Engineering and improvement of the efficiency of a chimeric [P450cam-RhFRed reductase domain] enzyme.

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Chemicals and Bacterial Strains- All chemicals were purchased from Sigma-Aldrich unless otherwise stated. *Escherichia coli* XL1 Blue supercompetent cells were obtained from Stratagene (La Jolla, CA) and *E. coli* BL21(DE3) was from Invitrogen Life Technologies (Carlsbad, CA).

Engineering the P450cam-RhFRed L1-L7 chimeric constructs

Engineering the P450cam(Y96A)-RhFRed L1-L4 chimeric constructs- Standard DNA manipulation procedures were used throughout (Sambrook et al., 2001). All constructs were sequenced to ensure no mistakes had been introduced during amplification. For clarity, references to the amino acid sequence of the reductase domain of P450RhF correspond to the numbering system for the full length protein. Our aim was to generate a recombinant chimeric protein consisting of P450cam at the N-terminus fused to the C-terminal reductase region of P450RhF (FMN plus FeS domains). Sequence analysis of P450RhF identified the likely linker region between the P450 domain and FMN domain to comprise residues 444 to 466 in the full length protein (Roberts et al., 2002). As a starting point, we used an expression construct encoding a truncated version of P450RhF that encoded only the C-terminal FMN-FeS domains (RhFRed). Two separate constructs were engineered, which included either all or just part of this linker region (i.e., residue 445 or 452 onwards).

The PCR primer pairs used to amplify RhFRed with or without the complete linker region are given in Table 1. Each forward primer was designed to incorporate an NdeI restriction site for subsequent cloning into pET14b. The reverse primer, which was common to both amplification reactions, included a BclI restriction site located just downstream of the stop codon. pAG03 (Roberts et al., 2002) was used as template for the PCR. The PCR comprised an initial denaturation step of 94°C for 2 min followed by 30 cycles of denaturation at 94°C for 1 min 30 sec, annealing at 60°C for 45 sec and elongation for 1 min 30 sec at 72°C. The PCR mix included *Vent* DNA polymerase (New England Biolabs, Beverly, MA), 10 mM Tris-HCl (pH 9.0 at room temperature), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each deoxynucleoside triphosphate, 40 pM each primer and ~5 ng of template DNA. Products of the anticipated size were obtained, digested with NdeI/BclI and cloned into the NdeI and BamHI sites of pET14b. The two constructs encoding the reductase domain RhFRed with and without the linker region were named pRhFRed(1) and pRhFRed(2), respectively.

Construct	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
pRhFRed(1)	CGGTGT**CATATG**CTGCACCGGCATCAA	AGGT**TGATCA**TTCAGAGTCGGAGGGCCAGCC
pRhFRed(2)	CGGTGT**CATATG**ACCATCGGAGAACCCGC	AGGT**TGATCA**TTCAGAGTCGCAGGGCCAGCC
pCAM(Y96A)	CATAG**CCATGG**GCACTGAAACCATACAAAG	CGGTGTC**CATATG**TACCGCTTTGGTAGTCG

Table 1. The relevant restriction endonuclease recognition sites used for the subsequent cloning steps are highlighted in bold (NdeI, NcoI or BclI).

The gene encoding the Y96A C334A double mutant of P450cam from *Pseudomonas putida* was amplified using primers shown in Table 1 for the generation of pCAM(Y96A). The forward primer was designed to incorporate an NcoI restriction site that overlaps the ATG start codon of the gene. The reverse primer included an NdeI restriction site immediately downstream of the most 3' codon, thereby removing the natural stop codon. A cloned version of the Y96A C334A mutant of P450cam in pRES18 (Speight et al., 2004) was used as template. PCR conditions were similar to those described earlier except the extension time was increased to 2 min. A product of the expected size was obtained and digested with NcoI/NdeI.

In order to facilitate cloning of the amplified product encoding P450cam (Y96A C334A) into both pRhFRed(1) and pRhFRed(2) it was first necessary to remove an internal NcoI site within the FMN-FeS encoding region of the two constructs. This was achieved by introducing a silent mutation into pRhFRed(1) and pRhFRed(2). In each case, codon A581 in the FMN domain was mutated (GCC to GCG) using the QuikChange® II Site-Directed Mutagenesis kit from Stratagene (La Jolla, CA). The mutated versions of pRhFRed(1) and pRhFRed(2) were digested with NcoI/NdeI to allow introduction of the P450cam (Y96A C334A) gene. The final constructs, which were derived from pRhFRed(1) and pRhFRed(2), were named P450cam(Y96A)-RhFRed L3 and P450cam(Y96A)-RhFRed L1, respectively.

