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Stereoselective hydroxylation of isophorone by variants of the cytochromes P450 CYP102A1 and CYP101A1

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Abstract

The stereoselective oxidation of hydrocarbons is an area of research where enzyme biocatalysis can make a substantial impact. The cyclic ketone isophorone was stereoselectively hydroxylated (≥95%) by wild-type CYP102A1 to form (*R*)-4-hydroxyisophorone, an important chiral synthon and flavour and fragrance compound. CYP102A1 variants were also selective for 4-hydroxyisophorone formation and the product formation rate increased over the wild-type enzyme by up to 285-fold, with the best mutants being R47L/Y51F/I401P and A74G/F87V/L188Q. The latter variant, which contained mutations in the distal substrate binding pocket, was marginally less selective. Combining perfluorodecanoic acid decoy molecules with the rate accelerating variant R47L/Y51F/I401P engendered further improvement with the purified enzymes. However when the decoy molecules were used with A74G/F87V/L188Q the amount of product generated by the enzyme was reduced. Addition of decoy molecules to whole-cell turnovers did not improve the productivity of these CYP102A1 systems. WT CYP101A1 formed significant levels of 7-hydroxyisophorone as a minor product alongside 4-hydroxyisophorone. However the F87W/Y96F/L244A/V247L CYP101A1 mutant was ≥98% selective for (*R*)-4-hydroxyisophorone. A comparison of the two enzyme systems using whole-cell oxidation reactions showed that the best CYP101A1 variant was able to generate more product. We also characterised that the further oxidation metabolite 4-ketoisophorone was produced and then subsequently reduced to levodione by an endogenous *Escherichia coli* ene reductase.

Keywords: cytochrome P450; isophorone; hydroxylation; stereoselective; regioselective

1. Introduction

Cytochromes P450 (P450s)[1] are a family of enzymes that carry out oxidative transformations including hydroxylations, epoxidations, heteroatom oxidations and dealkylations as well as other more complex reactions [1-3]. Most P450s catalyse the oxidation of their substrate via a high-valent iron-oxo radical cation intermediate which inserts an oxygen atom into unactivated carbon-hydrogen bonds using a radical rebound mechanism [4, 5]. The key steps of the catalytic cycle are substrate binding, electron transfer and dioxygen binding followed by the activation of the oxygen by delivery of a second electron. These electrons are sourced from a nicotinamide cofactor (NADH or NADPH) and delivered via electron transfer partners as required [6-8]. These enzymes offer advantages over traditional methods of synthesis, in that the process of carbon-hydrogen bond hydroxylation occurs with high regio- and stereoselectivity in a single step under ambient conditions. The combination of all of these factors result in P450s being attractive enzymes for biocatalytic applications which could be used to complement traditional synthetic chemistry [9-11].

$P450_{cam}$ (CYP101A1) is a bacterial P450 that catalyses the stereospecific hydroxylation of (1*R*)-camphor to 5-*exo*-hydroxycamphor [9, 12]. It uses a Class I electron transfer system, comprising of the FAD dependent, putidaredoxin reductase (PdR) and the [2Fe-2S] ferredoxin, putidaredoxin (Pdx) to obtain electrons from NADH [13, 14]. The development of active site mutants which catalyse the oxidation of alternative substrates highlighted the biocatalytic potential of CYP101A1 and this family of enzymes in general [9, 15-19]. For example, a number of active-site mutations introduced into CYP101A1 at phenylalanine 87, tyrosine 96, leucine 244 and valine 247, were found to improve its selectivity toward other monoterpenes, including *S*-limonene and (+)-α-pinene, enabling a route to

[1] *Abbreviations:* P450, cytochrome P450; $P450_{cam}$, CYP101A1 from *Pseudomonas putida*; $P450_{BM3}$, CYP102A1 from *Bacillus megaterium*; Pdx, [2Fe-2S] ferredoxin from *Pseudomonas putida*; PdR, flavin dependant ferredoxin reductase from *Pseudomonas putida*; EMM, *E. coli* minimal media; PFC, perfluorocarboxcylic acid, IPTG, Isopropyl β-D-1-thiogalactopyranoside; TLC, Thin layer chromatography, NADH, reduced nicotinamide adenine dinucleotide; NADPH, reduced nicotinamide adenine dinucleotide phosphate; DMSO, dimethyl sulfoxide.

the production of compounds for the flavour and fragrance industries [20, 21]. The enzyme has also been altered to oxidise substrates as varied as gaseous alkanes, such as ethane, and large polycyclic aromatic hydrocarbons, including pyrene [15, 17, 19, 20, 22-26].

The cytochrome P450 CYP102A1 (P450Bm3) from *Bacillus megaterium* oxidises fatty acid substrates close to the omega terminus at high activities [27, 28]. It and other members of the CYP102A subfamily are unusual in that the electron transfer partner domain is fused to the heme domain [29-32]. CYP102A1 is soluble, easy to produce and the self-sufficient nature and high activity overcome two of the major hurdles to the use of P450 enzymes in synthesis. CYP102A1 has a high specificity for NADPH over NADH as the electron source [33], and it has been used as a template for the design of selective oxidation biocatalysts through rational protein engineering and directed evolution [9, 17, 34-37]. For example, CYP102A1 isoforms have been created which enable the cyclopropanation, amination and aziridination of various substrates [38-42]. CYP102A1 variants, which enhance the oxidation activity for unnatural substrates but do not alter the product regioselectivity, have been identified [43-46]. These include the R47L/Y51F (RLYF) mutant of CYP102A1, where the two mutations are located at the entrance of the substrate access channel, which enhanced the oxidation of hydrophobic unnatural substrates such as polyaromatic hydrocarbons [47]. Studies of other generic accelerator mutants, such as I401P and KT2 (A191T/N239H/I259V/A276T/L353I), revealed that in the absence of substrate the rate of the first electron transfer step was comparable to that when a fatty acid is bound [44, 46]. Structural analysis of these variants has been used to rationalise this behaviour with the mutants having conformations which more closely resemble the fatty acid bound form of the enzyme and have longer heme-iron axial water interactions [44, 46]. These more catalytically ready conformations result in substrate binding-induced changes to the structure playing a less significant role in promoting the electron transfer steps. This accounted for their ability to oxidise non-natural substrates, including alkylbenzenes and alkanes, at elevated rates. The combination of the RLYF couple with rate

