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Evolving P450pyr hydroxylase for highly enantioselective hydroxylation at non-activated carbon atom†

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Directed evolution of a monooxygenase to achieve very high enantioselectivity for hydroxylation at non-activated carbon atoms is demonstrated for the first time, where a triple mutant of P450pyr hydroxylase is obtained *via* determination of enzyme structure, iterative saturation mutagenesis, and high-throughput screening with a MS-based *ee* assay to increase the product *ee* from 53% to 98% for the hydroxylation of *N*-benzyl pyrrolidine to (*S*)-*N*-benzyl 3-hydroxypyrrolidine.

Regio- and stereoselective hydroxylation at non-activated carbon atom is a useful reaction for the functionalization of alkanes and the preparation of valuable chiral alcohols. However, it remains a significant challenge in classic chemistry. Monooxygenase could catalyze this type of reaction with molecular oxygen as green oxidant. Many monooxygenase-containing microorganisms were reported for the hydroxylations,[1] and some monooxygenases (MMO,[2] AlkB,[3] P450[4]) were well characterized and engineered for special hydroxylations. Nevertheless, the enantioselectivities in many cases need further improvement for practical application. Recently, directed evolution has become a useful tool for improving enzyme enantioselectivity.[5,6] Pioneering work in biohydroxylation was performed on the directed evolution of P450BM3 monooxygenase,[7] a class II P450 with a single protein combining all three catalytic functions. Special difficulties in evolving a monooxygenase for highly enantioselective biohydroxylation are the involvement of enantiotopic discrimination and the lack of an applicable high-throughput *ee* assay. Thus, it is still a significant challenge to evolve a monooxygenase giving a hydroxylation product with $\geq 98\%$ *ee*, the required *ee* value for chiral pharmaceutical intermediates.

Recently, we discovered P450pyr hydroxylase[8] from *Sphingomonas* sp. HXN-200, a class I P450 requiring ferredoxin (Fdx) and ferredoxin reductase (FdR) for electron transfer (Fig. 1a),

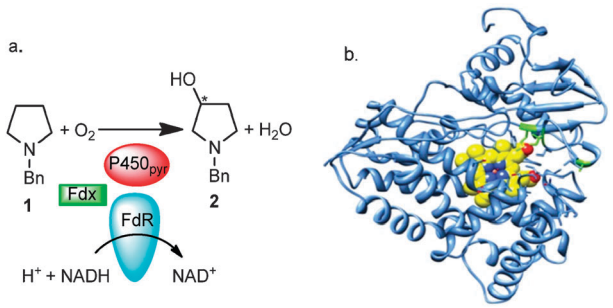

Fig. 1 (a) P450pyr-catalyzed enantioselective hydroxylation of *N*-benzyl pyrrolidine; (b) structure of P450pyr hydroxylase. Yellow: heme, green: the 3 mutation sites leading to the highest enantioselectivity.

as an active and selective catalyst for the regio- and stereoselective hydroxylation at non-activated carbon atom with a broad substrate range and broad applications.[9] However, in the case of hydroxylation of *N*-benzyl pyrrolidine[9a,b] to prepare (*S*)-*N*-benzyl 3-hydroxy-pyrrolidine as useful pharmaceutical intermediate,[10] the product *ee* was only 53%. To improve the enantioselectivity, we previously performed the directed evolution of P450pyr hydroxylase by identifying key amino acid residues for evolution based on a homology structure model and using a colorimetric *ee* screening assay.[11] P450pyr mutant N100S/T186I with reversed and improved enantioselectivity was obtained to give (*R*)-3-hydroxypyrrolidine in 83% *ee*.[11] Here we report our recent success in evolving P450pyr for the highly enantioselective hydroxylation of *N*-benzyl pyrrolidine to reach 98% *ee* of the (*S*)-product by structure-based selection of key amino acid residues, iterative saturation mutagenesis, and high-throughput screening with an accurate and sensitive MS-based *ee* assay.

