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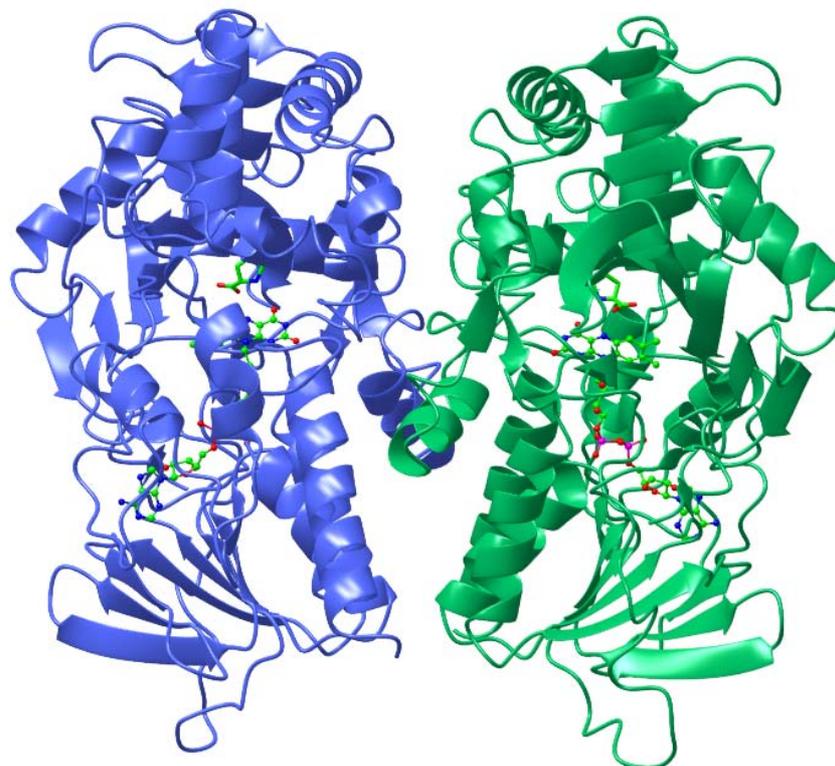
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Inverting the enantioselectivity of P450pyr monooxygenase by directed evolution†‡

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We report the first example of directed evolution of a P450 monooxygenase with inverted enantioselectivity for asymmetric biohydroxylation. The biohydroxylation product of the best mutant 1AF4A has an *ee* of 83% (*R*) compared to the wild type's *ee* of 43% (*S*).

Enantioselective hydroxylation of non-activated carbon atoms represents a significant challenge in classical organic chemistry.¹ However, nature has offered us a solution *via* biocatalysis with monooxygenases using molecular oxygen as oxidant. Cytochrome P450 monooxygenases (also known as CYP) constitute the largest family of heme-containing monooxygenases that can oxidize a broad range of substrates, often at non-reactive carbon centers.² Of particular interest is a novel P450pyr enzyme from *Sphingomonas* sp. HXN-200³ that belongs to the class I P450 proteins and requires the presence of an electron-delivering protein system (*i.e.* ferredoxin and ferredoxin reductase) to effect its monooxygenase activity. The P450pyr monooxygenase was found to catalyze the hydroxylations of *N*-substituted pyrrolidines, piperidines, azetidines, 2-pyrrolidinones, and 2-piperidinones with high activity, excellent chemo- and regioselectivity, and good to excellent enantioselectivity.⁴ The enzymatic hydroxylation of *N*-benzylpyrrolidine **1** to its corresponding (*R*)- and (*S*)-*N*-benzyl-3-hydroxypyrrolidines **2** is of great interest since these enantiomers are important pharmaceutical intermediates in the synthesis of a *k*-receptor agonist, an antibacterial agent, carbapenem antibiotics, and a 5-HT_{1Da} receptor agonist.³ Unfortunately, the enantioselectivity of the wild type (WT) P450pyr is unsatisfactory and therefore it is desirable to improve the P450pyr enantioselectivity *via* protein engineering.