accelerating CYP102A1 variants has also been found to further enhance their activity [46, 48]. In addition CYP102A1 variants, in which phenylalanine 87 in the enzyme active site has been mutated, have been reported to modify the substrate binding profile and product selectivity of the enzyme [36, 49-52]. The GVQ variant (A74G/F87V/L188Q) of CYP102A1 has been reported to be an effective biocatalyst for a range of organic molecules including aromatic hydrocarbons, alkanes and norisoprenoids [43, 53].

Chemically inert perfluorocarboxylic acid (PFC) decoy molecules have also been used to improve the activity of CYP102A1 [54]. These greatly promote the oxidation of unnatural substrates such as benzenes, xylenes and short chain alkanes by the wild-type (WT) enzyme [54-57]. They work as the shorter fluorinated fatty acid decoy molecule is used to fill part of the enzyme's active site resulting in conformational changes in the enzyme. However there is enough space in the vicinity of the heme to allow a substrate to bind and the regioselectivity of oxidation is largely unaffected [56]. We have shown that it is possible to use decoy molecules, in conjugation with the rate accelerating mutants of CYP102A1 to substantially enhance the rates of product formation for cyclohexane and benzene-derived substrates and improve the productivity of regio- and stereo-selective biocatalytic reactions [48, 57].

The selective oxidation of isophorone to 4-hydroxisophorone, which is an important flavour and fragrance compound as well as a synthetic intermediate for pigments and drug molecules, is a suitable target for biocatalytic oxidation [58-61]. Recently WT CYP102A1 was shown to be capable of generating 4-hydroxyisophorone on a kilogram scale [59]. During the preparation of this manuscript a report combining CYP101A1 variants with an alcohol dehydrogenase to generate 4-ketoisophorone was published [62]. Here we report the stereoselective oxidation of isophorone by mutant forms of CYP102A1 and CYP101A1. We show that the *in vitro* oxidation activity with the CYP102A1 variants was improved using these variants and decoy molecules. Whole-cell oxidation of isophorone was used to compare the activity of the class I CYP101A1 system with the self-

sufficient CYP102A1 variants. Using these systems we were also able to identify the major and minor products arising from moonoxygenase activity and those from further processing by *Escherichia coli*.

2. Materials and Methods

2.1 General

General reagents and isophorone were from Sigma-Aldrich, or VWR. Buffer components, NADPH, and isopropyl-β-D-thiogalactopyranoside (IPTG) were from Astral Scientific (Australia). Production and purification of full-length CYP102A1 variants for *in vitro* use was carried out as described previously [43, 57]. UV/Vis spectroscopy was performed on an Agilent Cary 60 spectrophotometer. Gas chromatography mass spectrometry (GC-MS) analyses were carried out on a Shimadzu GC-17A instrument coupled to a QP5050A MS detector using a DB-5 MS fused silica column (30 m x 0.25 mm, 0.25 μm) and helium as the carrier gas. Additional GC analysis and chiral chromatography were performed on a Shimadzu Tracera GC coupled to Barrier discharge Ionisation Detector (BID) detector using a Supelcowax column (30 m x 0.32 mm x 0.25 um) and a RT®-BDEXse chiral silica column (Restek; 30 m x 0.32 mm x 0.25 um), respectively. Helium was used as the carrier gas. The detailed GC methods used are given in the Supplementary Material.

2.2 Activity assays

CYP102A1 *in vitro* NADPH turnovers were run at 30 °C in 1200 μL of 50 mM Tris, pH 7.4 at 30 °C, containing 0.2 μM enzyme and 120 μg bovine liver catalase. The buffer was saturated with oxygen gas just before use and the assays were allowed to equilibrate for 1 min prior to the addition of the decoy molecule (100 μM) if present, and isophorone (1 mM substrate from a 100 mM stock in DMSO). Finally NADPH was added, from a 20 mg mL^{-1} stock, to a final concentration of ~320 μM (equivalent to 2 AU). A period of 10 seconds was allowed to elapse after NADPH addition before the rate of absorbance decay at 340 nm was measured. The reactions were allowed to run until all the NADPH was consumed. The NADPH turnover rate was derived using $\varepsilon_{340} = 6.22\ mM^{-1}\ cm^{-1}$. Similar experiments in which the concentration of the added isophorone was altered (0.1 – 2 mM) were performed to determine the catalytic activity of the enzyme.

2.3 In vivo metabolite generation and product identification

The CYP101A1 variants were screened using a plasmid system pCWSGB++ which contained the genes for PdR, Pdx and the relevant mutant (Table S1) [16, 17, 19, 20, 22, 23]. The DNA was transformed into competent DH5α *Escherichia coli* (*E. coli*) cells and a single colony was grown in 2 x LB media and protein produced as previously described [16]. The cell pellet was harvested by centrifugation and resuspended in *E. coli* minimal media (EMM; K_2HPO_4, 7 g, KH_2PO_4, 3 g, Na_3citrate, 0.5 g, $(NH_4)_2SO_4$, 1 g, $MgSO_4$, 0.1 g, 20% glucose, 20 mL and glycerol, 1% v/v per litre). Isophorone was added and the cells (30 mL in a 250 mL Erlenmeyer flask) were shaken at 200 rpm and 30 °C. Aliquots of the turnover (1 mL, supernatant and cells) were taken and extracted with ethyl acetate for analysis by GC-MS.

