1, 1.3%, 2.0-fold; R3, 0.85%, 1.3-fold; R4, 0.96%, 1.5-fold) of the holoenzyme (Table 1 and Figure S3). When combining these sites, it is found that a double-site mutant, MR1 (M612L/K774Y), had 4.9-fold and 4.2-fold improvements in TOF and coupling efficiency for tetralin hydroxylation, respectively.

Table 1. Catalytic properties of the redox domain-based mutants of P450$_{LaMO}$

Mutant	Mutation	Cytochrome *c* reduction activity (U/mg protein)[b]	TOF$_{tetralin}$ (min^{-1})[c]	CE$_{tetralin}$ (%)[d]
WT	–	7.7 ± 0.3	0.018 ± 0.002	0.7 ± 0.1
R1[a]	M612L	319 ± 39	0.028 ± 0.002	1.3 ± 0.3
R2[a]	Q684F	26 ± 3	0.036 ± 0.004	1.1 ± 0.03
R3[a]	N694D	139 ± 4	0.029 ± 0.002	0.85 ± 0.02
R4[a]	K774Y	80 ± 6	0.031 ± 0.008	0.96 ± 0.05
MR1	M612L/K774Y	298 ± 7	0.088 ± 0.002	2.8 ± 0.3
MR2	M612L/N694D	112 ± 10	0.078 ± 0.004	2.7 ± 0.6

[a] Redox domain-based mutants was obtained based on 7-ethoxycoumarin fluorescence initial screening method;

[b] Cytochrome *c* reduction activity was measured with spectrophotometric method, as described in the Supporting Information, where 1.0 U = 1 μmol$_{cyt\ c}$ min^{-1};

[c] TOF: Turnover frequency (TOF) was defined as the number of product molecules generated by each P450 enzyme molecule per minute, with the unit of nmol tetral-1-ol nmol P450^{-1} min^{-1};

[d] CE: Coupling efficiency (CE) was defined as the ratio of tetral-1-ol formation rate to NADPH consumption rate.

To characterize the electron transfer capability of the redox domain-based mutants (MR1 and MR2), we measured their kinetic parameters of cytochrome *c* (Cyt *c*) reduction (Table 2 and Figure S1, Supporting Information).[19] The k_{cat} of MR1 for cytochrome *c* was improved to 128 s^{-1} from 58.7 ± 1.4 s^{-1} of the wild type and k_{cat} of MR2 for cyt *c* was to 187 s^{-1} (2.2-fold and 3.2-fold enhancement over WT, respectively, see Table 2).[20] All of the single and double-site mutations in the redox domain contributed to an improvement in turnover frequency of the holoenzyme, suggesting that these variants offer a more efficient

redox partner, which is helpful for achieving higher activity of the holoenzyme. Meanwhile, the results of homologous modeling showed that three substitutions (Q684, N694 and K774) were clustered in the Fe_2S_2 domain, while M612 was located in the FMN domain. The Q684 situated mainly on the surface between the FMN and Fe_2S_2 subdomains (Figure 1A). In particular, residues N694 and K774 were close to the heme domain, and they may affect local changes in protein conformation to bridge the electron transfer between two distinct domains.

Table 2. Cytochrome *c* reduction kinetics of some redox domain-based mutants of $P450_{LaMO}$.

Mutant	K_M (cyt *c*, mM)	k_{cat} (s^{-1})[a]	k_{cat}/K_M ($s^{-1}mM^{-1}$)	Folds improvement[b]
WT	0.031 ± 0.03	58.7 ±1.4	189	1.0
MR1	0.21 ±0.03	128 ±9.0	610	3.2
MR2	0.63 ±0.04	187 ±7.1	297	1.6

[a] Kinetic parameters determined with different concentrations of cytochrome *c*, 0.5 mM NADPH, P450s 20 nM, 100 mM KPi buffer pH 8.0, by measuring the absorbance at 550 nm;

[b] Folds improvement was based on k_{cat}/K_M.

Mutagenesis in the Heme Domain of $P450_{LaMO}$

The redox domain beneficial mutations improved the coupling efficiency and turnover efficiency of holoenzyme (Table 1). However, the turnover frequency for the target substrate tetralin was still low (e.g., only 0.088 min^{-1} for MR1, Table 1). Therefore, we tried to perform directed evolution of the heme domain in order to accelerate both the electron accepting and substrate conversion processes.

