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Rapid identification of cytochrome $P450_{cam}$ variants by in vivo screening of active site libraries

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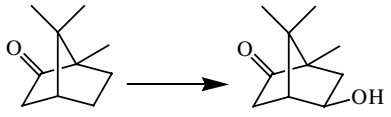
Abstract—The selection of cytochrome P450 enzymes from large variant libraries, and the subsequent use of these enzymes in preparative scale biotransformations, remains a formidable challenge due to the complexities of the associated electron transport systems. Here, a powerful approach for the generation and screening of $P450_{cam}$ libraries for new function is presented that is both flexible and robust. A targeted library was generated wherein only the $P450_{cam}$ active-site amino acids Y96 and F98 were fully randomized and biotransformations, using a novel $P450_{cam}$ whole-cell system, were screened by GC–MS for the hydroxylation of diphenylmethane. One in 50 of the reactions screened, including 16 different variants, produced 4-hydroxydiphenylmethane with up to 92% conversion observed in the case of the Y96A variant. These results demonstrate a primary example of the screening of $P450_{cam}$ libraries in a format that is compatible with extension to preparative scale reactions.

1. Introduction

The cytochrome P450 (CYP) enzyme family catalyzes the regio and stereospecific hydroxylation of a wide range of molecules, typically at nonactivated carbon atoms.[1] Such hydroxylations are particularly difficult using traditional chemical methods, suggesting that these enzymes could be highly useful biocatalysts. However, the application of CYP enzymes in biotransformations has been limited by (i) the requirement for heterologous electron transport proteins that utilize NAD(P)H in stoichiometric quantities and (ii) the substrate specificity, which is not usually optimized for relevant reactions. One solution for tailoring substrate specificity involves random genetic mutation followed by identification of proteins with the desired activity. Screening large CYP variant libraries is challenging due to the complexities of the required electron transport systems and the lack of high-throughput screens that are generally applicable, robust and powerful. Furthermore, since enzymatic hydroxylation reactions are in practice carried out using whole cell systems, it would be desirable to screen for activity using in vivo rather than in vitro systems.

Successful reports of directed evolution have been limited to the self-sufficient protein CYP102 ($P450_{BM-3}$)[2] and a variant of CYP101 ($P450_{cam}$) that circumvented the need for NADH and associated electron transport proteins by utilizing the peroxide shunt pathway.[3] However, $P450_{cam}$, which catalyzes the hydroxylation of camphor **1** to 5-*exo*-hydroxycamphor **2** (Scheme 1), is an attractive target for protein engineering due to the



1 **2**

Scheme 1. $P450_{cam}$ native activity, the hydroxylation of camphor **1** to 5-*exo*-hydroxycamphor **2**.

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availability of an X-ray crystal structure[4] and the diverse set of reactions that $P450_{cam}$ variants, generated by site-directed mutagenesis, can perform.[5] Herein, we report an efficient strategy for identifying $P450_{cam}$ variants based upon generating fully randomized active site libraries coupled with screening for product formation through in vivo biotransformations.

2. Results

To enable screening of variant $P450_{cam}$ activities present in whole cells we devised an in vivo system containing two different plasmids in each *Escherichia coli* cell (Fig. 1). The auxiliary proteins putidaredoxin (Pd) and putidaredoxin reductase (PdR) were expressed from one plasmid and $P450_{cam}$, or variants thereof, were expressed from the other with NADH supplied in vivo. Previously, CYP in vivo catalytic systems have been produced with CYP102,[6] engineered fusion proteins based on CYP102[7] and co-expression of the separate proteins,[8] including a tricistronic plasmid for $P450_{cam}$ expression and activity in *E. coli*.[9] However, we envisaged that our approach would allow the facile and high-efficiency introduction of $P450_{cam}$ plasmid libraries into a host strain that already contained plasmids encoding Pd and PdR to establish an efficient whole-cell biocatalyst.