To determine the structure of P450pyr hydroxylase, an *Escherichia coli* Top10 pTr99A-his-P450pyr was engineered to express P450pyr with a poly-histidine tag at the N-terminal. The his-tagged P450pyr was purified by fast protein liquid chromatography (FPLC) using a Ni-NTA column and a size-exclusion column, with >95% purity and a specific activity of 1595 U g^{-1} protein for the hydroxylation of *N*-benzyloxycarbonyl pyrrolidine in the presence of NADH, together with the purified his-tagged ferredoxin and his-tagged ferredoxin reductase.

His-tagged P450pyr was crystallized, and its structure was determined by X-ray diffraction (PBD ID 3RWL). As shown in Fig. 1b, the structure exhibits an overall fold typical of

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P450 hydroxylases. The heme is located at the bottom of a gorge leading to the active site. The large size of the pocket explains the broad substrate range for the biohydroxylation. The final model is lacking residues 88–96 for which no electron density is visible. These residues are located in a loop on the top of the gorge leading to the active site. The same disorder is observed in the equivalent loop of the structure of the cytochrome P450cam in the "open state" corresponding to the substrate-free state.[12] 20 amino acids located either in the vicinity of the active site or in the gorge leading to the heme were selected for mutation: A77, I82, I83, L98, P99, N100, I102, A103, S182, D183, T185, T186, L251, V254, G255, D258, T259, L302, M305, and F403.

A simplified version of Iterative Saturation Mutagenesis[5a] was applied to the evolution. Each of the selected amino acid sites was subjected to saturation mutagenesis; all mutants were screened for better enantioselectivity; and the best mutant was used as the template for the next round of evolution in which each of other selected residues was subjected to saturation mutagenesis and screening. For saturation mutagenesis, random mutations at the desired position of the P450pyr gene were generated by PCR using NNK degenerated codon. PCR products were then transformed in *E. coli* BL21(DE3) containing ferredoxin and ferredoxin reductase genes to create P450pyr mutants. To cover all possible 19 mutants at the selected site, 188 colonies were picked up and grown in two 96-deep well plates. For high-throughput screening of enantioselective mutants, our previously developed assay based on mass spectrophotometer[13] was applied (Fig. 2a). The cells in each well were harvested and then equally resuspended in potassium phosphate buffer in two wells.

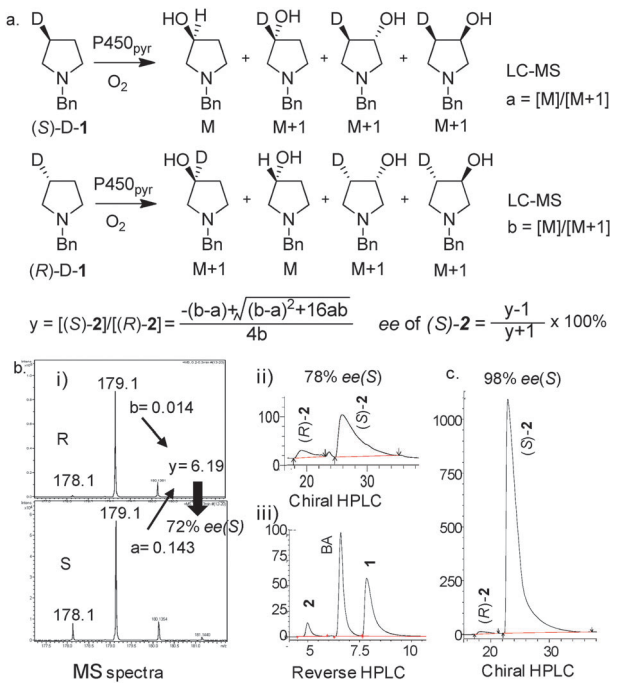

Fig. 2 (a) High-throughput *ee* assay based on the use of a deuterated substrate and MS detection;[13] (b) example of screening an enantioselective mutant (I83H). (i) MS spectra for fast *ee* determination, (ii) chiral HPLC chromatogram for accurate *ee* analysis, (iii) reversed-phase HPLC chromatogram for conversion analysis; (c) chiral HPLC chromatogram of the purified product **2** from biohydroxylation of *N*-benzyl pyrrolidine **1** with best mutant I83H/M305Q/A77S.