As a result, the heme domain-based mutagenesis also helped us improve the TOF value and coupling efficiency of the holoenzyme by about 7.8-fold and 3.0-fold, respectively (Table 3). The conversion by three mutants (H1 (V264A), H2 (V264A/V437G) and H3 (N119C/V264A/V437G)) was improved; in particular, H3 could efficiently transform the substrate tetralin. It is shown that mutations in the heme domain effectively promote the catalytic efficiency of the whole P450 enzyme.

Based on the molecular docking results, we could elucidate the approximate locations of the three mutation sites in the heme domain structure (Figure 1B), showing that site 264 was near the heme, site 119 was close to the entrance channel of substrate and the site 437 was on the surface of the protein. Moreover, the mutation at sites 264 and 119, might decrease the interaction with surrounding amino acid residues because of the reduced number of hydrogen bonds. However, the mechanism on the site G437 influence was still unclear.

Transdomain combination evolution of $P450_{LaMO}$

Via site-directed overlap extension,[21] we combined the beneficial mutations from the heme and redox domains. Among the resulting mutants, the best mutant HR1 exhibited significant improvements in TOF(0.18 min^{-1}) by 10-fold and in coupling efficiency (6%) by 9.1-fold compared to $P450_{LaMO}$ wild type

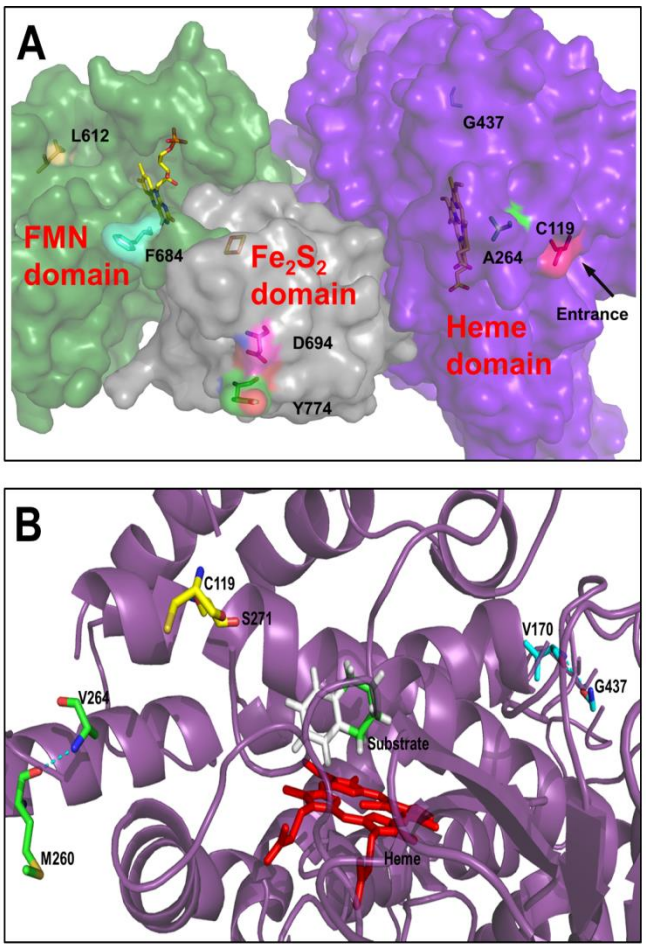

Figure 1. Mutation sites in the heme and redox domains of $P450_{LaMO}$. A) All beneficial sites were showned in the whole protein of $P450_{LaMO}$. Individual domains are shown with different colors: green for FMN domain, gray for Fe_2S_2 domain and purple for heme domain. B) The important residues in heme domain of $P450_{LaMO}$, C119 (yellow), A264 (green) and G437 (cyan) and surrounding interacting sites S271 (yellow), M260 (green) and V170 (cyan).

(0.018 min^{-1}, 0.7%, Table 3). These results indicated that simultaneously introducing mutagenesis into the heme and redox domains[14, 22] could efficiently improve the global activity and electron coupling efficiency of the holoenzyme. Additionally, mutations within the heme domain (based on M612L/K774Y and M612L/N694D) caused obvious improvements in thermostability, especially when considering the outstanding contribution of the single mutation K774Y. The resultant variants HR1 (+3.8°C) and HR2 (+5.3°C) enhanced the thermostability (Figure S2, as measured by T_{50}[10], Supporting Information) of the holoenzyme by 3.8°C and 5.3°C, respectively, as compared with the wild-type.