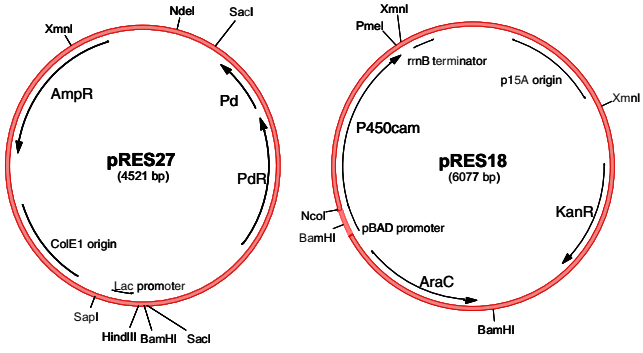


Figure 1. The two plasmids involved in the *E. coli* $P450_{cam}$ whole-cell catalyst. Pd and PdR were expressed from a *Pseudomonas putida* genomic fragment cloned into pUC18 (New England Biolabs) to produce pRES27. $P450_{cam}$ expression was induced with arabinose, in a concentration dependent manner, under the control of the pBAD promoter in a plasmid that is a hybrid of pACYC177 (New England Biolabs) and pBAD-Thio (Invitrogen).

To ensure that the engineered whole-cell biocatalyst could be used to screen variant libraries, the conversion of camphor **1** to 5-*exo*-hydroxycamphor **2** by native $P450_{cam}$ was carried out using growing cells of *E. coli* DH5α [pRES18][pRES27] with GC–MS analysis. Experiments were performed with substrate concentrations up to 10 mM and the effect of increasing $P450_{cam}$ expression was evaluated at increasing arabinose concentrations. Up to 80% substrate conversion was achieved in only 5 h, with most reactions proceeding to over 99% conversion after 20 h. Performing the reaction at 10 mM (1.52 g L^{-1} camphor) **1** yielded 5-*exo*-hydroxycamphor **2** in 61% yield after chromatographic purification.[10] These experiments suggested that any selected $P450_{cam}$ variants could be immediately transferred to preparative scale biotransformations without further development, a significant advantage of the current approach.

With a functional whole-cell catalyst in hand, we proceeded to devise a strategy for library generation and screening. In order to be able to screen generically for substrate to product conversions we elected to use LC–MS/GC–MS. Such methods have a modest throughput (ca. 150–300 samples/day) and hence are best used for screening smaller libraries. Although fully random approaches can lead to the generation of substitutions at unpredictable positions, away from the active site, there is a strong precedent for targeting only the $P450_{cam}$ substrate binding site (Fig. 2). In previous reports, the substrate specificity of $P450_{cam}$ has been extended to molecules as diverse as large polycyclic aromatics and propane by site directed mutagenesis of F87, Y96, F98, T101 and V247.[4] Herein, a library of 400 possible variants of $P450_{cam}$ was produced in which the two amino acids Y96 and F98 were simultaneously mutated to each of the remaining 19 amino acids. Y96 and F98 are both proximal to the substrate binding site of $P450_{cam}$ and a Y96A variant has previously been reported to catalyze the hydroxylation of diphenylmethane **3** to 4-hydroxydiphenylmethane **4** (Scheme 2).[11] In order to validate the biotransformation system, library generation and screening methodologies, the library was screened against diphenylmethane **3** for the selection of the Y96A variant along with the possibility of new or improved $P450_{cam}$ proteins.

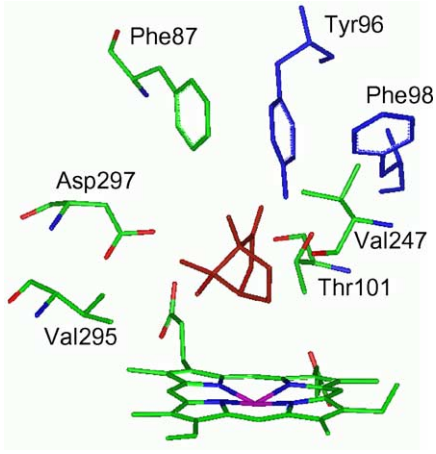
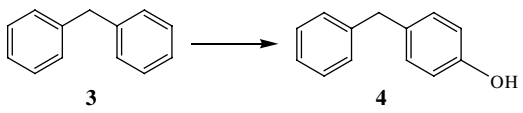


Figure 2. Structure of selected $P450_{cam}$ substrate binding pocket amino acids with camphor (red) and the randomized amino acids (blue).