To explore the effect of varying the linker region between P450cam(Y96A) and the reductase portion RhFRed we also engineered two further constructs by inserting an additional short segment of DNA into P450cam(Y96A)-RhFRed L3 and P450cam(Y96A)-RhFRed L2. This was achieved by linearising the construct at the unique NdeI site (corresponding to the junction between P450cam(Y96A) and RhFRed reductase) and then introducing a short double stranded segment of DNA formed by annealing two complementary oligonucleotides that generate NdeI compatible ends. The sequence of these oligonucleotides was 5'-TATGCGATTGGCCTCCACGCA-3' and 5'-TATGCGTGGAGGCCAATCGCA-3'. Initial screening led to the isolation of a single clone for P450cam(Y96A)-RhFRed L3 and P450cam(Y96A)-RhFRed L1 in which the oligonucleotide had successfully ligated to give an additional segment of seven amino acids (RLASTHM) positioned at the linker region between the P450cam domain and the RhF reductase domain of the chimeric translation product. The two constructs derived from P450cam(Y96A)-RhFRed L3 and P450cam(Y96A)-RhFRed L1 with the additional seven amino acid linker were named P450cam(Y96A)-RhFRed L4 and P450cam(Y96A)-RhFRed L2, respectively.

Engineering the P450cam-RhFRed L1-L4 chimeric constructs- We performed QuikChange® site-directed mutagenesis at position 96 (alanine to tyrosine) on the constructs P450cam(Y96A)-RhFRed L1-L4 in order to obtain the WT P450cam. The reactions were carried out as described in the QuikChange® site directed mutagenesis

protocol using the primers 5'-CCGGCGAAGCC**TAC**GACTTCATTCC-3' (forward) and 5'-GGAATGAAGTC**GTA**GGCTTCGCCGG-3' (reverse). The thermal profile cycle was: 95°C for 1 min 30 sec/annealing temperature for 2 min/68°C for 9 min (20 cycles).

	Annealing temperature	DNA template (ng)
P450cam-RhFRed L1	55°C	100
P450cam-RhFRed L2	60.8°C	100
P450cam-RhFRed L3	66°C	150
P450cam-RhFRed L4	63.5°C	150

Table 2. PCR conditions for the engineering of P450cam-RhFRed L1-L4

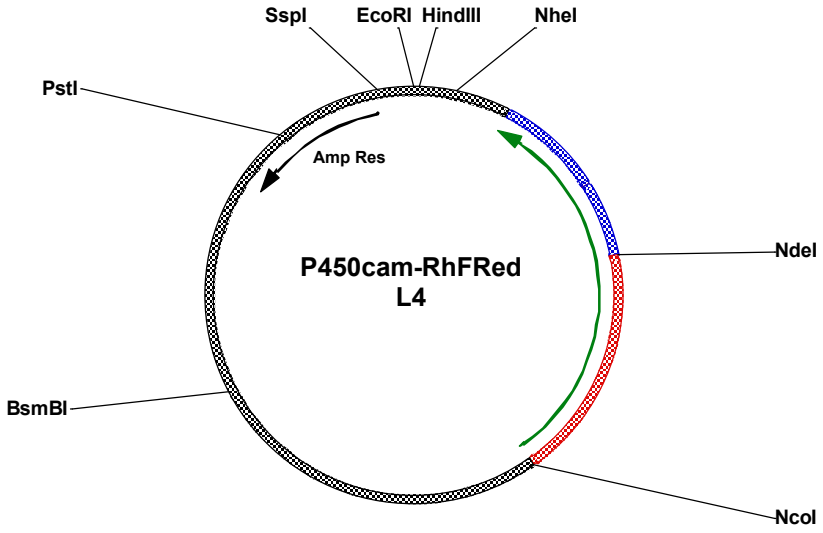

Figure 1-Schematic representation of P450cam-RhFRed L4. The portion of sequence corresponding to P450cam is highlighted in red. The portion of sequence corresponding to the reductase region RhFRed (i.e., FMN plus FeS domains) is highlighted in blue. The smaller open reading frame labelled Amp Res encodes β-lactamase, which acts as a selection marker.

Engineering the P450cam-RhFRed L5-L7 chimeric constructs We performed PCR to vary the length of the linker using primers constructed from sequences encompassing the insertion. The sequences of the oligonucleotides are listed in Table 2. The inserted amino

acids are underlined. The reactions were carried out as described in the QuikChange® site-directed mutagenesis protocol using the following thermal profile cycle (20 cycles): 95°C for 1 min 30 sec/annealing temperature for 2 min/68°C for 9 min.