By performing transdomain combinatorial mutation of $P450_{LaMO}$, we were able to excavate the holoenzyme's catalytic efficiency. Some literatures have shown that P450 systems have

different rate limiting steps.[25] Our results showed that the redox domain laid the foundation for electron transfer and acceptance, even affected the holoenzyme's catalytic performance,[26] but the heme segment is more important in electron utilization and the holoenzyme's activity.

Based on combinatorial mutation, the resulting mutant HR1 did enhance the coupling efficiency, TOF value and thermostability of the P450 monooxygenase. Moreover, it is of improved high spin content, which showed that this enzyme formed more efficient intermediates (Figure S5, Table S1, Supporting Information). From the view of electrochemistry, the redox potential was lowered from -27 mV of wild-type to -90 mV of HR1 (Figure S4), illustrating that the efficiency of the driving force for this P450's catalytic cycle is much more powerful than that of the native protein. Although the coupling efficiency of this monooxygenase was improved to varied extents, questions such as the destiny of the remaining electrons are not yet clear. Therefore, further exploration on the mechanism of the electron leakage in every process of catalytic cycle would be intriguing to improve the coupling efficiency.

Table 3. Catalytic properties of P450$_{LaMO}$ mutants toward tetralin 1-monooxygenation.

Mutant	Mutation	TOF (min^{-1})[a]	CE (%)[b]	Conv. (%)[c]
WT	–	0.018 ± 0.002	0.7 ± 0.1	10
H1[a]	V264A	0.030 ± 0.002	0.8 ± 0.2	13
H2[a]	V264A/V437G	0.049 ± 0.007	1.4 ± 0.03	22
H3[a]	N119C/V264A/ V437G	0.140 ± 0.020	2.0 ± 0.2	94
HR1	N119C/V264A/ V437G/M612L/ N694D	0.180 ± 0.010	6.0 ± 0.2	>99
HR2	N119C/V264A/ V437G/ M612L/K774Y	0.130 ± 0.010	5.8 ± 0.2	98

[a] Heme domain-based mutants was initially obtained based on NBT-PMS colorimetric screening method.

[b] TOF: Turnover frequency (TOF) was defined as the number of tetral-1-ol molecules generated from tetralin per each P450 enzyme molecule per minute, with a unit of nmol tetral-1-ol nmol $P450^{-1}$ min^{-1};

[c] CE: Coupling efficiency (CE) was defiined as the ratio of tetral-1-ol formation rate to NADPH consumption rate;

[d] Conversion was measured after 24 h reaction.

In terms of the redox domain, the k_{cat} of MR1 (for cytochrome *c*) was improved to 128 s^{-1} from 58.7 ± 1.4 s^{-1} of the wild type. It is suggested that engineering of redox domain could further strengthen the electron transfer and elevate the catalytic performance, which can be adopted not only for enhancing the productivity of other reactions catalyzed by P450$_{LaMO}$ such as *anti*-Markovnikov addition of styrene, but also helping other CYP116B members to achieve higher electron transfer efficiency.[6c,7,8,10a,13]

To test the performance of the best mutant HR1 as a biocatalyst, in the 4-hydroxylation of various 1,2,3,4-tetrahydronaphthalene derivatives **1a-e** (Table 4) were conducted.[23] The hydroxylation products **2a-e** were identified by mass spectra and compared with those of standards (Scheme S1, Table S3, Supporting Information). The results show that the TOF (0.18 min^{-1}) of HR1 for **1a** was improved by 4.2 folds over the wild type (0.043 min^{-1}). When halogen atoms were introduced into the substrates (**1b,** F-; **1c,** Cl-; **1d**, Br-; **1e**, I-), the HR1's TOF values (0.16 min^{-1}, 0.15 min^{-1}, 0.25 min^{-1} and 0.24 min^{-1}) are higher than the wild type's (0.062 min^{-1}, 0.06 min^{-1}, 0.042 min^{-1} and 0.067min^{-1}), with 2.6-fold, 2.5-fold, 6.0-fold and 3.6-fold improvements, respectively. Significant improvements were also observed for the conversion of tetralin derivatives, *e.g.*, 4.1-fold (**1a**), 2.7-fold (**1b**), 2.4-fold (**1c**), 5.7-fold (**1d**) and 3.7-fold (**1e**). Moreover, these hydroxylated products may be used to synthesize drugs for the treatments of cancer or dermatosis.[24]

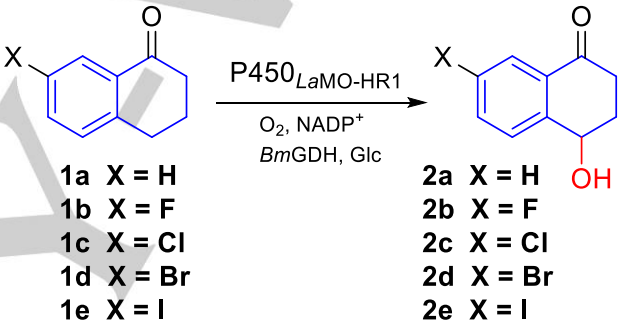

Table 4. Comparison of P450$_{LaMO-HR1}$ with its wild-type in enzymatic 4-hydroxylation of various tetrahydronaphthalene derivatives.