Directed evolution and rational design have been used to enhance the enantioselectivity of enzymes, such as lipases, esterases, hydantoinases, nitrilases, epoxide hydroxylases, phosphotriesterases, aminotransferases, aldolases, cyclohexanone and cyclopentanone monooxygenases, and monoamine oxidases.⁵ Previous directed evolution studies on P450

monooxygenases have focused on altering their catalytic efficiency,⁶ substrate specificity,⁷ regioselectivity,⁸ solvent tolerance,⁹ and thermostability.¹⁰ There have also been many successful attempts to engineer an enzyme for the reversal in enantioselectivity,¹¹ but none of these studies were done with the P450 monooxygenases and very few on asymmetric catalysis. While screening a library of self-sufficient P450 BM-3 mutants for changes in regioselectivity, Arnold and coworkers have also identified some mutants with altered enantioselectivity based on an activity assay.¹² Here, we report the first example where directed evolution, coupled with a high-throughput enantioselectivity screening system, has been applied to improve the biohydroxylation enantioselectivity of a class I P450 monooxygenase using a prochiral substrate.

In this work, we have evolved both (*S*)- and (*R*)-selective P450pyr by iterative targeted site-saturation mutagenesis using the prochiral substrate **1**. The development of an efficient high-throughput enantiomeric excess (*ee*)-screening method was no trivial task. In fact, most of the directed evolution studies found in the literature focused on improving the enantioselectivity in the kinetic resolution of the enzyme, and very few for asymmetric transformations due to the complexities in developing an *ee*-assay. A high-throughput two-enzyme based colorimetric assay was applied to semi-quantitatively determine the enantiomeric ratio of the racemic product. The concept of utilizing two complementary alcohol dehydrogenases has been described,¹³ but the colorimetric determinations make it the first example of a high-throughput enantioselectivity assay developed and used in the directed evolution of a monooxygenase for asymmetric transformations.

After an extensive literature search, we found two alcohol dehydrogenases, *i.e.* BRD¹⁴ from *Micrococcus luteus* and RDR¹⁵ from *Devosia riboflavina*, that were highly specific for (*S*)- and (*R*)-**2**, respectively. The genes encoding these two alcohol dehydrogenases were cloned and overexpressed in *Escherichia coli* BL21(DE3). The resulting purified enzymes were used in the high-throughput screening method (Scheme 1). We assayed these purified enzymes with different mixtures of (*S*)- and (*R*)-**2** and found that the *ee* correlated to the color intensity of the assay (See Fig. S1 in Supplementary Information†).

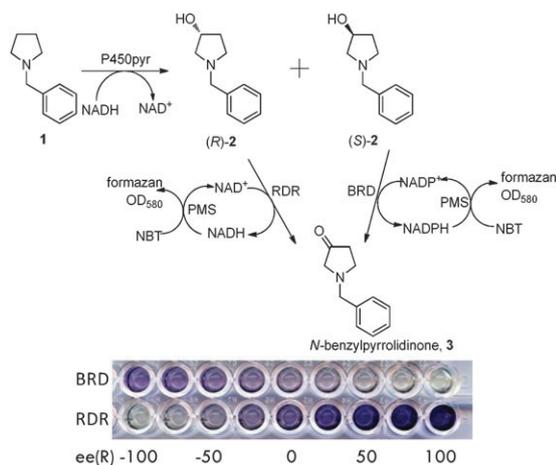
Whole cell assay was necessary in this case as our P450pyr system requires the close interaction of the ferredoxin and ferredoxin reductase to form an electron transport system, unlike P450BM3 which has its hydroxylase and reductase domains on a single polypeptide. Also, the whole cell assay approach removed the need for the cell lysis step, and the addition of NADH and other auxiliary protein components,

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Scheme 1 A high-throughput two-enzyme based colorimetric *ee* assay for asymmetric biohydroxylation of prochiral substrate **1** to (*R*)- and (*S*)-**2**.

thus simplifying our screening protocol. Previously, the *Pseudomonas putida* strain expressing the P450pyr together with the ferredoxin gene (Fdx) from *Sphingomonas* sp. HXN200 and the ferredoxin reductase gene (FdR) from *Mycobacterium* sp. HXN1500 was developed but showed low activity.¹⁶ Hence, we constructed a recombinant *E. coli* BL21(DE3) strain containing a dual plasmid system (pRSFDuet P450pyr and pETDuet Fdx FdR) which showed much higher hydroxylation activity and was thus designated as the parent strain.