	Linker sequence	PCR primers *(DNA template, mass DNA template, annealing temperature)*
P450cam-RhFRed L5	STHMRLASTHM	L1F 5'-CCAAAGCGGTATCCACGCATATGCGATTGGC-3' L1R 5'-GCCAATCGCATATGCGTGGATACCGCTTTGG-3' *(P450cam-RhFRed L4, 150 ng, 55.5°C)*
P450cam-RhFRed L6	LASTHMRLASTHM	L2F 5'-GACTACCAAAGCGGTATTGGCCTCCACGCATATGCGATTG-3' L2R 5'-CAATCGCATATGCGTGGAGGCCAATACCGCTTTGGTAGTC-3' *(P450cam-RhFRed L5, 150 ng, 60.8°C)*
P450cam-RhFRed L7	HMRLASTHMRLASTHM	L3F 5'-CCAAAGCGGTACATATGCGATTGGCCTCCACGC-3' L3R 5'-GCGTGGAGGCCAATCGCATATGTACCGCTTTGG-3' *(P450cam-RhFRed L6, 150 ng, 55.5°C)*

Table 2. PCR conditions and primers for the engineering of P450cam-RhFRed L5-L7.

Production of P450cam-RhFRed L1-L7 / Whole cell biotransformations for the conversion of camphor to (+)-5-exo-hydroxycamphor

Production of P450cam-RhFRed L1-L7-A single colony of *E. coli* BL21(DE3) cells containing plasmid P450camRhF F1-F4 was picked from overnight plates and used to inoculate 5 mL LB medium supplemented with 100 μg of ampicillin/mL. The overnight starter culture was used to inoculate 500 mL of M9 medium (Sambrook and Russell 2001) containing 100 μg ampicillin/mL, 0.4% of glucose and 0.05% of $FeCl_3$ in a 2 L flask. Cells were grown at 37°C to an optical density of 0.8. Following induction with β-D-thiogalactopyranoside (IPTG) at 0.4 mM and addition of 5-aminolevulinic acid hydrochloride (ALA) at 0.5 mM, the cells were grown for a further 16 h at 25°C. Expression of P450cam-RhFRed L1-L7 was detected by analysing cells extract with SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

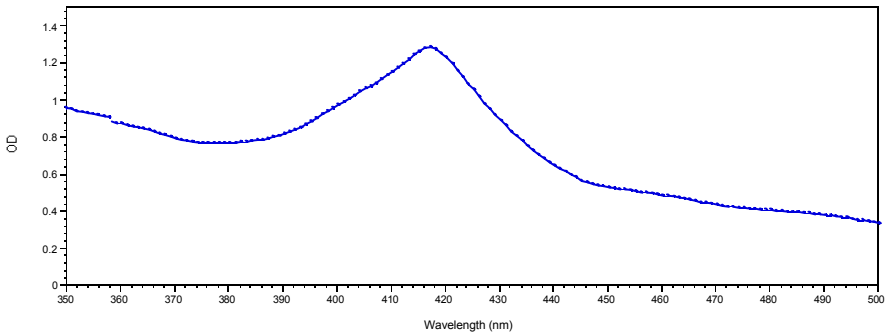

Figure 2. UV-visible absorption spectra of oxidized P450cam-RhFRed L4 (cell free extract)

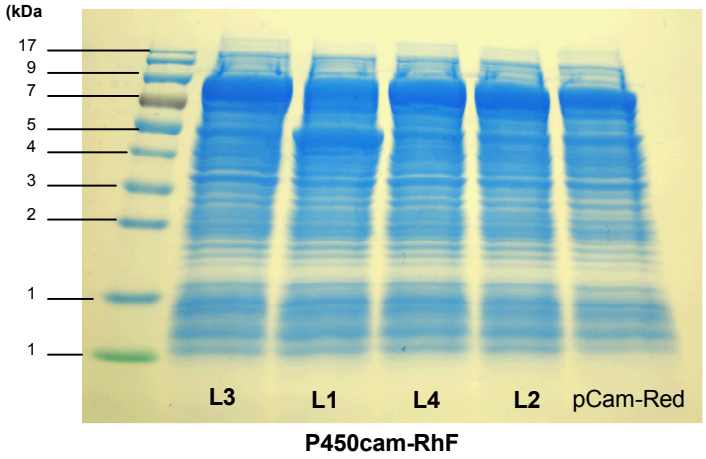

Figure 3. SDS-PAGE analysis of the soluble fractions showing the expression of P450cam-RhFRed L1-L4 and pCam-Red (Nodate et al., 2006) in *E. Coli*. The calculated molecular mass is about 81 kDa.