Entry	Substrate	Product	Enzyme	TOF (min^{-1})[a]	Conv. (%)[b]
1	**1a**	**2a**	WT	0.043 ± 0.005	8.7
2	**1a**	**2a**	HR1	0.180 ± 0.030	36
3	**1b**	**2b**	WT	0.062 ± 0.011	12
4	**1b**	**2b**	HR1	0.160 ± 0.010	32
5	**1c**	**2c**	WT	0.060 ± 0.002	12
6	**1c**	**2c**	HR1	0.150 ± 0.070	29
7	**1d**	**2d**	WT	0.042 ± 0.006	8.6
8	**1d**	**2d**	HR1	0.205 ± 0.010	49
9	**1e**	**2e**	WT	0.067 ± 0.001	13
10	**1e**	**2e**	HR1	0.240 ± 0.010	48

[a] TOF: turnover frequency [nmol product nmol $P450^{-1}$ min^{-1}];

[b] Conversion was measured after 24 h reaction.

Conclusions

In conclusion, the best mutant of $P450_{LaMO}$, HR1 (N119C/V264A/V437G/M612L/N694D), was formed by introducing the mutated sites from redox domain into mutated heme domain, yielding 9.1-fold improvement of coupling efficiency, 10-fold enhancement of turnover frequency and 3.8°C increase in thermostability. This resulting HR1 also showed higher hydroxylation activity for series of tetrahydronaphthalene derivatives, which can be used as important building blocks to synthesize biologically active molecules. Moreover, the transdomain combination evolution strategy is expected to be useful for engineering other members of the CYP116B subfamily, especially for improving the low NADPH coupling efficiency and the total reaction rate of unnatural substrates. Meanwhile, engineering of the redox domain could further strengthen the electron transfer and elevate the catalytic performance, which can be adopted not only for enhancing the productivity of other $P450_{LaMO}$-catalyzed reactions, such as *anti*-Markovnikov addition of styrene, *O*-dealkylation and thioether sulfoxidation.

Experimental Section

For full experimental details please refer to the Supporting Information.

Random Mutagenesis of the Heme or Redox Domain

Modified error-prone PCR was used for random mutagenesis of the heme and redox domains. The phosphatethioate-based gene cloning method was used for high positive ligation rates.[27] The $MnCl_2$ concentration was set at 75~100 μM for controlling the mutagenesis rate at 1~2 amino acid residues for both the heme and redox domains. Heme domain mutagenesis (1201 bp; 51% of the holoenzyme): modified PCR primers were as follows: 5'-CGGATCTT*CCTTGAAG*AGATG-3' as the forward primer (forward, designated as Lamo-1201-F, where '*' denotes the phosphorothioate modified position) and 5'-ATGCGGCC*GCAAGCTT*TTACAGCGCCAGCT-3' as the reverse primer (reverse, Lamo-PRSTduet1 R). The PCR mixture (50 μL) for amplifying the heme gene fragment was composed of KOD-neo-plus (1 U), 1×buffer for KOD-plus-Neo, 0.2 mM dNTP mix, 1 mM $MgSO_4$, 100 nM primers and 50 ng template (wild type, plasmid named prsfduet-$p450_{lamo}$). PCR cycling conditions were as follows: 94 °C for 2 min, 30 cycles [98 °C for 10 s, 56 °C for 30 s, 68 °C for 160 s], and 68 °C for 10 min. Redox domain mutagenesis (1148 bp; 49% of the holoenzyme): modified PCR primers were as follows: 5'-TCCGAATT*CGAGCTCG*ATGGAACGTACTGC-3' as the forward primer (designated as PRSFduet1-Lamo-F, where '*' denotes the phosphorothioate modified position) and 5'-CTTCAAGG*AAGATCCG*CATTTCCATGCGGGCG-3' as the reverse primer (Lamo-1201-R). The PCR mixture contained 1× r*Taq* buffer, 0.2 mM dNTP mix, 100 nM forward and reverse primers, 75 μM Mn^{2+}, 1 U r*Taq* DNA polymerase, and 50 ng plasmid template (plasmid prsfduet-$p450_{lamo}$). PCR cycling conditions were as follows: 95 °C for 5 min, 30 cycles [95 °C for 30 s, 58 °C for 30 s, 72 °C for 90 s] and 72 °C for 10 min. The PCR products were digested using *Dpn*I and purified using a DNA purification kit (Macherey-Nagel, Dueren, Germany). Iodine was used to treat the fragments at 70 °C for 10 min. The mixture (25 μL) was composed of 15 μL DNA (8 pmol), 5 μL cleavage buffer (0.5 M Tris-HCl, pH 9.0), 3 μL iodine stock solution (100 mM iodine in absolute ethanol), and 2 μL Milli-Q water. Subsequently, the purified iodine-treated DNA fragments were hybridized in a PCR cycler (80 °C for 3 min, 60 °C for 10 min, 20 °C for 20 min, ramp 0.1 °C/s, 0.1 °C/cycle, 4 °C for 10 min). Finally, 10 μL of each hybridization sample was transformed into competent cells *E. coli* BL21 (DE3).[28]