To isolate and identify the isophorone products, for which standards were not readily available, and to compare the CYP102A1 and CYP101A1 turnovers whole-cell oxidation systems utilising the plasmid pET28 containing the CYP102A1 gene of interest or the pCWSGB++ CYP101A1 system were used. The plasmids were transformed into competent BL21(DE3) cells and grown on LB plates containing 100 μg mL^{-1} ampicillin (pCW) or 30 μg mL^{-1} kanamycin (pET28). A single colony was inoculated into 500 mL 2 x LB broth in a 2 L flask and grown at 37 °C overnight or for smaller scale growths 50 mL in a 250 mL flask). Protein expression was induced by the addition of 60 μM IPTG (from a 0.4 M stock in H_2O), and the temperature was reduced to 20 °C and the shaker speed to 110 rpm. The growths were allowed to continue for another 16 h before the culture was harvested by centrifugation and washed with EMM. The cell pellet was resuspended in the same volume of media (EMM) and split into flasks as required. Isophorone (2 mM from a 100 mM stock in ethanol) was added to the resuspended cells (50 mL in a 250 mL flask or 200 mL in a 2 L baffled flask for large scale growths) the reactions were shaken at 150 rpm and 30 °C. A second aliquot of isophorone was added to the small scale whole-cell turnovers after 6 hours and the reactions were allowed to proceed for another 14 hours. For large scale turnovers two further aliquots of substrate were added after 3

and 6 hours. For both large and small scale reactions an aliquot of 20 x phosphate buffer (pH 7.4, 1/40th of the total reaction volume) was added to the mixture to help maintain the pH. The large scale reactions were also allowed to proceed for 20 hours.

For the comparison of the CYP101A1 and CYP102A1 variants, *in vivo*, the plasmid systems were transformed into the *E. coli* strain BL21(DE3) and grown in LB with the relevant antibiotics. The cells were harvested, resuspended in EMM and the biotransformations were performed in triplicate as described above (50 mL in 250 mL flask). The biomass yield was recorded as the cell wet weight and the concentration of P450 was determined by lysing the cells (via sonication) and recording the CO difference spectrum of the supernatant [63].

2.4 Product analysis

After the *in vitro* NADPH consumption assays were completed, 990 μL of the reaction mixture was mixed with 10 μL of an internal standard solution (*p*-cresol, 20 mM stock solution in DMSO). For whole-cell turnovers 990 μL aliquots of the turnover (including cells) were taken for analysis (after addition of the same internal standard). The mixture was extracted with 400 μL of ethyl acetate and the organic extracts were used directly for GC-MS or GC analysis. Products were initially identified by matching the GC-MS mass spectra to those expected for the metabolites (see Supplementary Material). The amount formed was determined by calibrating against isophorone and 4-ketoisophorone using the assumption that the hydroxyl isomeric products would give comparable responses e.g. 4-hydroxyisophorone and 7-hydroxyisophorone were presumed to give a similar detector response to isophorone.

To determine the identity of the unknown product observed in the GC-MS analysis of the GVQ CYP102A1 variant (which was expected to arise from non-P450 catalysed enzyme activity) a whole-cell turnover was performed with an *E. coli* containing an empty pET vector. The cells were grown as above and a 2 mM aliquot of 4-ketoisophorone was added. After 20 hours all the substrate had

been converted to a single product which was isolated and analysed by MS and NMR (Supplementary Material).

Chiral normal phase HPLC analysis was carried out on a Shimadzu system equipped with a DGU-20A5R degasser, 2 x LC-20AR pumps, SIL-20AC HT autosampler, SPD-M20A photodiode array detector and a CT0-20AC column oven. Separation of the chiral products was carried out using a CHIRALPAK® IG column (5 μm particle size, 4.6 mm diameter x 150 mm; Daicel Chemical Industries Ltd.) equipped with a Chiralpak® IG guard column (5 μm particle size, 4.0 mm diameter x 10 mm; Daicel Chemical Industries Ltd.) operating in isocratic mode with 95% hexane and 5% isopropanol at 0.5 mL min^{-1} for 38 min. The injection volume was 5 μL and the absorbance was monitored at 254 nm. Retention times were as follows: ketoisophorone, 14.8 min; (*R*)-4-hydroxyisophorone, 26.9 min, (*S*)-4-hydroxyisophorone 29.4 min.

2.5 Isolation of isophorone oxidation products

The supernatant (200 mL) from an *in vivo* turnover of isophorone was extracted in ethyl acetate (3 x 100 mL), washed with brine (100 mL) and dried with magnesium sulphate and the organic extracts were pooled and the solvent was removed by vacuum distillation and then under a stream of nitrogen. The products were purified by silica gel chromatography using a hexane/ethyl acetate stepwise gradient ranging from 80:20 to 50:50 hexane to ethyl acetate with 2.5 % increases every 100 mL. The composition of the fractions was assessed by TLC and GC-MS and those containing single products (≥95 %) were combined for characterisation. The solvent was removed under reduced pressure.

The purified product was dissolved in $CDCl_3$ and the organics characterised by NMR spectroscopy and GC-MS. NMR spectra were acquired on a Varian Unity-plus spectrometer operating at 500 MHz for ^{1}H and 126 MHz for ^{13}C. A combination of ^{1}H, ^{13}C, COSY, HSQC and HMBC experiments was used to determine the structures of the products. The specific rotation was determined using an Anton-Paar MCP 100 modular circular polarimeter.