Table 1 Directed evolution of P450pyr hydroxylase for hydroxylation of *N*-benzyl pyrrolidine **1** to (*S*)-*N*-benzyl 3-hydroxypyrrolidine **2**

Round	No. of sites saturated	No. of mutants screened	Positive mutant	*ee*[a] [%]	*ee*[b] [%]	Activity[c] [U g^{-1} cdw]	Rel. activity[d] [%]
Wild type	Nil	Nil	Nil	49	53	6.4	100
1	9	1692	I83H	72	78	5.3	83
			M305A	76	64	0.6	9
2	19	3572	I83H/ M305Q	>70	94	4.1	64
			I83H/ A77S	>70	85	6.2	97
3	5	940	I83H/ M305Q/ A77S	>70	98	5.2	81

[a] Determined by using LC-MS assay. [b] Determined by chiral HPLC analysis with a concentrated product sample from a 10 mL-scale biotransformation. [c] Determined from 30 min biotransformation of 10 mM substrate **1** with 1 g cdw L^{-1} of cells of *E. coli* (P450pyr or mutant) at 30 °C and 300 rpm. [d] Related to the specific activity of *E. coli* (P450pyr).

(*R*)- and (*S*)-deuterated *N*-benzyl pyrrolidines **1** (1 mM) were added, respectively, and the aqueous biotransformation product after 1 h was analyzed by LC-MS (1 sample per min) to determine the product *ee*. An example is shown in Fig. 2b(i). For promising mutants selected by the *ee* assay, further tests were performed by biotransformation of *N*-benzyl pyrrolidine **1** with the resting cells of the mutant on a 10 mL scale. The product after 1 h reaction was analyzed by chiral HPLC to determine the product *ee* [Fig. 2b(ii)] and by reversed-phase HPLC to obtain accurate conversion and regioselectivity [Fig. 2b(iii)]. As demonstrated in Fig. 2b(i) and (ii), the *ee* value obtained from LC-MS assay was nearly the same as the value determined by chiral HPLC, thus proving the accuracy of the LC-MS *ee* assay.

The results of directed evolution are summarized in Table 1. In the first round of mutagenesis, 9 of 20 selected amino acid positions were subjected to saturation since other positions were screened in a previous study.[11] Mutant I83H was found to increase the product *ee* to 78%(*S*) with 83% activity related to the activity of the wild-type P450pyr. This mutant was used as a template for the second round evolution in which all other 19 selected amino acid residues were subjected to saturation and screening, respectively. In this and later rounds, only mutants with >70% *ee*(*S*) by LC-MS assay were selected for further investigations. Mutant I83H/M305Q was found to give a product *ee* of 94%(*S*) with 64% relative activity. Thus, a big improvement of enantioselectivity from product *ee* of 53%(*S*) to 94%(*S*) was achieved after only 2 rounds of mutagenesis and screening of 5264 colonies. The third round evolution was conducted using mutant I83H/M305Q as template with saturation at only 5 amino acid residues A77, P99, N100, S182, and L302, respectively, since mutations at these positions gave good results in previous rounds (data not shown). Triple mutant I83H/M305Q/A77S was found to give the product in 98% *ee*(*S*) (Fig. 2c), with a specific activity of 5.2 U g^{-1} cdw (81% activity of P450pyr). No by-products were formed, suggesting also clean reaction and excellent regioselectivity for the triple mutant.