Screening of the Heme Domain Mutation Library Based on the NBT-PMS Assay

Single colonies were picked with toothpicks, inoculated and grown at 37 °C in 96-deep well plates with 300 μL LB medium per well, containing 50 mg/L kanamycin and using $P450_{LaMO}$ (WT) as the positive control. The screening was performed with the full length enzyme. The expression culture was inoculated by transferring 50 μL overnight culture into 850 μL Terrific Broth (TB) expression medium supplemented with 50 mg/L kanamycin. When the OD_{600} reached ~1.0, 0.2 mM IPTG (isopropyl-*β*-D-thiogalactopyranoside) and 0.2 mM ALA were added to each well and the temperature was decreased to 16 °C for another 24 h. The cells were harvested by centrifugation (3220 *g*, 10 min). The cell pellets were then resuspended in 190 μL potassium phosphate (KPi) buffer (100 mM, pH 8.0, saturated with oxygen) and transferred into 96-well microplates.

The reaction (200 μL) containing whole cells expressing $P450_{LaMO}$ (1.6 g CDW/L), 0.5 mM 1,2,3,4-tetrahydronaphthalene, and 100 mM KPi buffer (pH 8.0) in 96-deep well plates was performed at 220 rpm for 4 h. Then, the mixture was separated by centrifugation (3220 *g*, 15 min) and 80 μL supernatant was taken to carry out the further dehydrogenation of the hydroxylation product into an aldehyde. This dehydrogenation was catalyzed by the reductase *Ss*CR from *Scheffersomyces stipitis* CBS 6045 with high activity but low enantioselectivity (Scheme 1B, Table S2, Figure S6, Figure S7, Supporting Information) toward the hydroxylation product. The dehydrogenation was started by adding 10 μL *Ss*CR (0.5 U) and 10 μL mixed NBT-PMS solution (NBT 2 mg/mL, PMS 0.1 mg/mL and $NADP^+$ 0.3 mM), and then the change in absorbance at 580 nm was monitored after the reaction was kept at 30 °C for 1 h away from light.

Screening of the Redox Domain Mutation Library with the 7-Ethoxycoumarin-based Fluorescence Method

The redox domain of $P450_{LaMO}$ as an electron partner affects the electron transfer capability and the total catalytic efficiency of the holoenzyme. A fluorogenic substrate, 7-ethoxycoumarin, was used for preliminary high-throughput screening of positive mutants from the redox domain mutagenesis library. The screening was performed with holoenzyme. After collecting the wet cells, 200 μL 100 mM KPi buffer (pH 8.0) was added to resuspend them. The preliminary screening mixture, containing 190 μL cell suspension (final conc., 1.6 g CDW/L) and 10 μL 7-ethoxycoumarin in DMSO (10 mM) stock solution (final conc., 0.5 mM), was incubated at 30 °C for 4 h. After centrifugation (3220 *g*, 10 min), the supernatant was transferred into a new microplate to measure the fluorescence intensity of the indicating product (7-hydroxycoumarin, excitation at 398 nm, emission at 458 nm, Scheme 1A). The measurements were performed for nine cycles with 90 s intervals. After the initial screening using whole cells and fluorogenic substrate, all potential hits were purified to measure the specific activity and coupling efficiency using the target substrate 1,2,3,4-tetrahydronaphthalene.