Whole cell biotransformation - 300 mg of wet cells were suspended in 5 mL of a 50 mM phosphate buffer (pH = 7.0) containing 0.5% of glycerol (added for the stabilization of the enzyme) and 3 mM of (+)-camphor. The reaction was performed at 25°C or 4°C in a falcon tube with shaking. 200 µL aliquots of the reaction mixture were collected after 0, 3, 6, 24 and 48h and extracted with 500 µL of ethyl acetate and 30 µL of HCl 3N. The organic phase was then analysed by GC/MS or GC/FID.

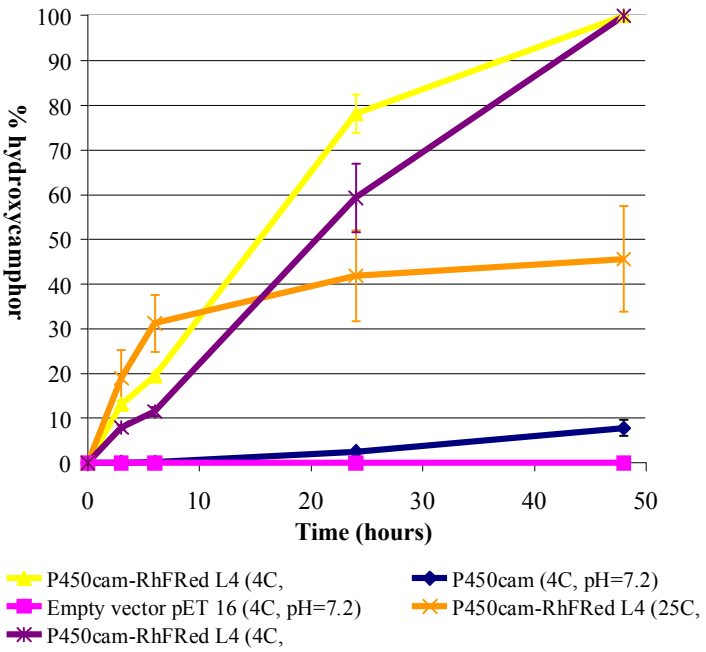

Figure. 4. Conversion of (+)-camphor to 5-*exo*-hydroxycamphor using P450cam-RhFRed L4 at 25°C (pH=7.0) and 4°C (pH=7.0 and pH=7.2). Control reactions were performed using P450cam (without the reductase domain RhFRed) and an empty vector pET16b (4°C, pH=7.2). All experiments were done in triplicate.

GC analysis of camphor and its metabolites- Analysis were performed on an Agilent 6850 GC/FID. Helium was used as the carrier gas at flow rate of 1.6 mL/min. The GC was equipped with an Agilent HP-1 column (30m, 0.32 mm i.d, 0.25 µm film thickness). The temperature program was 80-130°C at 4°C /min and then 130-230°C at 30°C/min. The temperatures were 270°C for the injection and 230°C for the detector. A sample of 1 µL was injected in a 10:1 split ration onto the column.

Protein purification of P450cam-RhFRed L4 / Spectral binding titration

Cloning of P450cam-RhFRed L4 into the pET-YSBLIC 3C VECTOR-The P450cam-RhFRed L4 coding sequence, previously cloned in the pET-14 plasmid (non his-tagged), was sub-cloned into the pET-YSBLIC 3C vector, which was especially optimized for the ligation independent cloning (LIC) and is also suitable for protein expression in *E. coli*

hosts (Bonsor D. et al., 2006). The PCR amplification of the insert was performed in a 50 µl reaction containing Phusion DNA polymerase (New England BioLabs) with a PCR cycle of 94°C for 30 seconds and 35 cycles of 94°C for 10 seconds, 65°C for 30 seconds and 72°C for 1 minute 30 seconds. The following oligonucleotides were used for the PCR:
5`-CCAGGGACCAGCAATGGGCACTGAAACCATACAAAGCAACG-3` (forward) and 5`-GAGGAGAAGGCGCGTCAGAGTCGCAGGGCCAGCC-3` (reverse).
5 µg of circular pET-YSBLIC 3C plasmid were cut with BseRI restriction enzyme (New England BioLabs) and the linearized vector ran on a agarose gel and purified using the ® SV Gel and PCR Clean-Up System (Promega). The linearized vector and the amplified product were treated with T4 Polymerase (Novagen/Merck) and the appropriate dNTP (dTTP and dATP respectively) to produce complementary overhangs. Small aliquots of T4-treated plasmid and insert were mixed at room temperature and the annealing product was used to transform Nova Blue Singles competent cells (Novagen/Merck) through heat-shock. Colonies were grown at 37°C on LB agar containing kanamycin and transformants were checked for the presence of the recombinant plasmid using either colony PCR or diagnostic digestion of the plasmid with appropriate restriction enzymes. DNA samples from positive clones were finally submitted for sequencing.