The epoxidation of isophorone and 4-ketoisophorone was undertaken using the method of Fioroni and coworkers [64]. In summary, 250 µl of an aqueous solution of 2 M NaOH was added to 1.0 mmol of substrate. The mixture was dissolved in 1.62 mL of water at 0–2 °C with stirring. After 10 minutes 0.15 mL of 30 wt % aqueous hydrogen peroxide was added. The product was extracted after 6 h with ethyl acetate. Using this method approximately 30% of the isophorone was oxidised to isophorone oxide while the conversion for 4-ketoisophorone epoxidation was ≥ 99%.

The reduction of 4-ketoisophorone to generate a racemic mixture of 4-hydroxyisophorone was undertaken using the method of Ishihara [65]. 4-Ketoisophorone (0.4 mmol) was reduced with sodium borohydride (0.1 mmol) in methanol at -5 to 0 °C to afford the product which was confirmed by GC coelution and MS analysis with the sample isolated from the whole-cell oxidation system which had been characterised by NMR. This sample was used for chiral HPLC analysis.

2.6 NMR data

4-hydroxyisophorone: ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.86 (s, 1H, H2), 4.03 (s, 1H, H4), 2.41 (d, *J* = 16.3 Hz, 1H, H6), 2.21 (d, *J* = 16.3 Hz, 1H, H6), 2.06 (s, 3H, H7), 1.07 (s, 3H, H8/H9), 1.02 (s, 3H, H8/H9); ^{13}C NMR (126 MHz, $CDCl_3$): δ 202.12 (C1), 164.61 (C3), 128.64 (C2), 79.28 (C4), 51.62 (C6), 41.17 (C5), 29.57 (C8/C9), 24.24 (C8/C9), 24.01 (C7).

7-hydroxyisophorone: ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.15 (s, 1H, H2), 4.22 (s, 2H, H7), 2.27 (s, 3H, H6), 2.14 (s, 2H, H4), 1.05 (s, 6H, H8 & H9). ^{13}C NMR (126 MHz, $CDCl_3$) δ 200.09 (C1), 162.22 (C3), 122.11 (C2), 65.03 (C7), 51.56 (C6), 40.26 (C4), 33.66 (C5), 28.23 (C8 & C9).

levodione: ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.03 (m, 1H, H6), 2.77 (d, 1H, H5), 2.74 (d, 1H, H3), 2.52 (d, 1H, H3), 2.35 (d, 1H, H5), 1.21 (s, 3H, CH_3), 1.15 (d, 3H, CH_3) 1.12 (s, 3H, CH_3). ^{13}C NMR (126 MHz, $CDCl_3$): δ 216.73 (C1), 210.60 (C4) 55.38 (C3), 47.51 (C5), 46.85 (C2), 42.47 (C6), 29.16 (C8), 28.21 (C9), 17.21 (C7).

2.7 Substrate binding assays

UV/Vis spectroscopy was performed on an Agilent Cary 60 spectrophotometer. For substrate binding the CYP102A1 enzymes were diluted to ~1 - 2 µM in 50 mM Tris, pH 7.4. After addition of the isophorone or the PFC10 decoy molecule (as 1 µL aliquots from a 100 mM stock in DMSO or ethanol) the spectra was recorded.

3. Results

3.1 CYP102A1 catalysed oxidation of isophorone

WT CYP102A1 has been shown to be capable of selective hydroxylation of isophorone but no enzymatic turnover data was reported [59]. We have shown that certain generic accelerator variants of this enzyme, which combine mutations outside of the distal active site pocket with the R47L and Y51F mutants at the entrance to the substrate access channel, can facilitate the oxidation of non-physiological substrates while maintaining the selectivity. These include the R19 (R47L/Y51F/H171L/Q307H/N319Y) and RP (R47L/Y51F/I401P) variants which have been shown to be effective at oxidising hydrophobic molecules such as alkylbenzenes. Decoy molecules, such as perfluorocarboxylic (PFC) acids, perfluorononanoic acid (PFC9) and perfluorodecanoic acid (PFC10), can also be combined with CYP102A1 variants to enhance the rate of oxidation of similarly sized substrates to isophorone [48, 55]. In these previous studies PFC10 resulted in the highest product formation rates due to a large increase in the turnover of the catalytic cycle whereas PFC9 generally resulted in a higher coupling efficiency of the reducing equivalents to product formation. Therefore both PFC9 and PFC10 were tested with the CYP102A1 variants to see if isophorone oxidation could be improved.

We determined the rates of NADPH oxidation and product formation and the coupling efficiency for isophorone oxidation with the purified CYP102A1 variants, in the presence and absence of the polyfluorinated carboxylic acids (Table 1). All the variants tested gave rise to a single major oxidation product using GC-MS analysis. In each turnover there were additional minor products, which from coelution experiments and analysis of the MS spectra were consistent with the further oxidation product 4-ketoisophorone and the epoxidation metabolite, isophorone oxide (Fig. 1). The oxidation of isophorone by WT CYP102A1 was slow (PFR; 0.2 nmol(nmol-CYP)$^{-1}$min^{-1}; henceforth abbreviated to min^{-1}) and generated low levels of the hydroxylated metabolite. Addition of both PFC9 and PFC10 improved the overall product yield. PFC10 increased the rate of production

formation 29-fold as a result of increasing the NADPH oxidation rate and the coupling efficiency. The rate accelerating variants all increased the amount of product formation arising from isophorone oxidation. The product formation decreased in the order RLYFIP > R19 > KT2 > WT with the highest PFR being 45 min^{-1} (Table 1).