Our high-throughput screening protocol is described as follows. Firstly, the mutants were inoculated and expressed in TB medium in 96-deep well plates, in the presence of δ -aminolevulinic acid (δ -ALA) to increase the expression level of P450pyr. The biohydroxylation assay to convert **1** to (*R*)- and (*S*)-**2** was performed with resting cells in 96-deep well plates with shaking at 1100 rpm to facilitate better mixing and mass transfer. When the biohydroxylation reaction was carried out at low shaking speed of 250 to 500 rpm, negligible substrate conversion was observed. After the biohydroxylation with whole cells, the deep well plates were centrifuged and aliquots (80 μ l) of the supernatant containing the unreacted **1** and its racemic product **2** were pipetted into two separate 96-well microtiter plates. In each plate, purified BRD or RDR and its corresponding cofactor were added. By utilizing the nitro blue tetrazolium (NBT)-phenazine methosulfate (PMS) colorimetric assay,¹⁷ the production of NAD(P)H was monitored by observing the formation of formazan at OD₅₈₀, which corresponded to the activity of the dehydrogenases that in turn correlated to the concentration of each enantiomer in the racemic product. Hence, the estimated *ee* of each mutant could be compared against the WT P450pyr. Mutants that showed a greater *ee* than that of the WT were selected and subjected to a larger-scale biohydroxylation assay in shaking flasks to verify the enantioselectivity of the mutants by chiral HPLC analysis.

To identify the residues for directed evolution, we used molecular modeling to build a structural model of P450pyr based on the crystal structure of CYP119 from *Sulfolobus solfataricus* (1F4T)¹⁸ and P450st from *Sulfolobus tokodaii* (1UE8).¹⁹ The mutant library was created using iterative targeted site-saturation mutagenesis.²⁰ Based on the P450pyr

homology model, 17 residues were identified within 5 Å of the heme-docked substrate (See Fig. S2(a) in Supplementary Information†) and were subjected to individual site saturation mutagenesis. C366 and G256 were not subjected to randomization because they are highly conserved residues involved in the heme coordination and catalytic activity of the P450pyr, respectively. For each site, 32 distinct variant possibilities exist (32 possible codon substitutions). Hence, the screening of 180 transformants per site should be more than enough to provide comprehensive coverage of all created variants. This protein engineering approach involves screening very manageable size of mutant libraries which target the residues within the substrate binding pocket.

In the first round of saturation mutagenesis, using the WT P450pyr as a template, we screened 180 mutants per site, which translates into a total of 3060 mutants (17 sites) screened. Two interesting mutants with preference for (*S*)- and (*R*)-**2** were identified (Table 1). The (*S*)-selective mutant 11BB12 (F403L) displayed an *ee* of 65% which was a 1.5 times improvement over that of the WT P450pyr. Mutant 1AF4 (N100S), on the other hand, displayed a complete reversal of enantioselectivity with an *ee* of 42% with a preference for (*R*)-**2**. In fact, it was identified twice in the screening of the same 3060-mutant library. It was intriguing that only one mutational change at position 100 (replacing asparagine with serine) could induce a total inversion of the P450pyr enantioselectivity.

Encouraged by the success in the first round, F403L was used as a template for a second round of saturation mutagenesis on the remaining 16 residue sites. Unfortunately, we failed to discover any (*S*)-selective mutants with significantly higher *ee* than the parent 11BB12. Hence, we decided to identify more (*R*)-selective mutants instead. Using N100S as a template, a third round of saturation mutagenesis was conducted on the remaining 16 residue sites. In this round, we discovered four mutants with improved (*R*)-enantioselectivity ranging from *ee* of 60% to 83%. The best mutant, 1AF4A, was sequenced and it was found that the mutational change at position 186 (isoleucine substitution of threonine) was responsible for the further increase in the (*R*)-enantioselectivity of the mutant P450pyr.