Cell Cultivation and Protein Purification

Single colonies of *E. coli* BL21 (DE3) were picked and inoculated into 4 mL LB medium (50 mg/L kanamycin) and cultivated at 37 °C for 12 h. Then, 1 mL of the pre-culture was inoculated into 80 mL TB medium containing 50 mg/L kanamycin. The cells were grown at 37 °C and 180 rpm until an OD_{600} of *approx.* 1.0, and then the enzyme was induced by adding 0.2 mM IPTG and 0.2 mM ALA. The culture was continued by

incubation at 16 °C and 180 rpm for another 24 h. More details of the protein purification can be found in a previous work.[12] The purified protein was concentrated via ultrafiltration tubes for further use (30 kDa, Millipore, MA).

Assay of Enzyme Activity and Coupling Efficiency

All of the enzyme catalytic reaction analysis was performed in 1-mL reaction tubes on a mini-shaker (HLCBioTech, MHR23) at 600 rpm and 20°C. The reaction mixture (1 mL) was composed of 4.5 μM P450s (P450 concentration was measured by CO-difference spectra, Supporting Information),[29] 0.3 mM NADPH and 0.3 mM tetralin. Ethyl acetate was used to extract the reaction product and the resulting organic extract was analyzed by GC-MS.

The electron coupling efficiency of $P450_{LaMO}$ was calculated as the ratio of the tetral-1-ol formation rate to the NADPH consumption rate. The P450 enzyme sample (10 μL, 11 μM) was mixed with 930 μL of KPi buffer (0.1 M, pH 8.0) containing 10 μL of substrate stock solution (ethanol, 50 mM). After standing for 5 min, 50 μL of 10 mM NADPH solution was added, followed by standing at 30 °C for 10 min to detect the absorbance of NADPH at 340 nm within a linear range of variation. Then, 500 μL ethyl acetate was used (with 0.5 mM guaiacol as an internal standard) to extract the sample twice, and the organics were dried over anhydrous Na_2SO_4. Subsequently, the extract was concentrated to 50 μL to perform GC-MS analysis for quantitation of the reaction product.

GC-MS Analysis

Product analysis was carried out by GC/MS (Shimadzu GCMS-QP2010, HP-5 MS, column length 30 m, internal diameter 0.25 mm, film thickness 0.25 mm) with helium as the carrier gas. Mass spectra were collected after 4 min (solvent cut time) by electron ionization with the scan mass range of 40-300 (m/z). The GC-MS retention times for tetralin, tetralol and the internal standard were 10.5 min, 13.5 min, and 9.3 min, respectively.

Molecular Docking and Mutation Site Exhibition

A structural model of $P450_{LaMO}$ wild-type was constructed based on the crystal structures of its homologue enzymes (PDB IDs: 1CPT, 1Z8O, 1JIP and 2UUQ). The on-line SWISS-MODEL web server (http://www.swissmodel.expasy.org/) was used to perform the homology modeling. The 3D structure of substrate tetralin was generated by energy minimization. Then the substrate structure was docked into the binding pocket of the constructed $P450_{LaMO}$ model (AutoDock 4.0). The best scoring results were selected for structural comparison and catalytic mechanism analysis. After introducing mutagenesis, the mutation sites were shown by the visual software Pymol 2.5.

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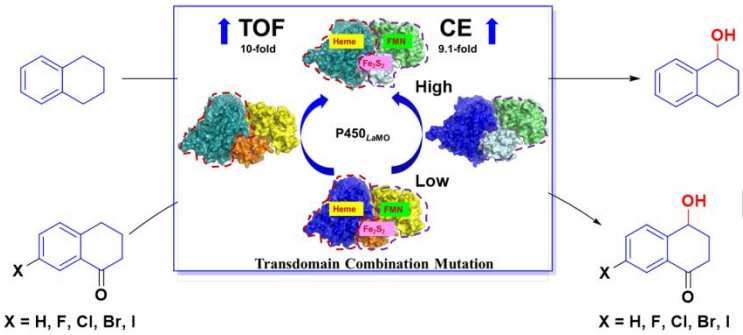
CYP116B4 monooxygenase was hindered by poor reaction rate and electron coupling efficiency. Via transdomain combination mutagenesis, the resulting variant improves TOF and CE for 1-hydroxylation of tetralin and other derivatives by up to 10- and 9.1-folds.



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Enhancing the catalytic performance of a CYP116B monooxygenase by transdomain combination mutagenesis