The oxidation of isophorone by all of these variants was also enhanced by adding the PFC9 and PFC10 decoy molecules. This arose due to an increase in both the NADPH oxidation activity and coupling efficiency resulting in more oxidation metabolites being formed in a shorter amount of time (Fig. 1, Table 1, Fig. S1). The product formation rate was always greater with PFC10 (Table 1). In line with previous studies the overall enhancement induced by the decoy molecules in rate accelerating variants was lower than that observed with the WT enzyme ranging from 30-fold for KT2 and 4-fold for RLYFIP. However the combined effect of the rate accelerating variants and decoy molecules produced the highest levels of isophorone oxidation. The RLYFIP variant was better than R19, both in the absence and presence of the decoy molecules (Table 1). The RLYFIP variant, which contain the RLYF couple at the entrance of the substrate access channel, was better than KT2 predominantly due to improved coupling efficiencies (Table 1). The RLYFIP and PFC10 combination resulted in the highest product formation rate for isophorone, 180 min^{-1} (Table 1).

In order to compare the effect of the decoy molecule on the kinetic parameters we determined the k_{cat} and K_m for the CYP102A1 enzyme variant in the presence and absence of PFC10. It was not possible to determine these values accurately for the WT enzyme but for each variant the addition of the decoy molecule resulted in an increase in k_{cat} and a decrease in K_m (KT2: k_{cat}; ~20 min^{-1}, K_m >2 mM, KT2/PFC10: k_{cat}; 202 ± 6 min^{-1}, K_m 380 ± 10 μM; R19: k_{cat}; 22 ± 1 min^{-1}, K_m 650 ± 40 μM, R19/PFC10: k_{cat}; 150 ± 30 min^{-1}, K_m 440 ± 110 μM and RLYFIP: k_{cat}; 155 ± 6 min^{-1}, K_m 750 ± 30 μM, RLYFIP /PFC10: k_{cat}; 330 ± 60 min^{-1}, K_m 440 ± 150 μM). The kinetic parameters were also in

accord with lower improvements the decoy molecule induced with the R19 and RLYFIP variants compared to the enhancement for KT2 (Table 1, Fig. S1).

We also tested the A74G/F87V/L188Q (GVQ) mutant with isophorone. This variant contains three mutations in the substrate access channel and active site of the enzyme. As such it alters the enzyme activity for more hydrophobic substrates and changes the selectivity of the enzyme. The oxidation of isophorone by this variant gave rise to the same major and minor products as the WT enzyme alongside an additional minor product at a later retention time (Fig. 1). The oxidation with the GVQ variant was more efficient, 57 min^{-1}, than RLYFIP and the other variants. This was predominantly due to a higher coupling efficiency (Table 1). However while the addition of the decoy molecules enhanced the NADPH oxidation rate with the GVQ mutant there was a significant reduction in the coupling efficiency and as a consequence lower levels of product formation with isophorone and this variant (Table 1).

To explore the different behaviour of the GVQ variant with the decoy molecules we determined the spin state shift of the ferric heme after addition of PFC10 to the different CYP102A1 mutants. With the WT and rate accelerating variants minimal alteration in the spin state from the low-spin to the high-spin (HS) form, as measured by UV-Vis, was observed (≤15% HS). A small shift was seen with the rate accelerating variants following addition of isophorone after PFC10 (Fig. S2, 40% HS). PFC10 did induce a shift with GVQ (55% HS) indicating perturbation of the water bound to the heme-iron. Isophorone addition to GVQ resulted in a small shift (≤20% HS). This increased to 50% HS when PFC10 and isophorone were added. Overall the data shows that the fatty acid based decoy molecule is bound closer to the heme in the GVQ variant as it can displace the heme iron-bound water molecule.

3.2 Identification of the products arising from CYP102A1 oxidation of isophorone

A single major product (>90%) was detected by GC-MS in the *in vitro* oxidation of isophorone by all CYP102A1 variants. The catalytic oxidation of isophorone by the CYP102A1 variants was performed, in the presence and the absence of decoy molecules, using a whole-cell system to generate greater quantities of this product for characterisation (Fig. S3). No material change in the levels of product formation was observed *in vivo* for either variant or when decoy molecules were added. Significantly more product was generated in turnovers using the variants when compared to the WT CYP102A1 enzyme (Fig. S4). The whole-cell turnovers with the R19 and RLYFIP variants were the most selective and the overall conversion of the isophorone into product was approximately 30% after 20 hours resulting in approximately 1-1.2 mM product (as determined by the amount of product formed). The turnovers were combined extracted and the product was isolated, purified (~6 mg after purification) and identified as 4-hydroxyisophorone (4-hydroxy-3,5,5-trimethyl-2-cyclohexen-1-one), by NMR and the MS fragmentation pattern (Fig. S3 and Supplementary Material). The GVQ variant was less selective generating the minor metabolites observed in the *in vitro* turnovers in greater quantities (Fig. S4). The minor products were not isolated in sufficient yield or purity, from any of the variants, for NMR characterisation. Analysis of the MS fragmentation pattern suggested that the as yet unidentified later eluting product was 7-hydroxyisophorone (see Supplementary Material). Further oxidation to 4-ketoisophorone was also observed in the GVQ turnovers as was the formation of another as yet unidentified metabolite (*vide infra*).

3.3 CYP101A1 catalysed oxidation of isophorone

While there were significant improvements in the product formation of 4-hydroxyisophorone using the CYP102A1 variants, without and when combined with the decoy molecules, the coupling efficiency was moderate (Table 1). We therefore wanted to assess if other P450 enzymes could selectively oxidise this substrate. Previously we have shown that CYP101B1 from *Novosphingobium aromaticivorans* was able to generate three products, including 4-hydroxyisophorone, but in low yield

[66]. As isophorone is similar in chemical composition and size to camphor we decided to screen its oxidation with WT CYP101A1 and a selection of mutant forms (Table S1). We used a small library (fourteen including the WT enzyme) of CYP101A1 mutant enzyme encoding genes, which had previously been generated and cloned into a whole-cell oxidation system with the physiological electron transfer partners, putidaredoxin (Pdx) and putidaredoxin reductase (PdR) [16]. These mutants were chosen as they previously had shown good activity and selectivity with hydrocarbon and terpenoid based substrates such as isomers of limonene and pinene, which are of a similar size to isophorone [15, 19, 20, 67, 68].