The conversion of **1** by the P450pyr mutants is comparatively lower than that of the WT P450pyr (Table 1). The conversion of the best mutant 1AF4A was more than 50% lower than the WT P450pyr. The introduction of the mutation in the active pocket of the P450pyr has probably introduced a destabilizing effect in the enzyme, thus negatively affecting its activity, although the enantioselectivity of the enzyme has been significantly improved. For future work, we could perhaps take a step back and apply error-prone PCR (epPCR) to the full-length gene encoding the 1AF4A mutant to improve its activity to a level equal to or higher than the WT P450pyr.

The roles played by individual mutations towards altering the enantioselectivity of the P450pyr were not clear. The inversion of enantioselectivity can arise by the insertion of the O-atom either from the opposite direction into the C–H bond at the original 3-position or from the same direction into the C–H bond at the adjacent position due to the special C(2v) symmetry nature of the pyrrolidine substrate. From the

Table 1 Hydroxylation of *N*-benzylpyrrolidine **1** by engineered cytochrome P450pyr variants

Description	Mutant	Amino acid changes	Conversion (%) ^a	ee (%) ^b
WT P450pyr	NA	NA	55	43 (S)
Round 1	1AF4	N100S	33	42 (R)
	11BB12	F403L	47	65 (S)
Round 2	No significantly improved mutants			
Round 3	1AF4A	N100S, T186I	23	83 (R)
	1AF4B	N100S, T259S	16	63 (R)
	1AF4C	N100S, L302V	19	71 (R)
	1AF4D	N100S, V404K	27	60 (R)

^a Conversion determined based on substrate consumption with whole cell assay (10 g cell dry weight (cdw) per liter) using starting substrate concentration of 5 mM. Biohydroxylation time was 4 h. ^b Determined by chiral HPLC analysis using the Chiralcel OB-H (250 mm × 4.6 mm) column. All verification experiments were carried out in shaking flasks in triplicate.

homology model, a large cavity allowing substrate access to the heme site was observed, with hydrophobic residues lining the entrance to the cavity. An interesting observation was that the mutation sites 100, 403, and 186 were located near the entrance of the cavity, suggesting that a benzyl ring–protein interaction furthest from the heme may induce enantioselectivity towards **1** (See Fig. S2(b) in Supplementary Information†). The single amino acid substitution of the Asn100 residue by Ser that caused a complete inversion of the P450pyr enantioselectivity is of great interest. We reasoned that changing the bigger hydrophilic Asn residue to the smaller neutral Ser residue would increase the active-site volume and alter the conformation of the helix containing the N100S upon substrate binding, which may be responsible for the reversal of enantioselectivity. When the neutral Thr residue was also mutated to a hydrophobic Ile, the cooperative effect of the two mutations increased the enantioselectivity towards the (*R*)-enantiomer by almost 2 fold. Interestingly, in both cases (N100S and T186I), the residue was replaced by a more hydrophobic residue. The Phe403 in the WT P450pyr is located in the middle of the cavity to the active site pocket, thus providing a hydrophobic barrier for the hydrophilic directing groups in incoming substrates. In the case of the (*S*)-enantioselective mutant, 11BB12, the aromatic hydrophobic side chain of Phe was replaced by the aliphatic side chain of Leu, thus slightly reducing the hydrophobicity which may have directly or indirectly increased the mutant's enantioselectivity towards the (*S*)-enantiomer due to conformational changes at the entrance to the enzyme's active site.

In summary, we have demonstrated the first example of improving the biohydroxylation enantioselectivity of a class I P450 monooxygenase by iterative targeted site-saturation mutagenesis combined with a colorimetric *ee* high-throughput screening assay. Interestingly, one mutation at position 100 was all it took to invert the enantioselectivity of the P450pyr.

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