To investigate the kinetics, P450pyr triple mutant, ferredoxin, and ferredoxin reductase, all with polyhistidine at the N-terminal,

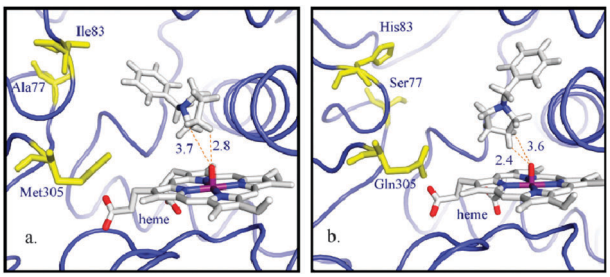

Fig. 3 Substrate–enzyme binding pose. (a) Wild type P450pyr; (b) triple mutant I83H/M305Q/A77S. The mutation sites are illustrated in yellow. The distance between the hydrogen atom of substrate **1** and the heme-oxygen atom is denoted by a dashed line and represented in angstrom.

were engineered, produced and purified. Hydroxylation of *N*-benzyl pyrrolidine **1** at different concentrations was performed with P450pyr mutant or P450pyr, ferredoxin, and ferredoxin reductase at a molar ratio of 1 : 10 : 1 in the presence of NADH for 5 min to obtain the kinetic data. The P450pyr mutant gave slightly higher apparent k_{cat} (26.9 *vs.* 21.9 min^{-1}) and K_m (15.6 *vs.* 13.6 mM) than P450pyr and nearly the same catalytic efficiency (k_{cat}/K_m) as P450pyr. Thus, the triple mutant is as efficient as P450pyr for the target hydroxylation. The product *ee* obtained with the triple mutant *in vitro* was also found to be 98%(*S*).

Docking models of *N*-benzyl pyrrolidine on the MD simulated structures of P450pyr and the triple mutant were established. In P450pyr, the benzene ring of the substrate is stabilized by hydrophobic I82, I102 and L251, while the pyrrolidine ring has only weak interaction with L302 and thus certain flexibility (Fig. S18, ESI†). Through the mutation of I83H and A77S, hydrogen bonds are formed within a big loop (S75–D105), which moved the loop closer to the heme with I102 occupying the original binding position of the benzene ring of the substrate (Fig. S19, ESI†). In addition, the mutation of M305Q moves L302 and V404 closer to the heme. As a result, the benzene ring of the substrate is located within a hydrophobic cleft (V254, V404) above the heme and the pyrrolidine ring is restrained by I102 and L302 in the vicinity of the heme in the mutant (Fig. S20, ESI†). As shown in Fig. 3, the distance between the heme O-atom and the pro-*S* or pro-*R* H-atom at C(3) of the substrate is 2.8 Å or 3.7 Å for P450pyr and 2.4 Å or 3.6 Å for the triple mutant, respectively. Thus, the preference of inserting an O-atom into the pro-*S* C–H bond over the pro-*R* C–H bond is much higher in the mutant than in P450pyr. This explains the significantly improved enantioselectivity for the hydroxylation *via* only three mutations.

A highly enantioselective P450pyr hydroxylase has been engineered for the hydroxylation of *N*-benzyl pyrrolidine to give the (*S*)-3-hydroxypyrrolidine in 98% *ee*. This is a breakthrough in directed evolution of an enantioselective monooxygenase for asymmetric hydroxylation at non-activated carbon atom: for the first time, a monooxygenase has been obtained *via* evolution to give very high product *ee* ($\geq$ 98% *ee*) and clean biohydroxylation; both high enantioselectivity and high whole-cell activity have been achieved with the engineered P450pyr triple mutant. The X-ray structure of P450pyr has been obtained and successfully used to guide the evolution. It provides with a solid basis for further engineering of P450pyr monooxygenase for enantioselective hydroxylations of other types of substrates as well as for other types of enantioselective oxidations. The high-throughput MS-based *ee* assay has been proven to be practical for evolution and will become a valuable tool for evolving other enantioselective hydroxylating enzymes.

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