The screen was performed using the available whole-cell system to determine which variants would hydroxylate isophorone in a selective manner. WT CYP101A1 catalysed oxidation of isophorone generated two detectable products by GC-MS. The major product (89%) co-eluted with the 4-hydroxyisophorone product with the second having a later elution time, which was the same as that of the minor product observed in the oxidation of isophorone by the CYP102A1 GVQ variant. Of the CYP101A1 mutants screened with isophorone two variants were promising in that they generated more product and were more selective for 4-hydroxyisophorone than the WT enzyme. These were F87W/Y96F/V247L and F87W/Y96F/L244A/V247L which produced 4-hydroxyisophorone at 93% and 98%, respectively (Fig. 2a). The remaining mutants tested generated lower levels of product and were in general less selective, though 4-hydroxyisophorone was the major product in all cases (Fig. 2b).

Both products were extracted from a larger scale whole-cell oxidation turnover using the F87W/Y96F/V247L mutant (Fig. S5). They were purified and isolated by silica chromatography and the major product from both enzyme turnovers was confirmed as 4-hydroxyisophorone (35 mg; Fig. S3). The second less abundant product was characterised as 7-hydroxyisophorone (12 mg; 3-hydroxymethyl-5,5-dimethyl-2-cyclohexen-1-one, Fig. S3).

3.4 Enantioselectivity of 4-hydoxyisophorone formation with CYP102A1 and CYP101A1.

4-Hydroxyisophorone can be generated as two enantiomers. To analyse the enantioselectivity of the biocatalytic hydroxylations the turnovers were analysed by chiral chromatography. In all cases a single peak was observed by GC (Fig. 2b). Analysis by chiral HPLC also resulted in the observation of a single major peak from the enzyme catalysed reactions. The reduction of 4-ketoisophorone by sodium borohydride generates a racemic mixture of 4-hydroxyisophorone and this mixture was separated into two peaks by chiral HPLC but not GC (Fig. S6). The optical rotation of the purified 4-hydroxyisophorone from the whole-cell turnovers of the CYP101A1 and CYP102A1 variants was measured in methanol. The specific rotation $[\alpha]_D^{20}$ = +113.2 (c = 1.00, methanol) – was found to be in agreement with that reported in the literature for the pure (*R*)-enantiomer [58, 59], which is also in agreement with the results reported by Turner and coworkers [62].

3.5 Comparison of CYP102A1 and CYP101A1 systems

CYP102A1 and it variants are often the P450 of choice for biocatalytic reactions due to their self-sufficient nature, level and ease of production and high levels of activity. The CYP101A1 system requires additional electron transfer proteins but whole-cell oxidation systems of this and other class I systems have been generated. These also have high oxidation activity [16, 69, 70]. The CYP101A1 variants tested here generated more product for characterisation than the CYP102A1 systems using the whole-cell oxidation system. A direct comparison between the two systems is not trivial due the disparate electron transfer systems (FMN versus iron sulfur ferredoxins, Class I versus Class III fused system), cofactor utilisation (NADH versus NADPH) and the different requirements of the plasmid systems used (pET28 versus pCWori). However we assessed if the CYP101A1 system would still give rise to increased levels of product when both systems were grown under similar conditions. The F87W/Y96F/V247L/L244A CYP101A1 system was first compared in DH5α and BL21(DE3) cells to see if this made a difference to the overall levels of product formation. In both types of cells almost all of the added substrate (4 mM) was consumed and similar levels of product (≥2.5 mM) were

generated after a 20 hour reaction suggesting the cell type is not of major significance when comparing the two systems (Fig. 3).

Next the WFAL CYP101A1 variant was compared with the RLYFIP and R19 variants of CYP102A1. The whole-cell oxidations were carried out in BL21(DE3) cells. Both systems generated similar levels of cell biomass (8-10 grammes of cell wet weight per litre of culture, Table S2). The amount of P450 detected in the CYP102A1 variants was lower than for the WT CYP102A1 system (88 – 183 nM) but was substantially higher than those of the CYP101A1 system (25 – 54 nM, Table S2). After seven hours the level of product formed with the R19 (~570 ± 100 μM) was lower than that with RLYFIP (~650 ± 100 μM) which was lower again than that of the CYP101A1 system (~935 ± 10 μM, Fig. 4a). After leaving the samples for a further 13 hours the CYP101A1 system was able to convert almost all of the substrate (4 mM) to product; 2.8 – 4 mM of the major metabolite could be detected at the end of the reactions (Fig. 3). Despite the higher P450 expression levels the conversion in the CYP102A1 turnovers were lower and the amount of product detected was ~1200 ± 50 μM and 1200 ± 100 μM for the RLYFIP and R19 variants, respectively (Fig. 4a). Overall the whole-cell turnover of isophorone with the CYP101A1 variants seems to be better than with CYP102A1 (>50000 total turnovers versus 11000 for the CYP102A1 variants). The GVQ CYP102A1 variant was also tested and the maximum levels of product formation were similar to those observed with the RLYFIP variant (Fig. 4b and Fig. S6). Additional oxidation products were detected during the whole-cell oxidations with CYP102A1 variants (Fig. S4 and Fig. S7). Two of these were assigned from the MS fragmentation patterns as the epoxides of isophorone and 4-ketoisophorone (Fig. 5). This was confirmed by coelution experiments with the products from the reactions of isophorone and 4-ketoisophorone with hydrogen peroxide in an alkaline solution, which generates the epoxides (Materials and Methods). The other metabolite, which was detected during the CYP102A1 turnovers, had an MS fragmentation pattern consistent with that of levodione. This is formed by reduction of the alkene double bond of 4-ketoisophorone (Fig. 5) [62]. To verify the identity of this product we

ran a control reaction with 4-ketoisphorone in *E. coli* containing an empty pET vector. The 4-ketoisophorone (2 mM) was completely converted in less than 20 h into the same metabolite observed in the enzyme turnovers. This was extracted, isolated and identified by NMR as levodione (Fig. S3, Supplementary Material).