A total of 1056 biotransformations in 96-well microtitre plates, each containing pRES27 and a distinct pRES18 library member, were analyzed by GC–MS. A total of 21 reactions were found to produce 4-hydroxydiphenylmethane **4**, a hit rate of ca. 1 in 50. Those variants that showed activity were subsequently grown on a 10 mL scale, to quantify the conversion of each reaction, fol-



Scheme 2.

lowed by DNA sequencing to identify the specific mutations.

The results shown in Figure 3 reveal that a range of variants possessed the ability to hydroxylate diphenylmethane **3** including the expected Y96A mutant, which had the highest activity. Phe at position 98 was frequently maintained, especially in the high activity mutants, and this was typically coupled with a small amino acid (e.g., Gly) at position 96, possibly to help accommodate one of the phenyl rings of the substrate. In addition, leucine at position 98 was a common substitution, especially with the lower activity mutants. A particular variant of interest had the substitutions Y96F and F98G wherein the positions for space generation and π–π stacking with the substrate are reversed as compared to the Y96A protein. In addition, some variants were selected more than once, including Y96C (3 times), Y96S (3) and Y96M F98L (2).

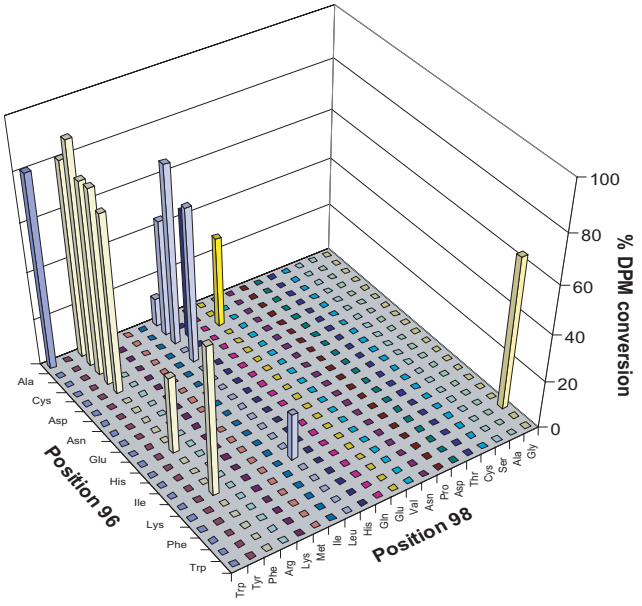


Figure 3. Hydroxylation of diphenylmethane (DPM) **3** by $P450_{cam}$ variants. The different colours of the bars are used for clarity to group variants that have a common residue at position 98. Only those clones that showed >5% conversion of **3** were sequenced and hence included in this analysis. A total of 1056 clones was analyzed in order to ensure that the library had a >99% chance of containing all 400 possible variants.

These results demonstrate a novel method for the generation and rapid screening of libraries of $P450_{cam}$ variants for new function that is both flexible and robust. The high hit rate and variety of mutations that enabled the hydroxylation of diphenylmethane demonstrates that combinatorial mutagenesis of active site amino acids, coupled with an in vivo biotransformation system and GC–MS/LC–MS screen, is a powerful method for discovering useful $P450_{cam}$ variants. As this approach is much faster than rational site-directed mutagenesis, it should be possible to extend these experiments to the identification of a variety of new activities in a much shorter time. Targeting three or more amino acids coupled with increased screening throughput, for example using capillary array electrophoresis,[12] should further accelerate this discovery process.

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