Protein purification of P450cam-RhFRed L4 - Approximately 2g of cell paste was suspended in 10 mL of ice-cold buffer A (25mM Tris-HCl, pH 7.8, 300 mM NaCl, 10% (v/v) glycerol) containing 0.1 mM phenylmethylsulfonyl fluoride and 200 µL of protease inhibitor mixture (Sigma-Aldrich). The cells were disrupted by sonification using a Soniprep 150 sonicator. The cell suspension was sonicated with a 15 s burst followed by a 45 s interval. This process was repeated 12 times. The resulting cell extract was centrifuged at 18000 rpm for 20 min at 4°C. The cell free extract was removed and filtered through a 0.45-micron filter unit. The clarified extract was then loaded onto a 1-mL Nickel HiTrap™ chelating HP column (GE Healthcare), previously equilibrated with buffer A, at a flow rate of 1 mL/min. The column was first washed with 10 volumes of buffer A to remove unbound material. His-tagged P450RhF F3 was eluted using a linear gradient (0-100%) of buffer B (30 mM imidazole in buffer A) over 60 column volumes at

a flow rate of 1 mL/min. The buffer was exchanged using a PD-10 desalting column (GE Healthcare) which was equilibrated in buffer C (50 mM Tris-HCl, pH 7.8, 10% (v/v) glycerol, 0.5 mM dithiothreitol).

Contaminating proteins were removed by anion exchange chromatography. The de-salted protein sample in buffer C (3.5 mL) was loaded onto a Resource^{TM} Q column (1 mL, GE Healthcare) at a flow rate of 2 mL/min. The column was then washed with 10 column volumes of buffer B. Protein was eluted using a linear gradient (0-50%) of buffer D (1 M NaCl in buffer C) over 20 column volumes at a flow rate of 2 mL/min.

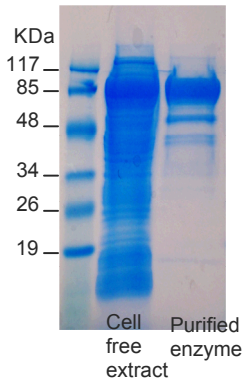

Figure. 4. SDS-PAGE of P450cam-RhFRed L4 after purification.

Spectral binding titration- Dissociation constant was determined at 25°C by titration of 1 mL of substrate-free P450cam RhF F3 (1 μM) in 40 mM phosphate buffer (pH=7.4) with (+)-camphor in ethanol. The spectroscopic changes (350-500 nm) associated to the sequential addition of camphor (in the range of 0.1 to 20 μM) were recorded on a Varian Cary 50 Bio. The difference in absorbance between the wavelength maximum (418 nm) and minimum (393 nm) was plotted *versus* the substrate concentration into a Michaelis-Menten equation to estimate K_d.

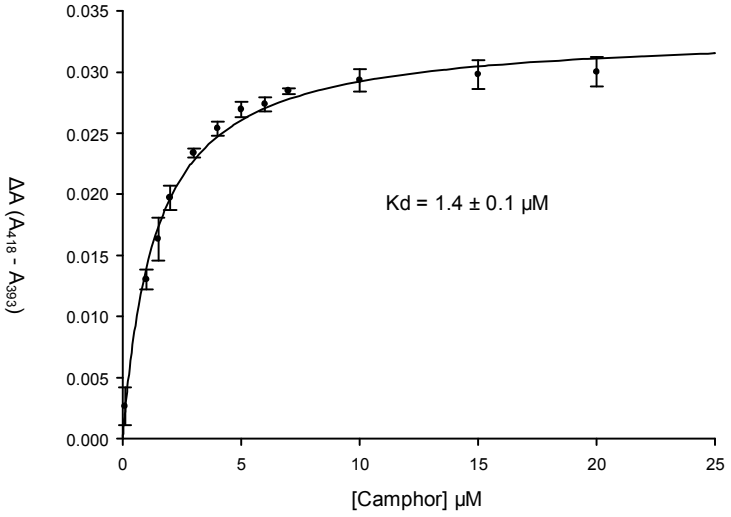

Fig. 4. Substrate binding affinity (K_d) of P450cam-RhFRed L4 toward (+)-camphor.

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