4. Discussion

Overall the formation of 4-hydroxyisophorone by CYP102A1 was significantly increased using generic rate accelerator variants and the GVQ mutant. The regio- and stereoselectivity of the reaction was largely unaffected with all reactions giving rise to the (*R*)-enantiomer of 4-hydroxyisophorone as the major product. The most efficient *in vitro* turnovers contained the decoy molecule PFC10 and variants with the R47L/Y51F couple. However compared to smaller substrates such as cyclohexane and ethylbenzene the coupling efficiency of the optimal systems was moderate. The selective nature of the turnover suggests that the substrate is held in enzyme active site with only one C–H bond close to the reactive iron-oxo cation radical intermediate but it must be far enough away to make the oxidation relatively inefficient. The larger size of isophorone may be one reason for the less efficient oxidation but smaller decoy molecules did not improve the levels of product formation and mutagenesis around the CYP102A1 active site may be required to optimise the hydroxylation further.

One important observation from this work is that the decoy molecules did not improve isophorone oxidation with the A74G/F87V/L188Q CYP102A1 variant. This may arise as mutations at these positions in the active site and access channel of the enzyme may alter the position of fatty acid binding, as evidenced by the different behaviour of the spin state of this variant upon addition of PFC10. This would result in the decoy molecules binding closer to the heme and interfering with rather than enhancing the oxidation of non-natural substrates. As such the perfluorinated decoy molecule could now be positioned between the isophorone and the heme, displacing the iron-bound water and increasing the NADPH oxidation rate but resulting in a reduced C-H bond abstraction and coupling efficiency. The addition of the decoy molecules did not improve the whole-cell oxidation activity. This may be due to reduced uptake of the perfluorinated fatty acids by the cells, though further optimisation of the relative amounts of decoy molecule and substrate in whole-cell reactions may improve this [71, 72].

Isophorone is of a similar size to camphor and the WT CYP101A1 enzyme was able to oxidise isophorone but this reaction was not as selective as WT CYP102A1. The oxidation of isophorone using a library of CYP101A1 mutants, derived from previous rational protein engineering studies (Supplementary Material), revealed that certain variants catalysed the selective formation of (*R*)-hydroxyisophorone. All of these variants modified the tyrosine 96 residue which interacts with the camphor carbonyl group. The more hydrophobic active site of the F87W/Y96F/V247L and F87W/Y96F/L244A/V247L (WFAL) mutants must able to bind isophorone to enable more efficient and selective hydroxylation. The WFAL mutant; designed by rational mutagenesis for pinene and chlorinated benzene hydroxylation, was found to be the best CYP101A1 variant for isophorone oxidation obtained by us and also by Turner and coworkers who used a library constructed using saturation mutagenesis at fourteen residues in the enzyme active site [19, 20, 62].

A comparison between the whole-cell oxidation activities of the best CYP101A1 and CYP102A1 variants showed that, despite lower levels of P450 production, the class I CYP101A1 system was superior over a longer period of time in shake flask conversions (385 mg L^{-1}). A direct comparison of the three component class I system versus the single component self-sufficient CYP102A1 system is difficult. The coupling efficiency and oxidation rates of the CYP102A1 system may be lower than that of the CYP101A1 variants [73]. Alternatively the concentration and rate of regeneration of cellular NADPH required by CYP102A1 may be different to that of NADH utilised by the CYP101A1 system [74-77]. The level of P450 holoprotein production of both systems in *E. coli* is known to be high but perhaps the fused CYP102A1 system is less stable under the turnover conditions used. The improvement in the CYP102A1 variants over the WT enzyme and the better whole-cell oxidation of isophorone by the CYP101A1 system should lead to refinements in the up sizing of this reaction. Recently researchers at DSM have scaled up the activity of WT CYP102A1 in a fermentor system. Using high cell densities, thirty times the amount used in our experiments, and the addition of additional NADPH enabled 4-hydroxyisophorone to be produced at 10 g L^{-1} and at

space-time yields of 1.5 g L^{-1} h^{-1} [59]. Given the low activity of the WT enzyme for this reaction this level of product formation is encouraging for the use of the CYP102A1 variants reported here. Whole-cell double oxidation of 10 mM α-isophorone to 4-ketoisophorone, using twenty times the amount of cells in our experiments on a 1 ml scale, was used to obtain a 65% yield of the product [62]. While a direct comparison of these systems is challenging due to differences in scale, biomass, redox partner utilisation, cofactor regeneration and the expression systems used the amount of product obtained from each relative to the amount of cell biomass is comparable (this work 45 mg g^{-1} L^{-1}, DSM 33 mg g^{-1} L^{-1}, double oxidation 5 mg g^{-1} L^{-1}). Taken together these systems show promise for the future scale up of metabolites from isophorone oxidation using bacterial cytochrome P450 systems.

5. Conclusion

Variants of both the CYP101A1 and CYP102A1 cytochrome P450 enzymes were able to regio- and stereo-selectively hydroxylate isophorone to (*R*)-4-hydroxyisophorone. Decoy molecules were shown to improve the rate of product formation with the rate accelerating CYP102A1 variants but inhibited the performance of the A74G/F87V/L188Q variant which has mutations in the active site and substrate access channel. Whole-cell oxidation reactions with the optimal CYP101A1 variants were more productive than the best CYP102A1 mutants. The addition of decoy molecules to the whole-cell turnovers did not have a major effect on the levels of product formation. Further protein engineering of both systems could lead to more efficient systems for the oxidation of isophorone. Increasing the coupling efficiency of the CYP102A1 variants and improving the CYP101A1 whole-cell oxidation system would enhance the productivity.

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Tables

Table 1. NADPH oxidation rate (N), product formation rate (PFR) and coupling efficiency (C) for purified CYP102A1 variants with isophorone. The assays were conducted using 1mM substrate, ~320 μM NADPH and 0.2 μM CYP enzyme in 50 mM Tris, pH 7.4. Coupling efficiency is the percentage of NADPH utilised for the formation of isophorone products. N and PFR are reported as mean ± S.D. ($n \geq 3$) and given in nmol(nmol-CYP)$^{-1}$min^{-1}. Examples of the NADH oxidation assays and the GC chromatogram of the product areas are provided in Fig. S2).

	isophorone		
	N	**C**	**PFR**
WT	57 ± 2	0.3 ± 0.1	0.2 ± 0.1
WT/PFC9	98 ± 3	3.4 ± 0.3	3.3 ± 0.2
WT/PFC10	318 ± 2	1.8 ± 0.1	5.8 ± 0.2
KT2	65 ± 1	4.4 ± 1	2.8 ± 0.7
KT2/PFC9	384 ± 2	6.0 ± 0.3	23 ± 1
KT2/PFC10	1260 ± 10	12 ± 1	150 ± 5
R19	148 ± 38	8.8 ± 0.5	13 ± 4
R19/PFC9	346 ± 8	14 ± 1	49 ± 2
R19/PFC10	497 ± 34	16 ± 1	83 ± 6
RLYFIP	425 ± 28	11 ± 1	45 ± 2
RLYFIP/PFC9	894 ± 6	16 ± 1	142 ± 4
RLYFIP/PFC10	1090 ± 30	16 ± 1	180 ± 8
GVQ	365 ± 3	16 ± 2	57 ± 5
GVQ/PFC9	1530 ± 20	2.5 ± 0.2	38 ± 3
GVQ/PFC10	1880 ± 30	1.0 ± 0.1	18 ± 1

List of Figures

Figure 1 (a) GC-MS analysis of the *in vitro* turnover of isophorone by the CYP102A1 variants R19 (grey) and GVQ (black). Note the chromatograms have been offset along the x-axis for clarity. Impurities are labelled (*), as are isophorone (RT 6.2 min), 4-hydroxyisophorone (RT 9.15 min) and 7-hydroxyisophorone (KIP; RT 10.6 min). A small amount of the further oxidation product 4-ketoisophorone (RT 6.6 min) was identified in both reactions. 7-Hydroxyisophorone was only observed in the GVQ turnover. (b) GC analysis (wax column) in the region of the 4-hydroxyisophorone product (RT 15.3 min) for different *in vitro* CYP102A1 turnovers. The chromatograms are labelled from those which generate the lowest amount of this product to the highest; WT, WT+PFC10, R19, GVQ and RLYFIP (RP). See Table 1 for turnover parameters.

Figure 2 (a) GC-MS analysis of the whole-cell turnovers of isophorone by WT CYP101A1 (black) and the variants WFAL (light grey) and WFL (grey). 4-Hydroxyisophorone (RT 9.0 min) and the minor product (7-hydroxyisophorone at RT 10.45 min) are labelled. Note the chromatograms have been offset along the y-axis for clarity. (b) The GC (chiral) chromatogram in the region of the 4-hydroxyisophorone product (RT 18.7 min). The amount of product generated by the different whole-cell CYP101A1 turnovers, from lowest to highest, was WT, F87W/Y96F/L244A, WFL and WFAL. Note the enantiomers of 4-hydroxyisophorone are not separated using this method.

Figure 3 GC chromatograms (wax column) of the whole-cell turnover of the CYP101A1 variant WFAL after 7 (grey) and 20 hours (black). Shown are the isophorone substrate (5.8 min), internal standard (IS; 12.8 min) and 4-hydroxyisophorone product (15.3 min). The chromatograms have been slightly offset along the x- and y-axes for clarity. There was an increase in the amount of product (935 ± 10 μM to ~2.5 mM) from 7 to 20 hour samples and the majority of the added substrate (4 mM) was consumed at the end of the turnover.

Figure 4 (a) GC chromatograms (wax column) of the whole-cell turnover of the CYP102A1 variant RLYFIP after 7 (grey) and 20 hours (black). Shown are the isophorone substrate (5.8 min), internal

standard (IS; 12.8 min) and 4-hydroxyisophorone product (15.3 min). The chromatograms have been slightly offset along the x- and y-axes for clarity. Compared to the CYP101A1 WFAL whole-cell turnovers there were lower levels of product at both 7 (650 ± 100 μM) and 20 hours (1100 ± 100 μM, see Fig. 3). Note that there is a significant amount of unconverted substrate after 20 hours (~1.5 mM). (b) GC-MS chromatograms of the turnover of isophorone with the CYP101A1 variant WFAL (grey) and the CYP102A1 variant GVQ (black). The retention times are the same as those in Figure 1. Additional metabolites can be seen in the GVQ turnover and include isophorone oxide (IPO; RT 5.9 min), 4-ketoisophorone epoxide (ket ox, RT 6.9 min), 4-ketoisophorone (KIP, RP 6.2 min) and levodione (LD 7.1 min). See the Supplementary Material for MS analysis of the metabolites.

Figure 5 The turnover of isophorone to (*R*)-4-hydroxyispophorone. The structures of the minor and further products which were generated in certain turnovers are also shown.