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Increasing the activity and efficiency of stereoselective oxidations using decoy molecules in combination with rate enhancing variants of P450Bm3

Samuel D. Munday,[a] Shaghayegh Dezvarei,[a] and Stephen G. Bell*[a]

Abstract: The use of rate accelerating variants of P450Bm3 coupled with decoy molecules is described resulting in improved catalytic activity of hydroxylation and epoxidation reactions. Prochiral substrates were investigated to ascertain the effect of the mutant enzymes and the decoy molecules on the regio- and enantioselectivity of the oxidations. For the alkyl and alkenyl substituted benzene substrates tested large improvements in the product formation activity over the wild-type enzyme were obtained. The product formation rates for the substrates tested ranged from 660 to 2210 nmol (nmol-P450)−1 min−1 for variants containing the R47L and Y51F mutations. While the regioselectivity was not significantly altered in most of the turnovers some adjustment in the enantioselectivity was observed with the smaller substrates. The addition of decoy molecules often resulted in improved enantioselectivity and counteracted reductions arising from the rate accelerating mutants.

Introduction

Cytochrome P450s (CYPs) are heme-dependent enzymes which are able to insert a single oxygen atom from molecular dioxygen into a carbon–hydrogen bond to give the corresponding alcohol or oxidise an alkene to yield an epoxide.[11] Nature has evolved many of these enzymes so that these reactions occur with total regio- and stereoselectivity.[2] The selective oxidation of unactivated C–H bonds by chemical methods is energy-intensive, non-selective and generates unwanted side products and toxic wastes. Biocatalytic asymmetric epoxidations are also of great interest. These CYP-mediated oxidation reactions have the potential to provide efficient biocatalysts for stereoselective oxidations to form alcohols and epoxides.[3,4] CYP102A1 (P450Bm3) from Bacillus megaterium is a self-sufficient enzyme in which the electron transfer reductase domain is fused to the heme domain.[35] It is soluble, easy to produce and requires only a substrate, NADPH and oxygen to operate. Its active state is a dimer which is capable of hydroxylating fatty acid substrates, at sub-terminal positions, with unusually high activity.[14] The fusion protein nature and high activity of P450Bm3 overcome two of the major hurdles to the use of these enzymes in synthesis.[36, 37] Wild-type P450Bm3 (WT) oxidises the majority of unnatural substrates at very slow rates if at all. However it has been used as a template for the design of biocatalysts through protein engineering.[38, 39, 40] These studies have resulted in variants which are capable of selective oxidation reactions and recently even non-physiological functions such as cyclopropanation, amination and aziridination have been engineered into this enzyme.[41, 42]

P450Bm3 variants, such as I401P and KT2 (A191T/N239H/I259V/A276T/L353I) have been identified, which enhance activity for unnatural substrate oxidation but maintain the product regioselectivity of the WT enzyme.[43, 44] The substrate free forms of these generic accelerator mutants have been shown to have conformations which are more similar to the fatty acid bound form of the enzyme (PDB: 1JPZ).[45] This is especially true in important regions such as the L helix which is involved in the mechanism of oxygen binding and cleavage to generate the reactive intermediate.[46, 47] In the crystal structure of the substrate-free form of the KT2 (PDB: 3PSX) and I401P (PDB: 3HF2) variants the heme axial water interactions were weakened. Kinetic studies also showed that the rate of the first electron transfer step for these variants in the absence of substrate were comparable to that when substrate is bound. Therefore it has been proposed that KT2 and I401P have catalytically ready conformations, such that substrate-induced changes to the structure play a less significant role in promoting the electron transfer steps resulting in its ability to oxidise non-natural substrates at elevated rates.

Other variants include the R47L/Y51F (RLYF) mutant of P450Bm3 which has been shown to promote the oxidation of a range of unnatural substrates.[48, 49] It has been hypothesised that the RLYF couple allows better recognition and entry of more hydrophobic substrates. Mutations at these sites are known to reduce the affinity for the fatty acid substrate. However this pair of amino acids is not conserved in many other members of the CYP102A subfamily which bind and hydroxylate fatty acids.[50] The RLYF couple has been added to rate accelerating P450Bm3 variants and has been found to further enhance their activity.[6a, 8a] For example the variant R19 (R47LY51F/H171L/Q307H/N319Y) has the RLYF couple to KSK19 variant (minus the F87A mutation).[51] The RLYFIP (R47LY51F/I401P) variant contains the I401P mutant which is on the proximal side of the heme and is known to be an effective rate accelerating variant for several substrates.[8a, 8b]

In an alternative approach to improve the activity of P450 enzymes, chemically inert perfluoroacarbonyl acids (PFCs) have been used as decoy molecules.[13] These greatly promote the oxidation of unnatural substrates such as benzenes, xylenes and short chain alkanes. They work as the inert decoy fills part of the enzyme’s active site causing conformational changes in the enzyme and constraining substrates to bind closer to the heme.[14] The structure of a PFC9-L-Trp-bound WT (PDB: 5WSP) showed that the decoy molecule (the N-perfluoroacyl amino acid, PFC9-
L-Trp) filled the access channel but left enough space close to the heme to bind a substrate. These enzyme-substrate interactions in the vicinity of the heme remain relatively unaffected allowing the reaction to proceed with the same selectivity of product formation.

It has been shown that incorporating the use of the decay molecules, perfluorocarboxylic (PFC8), perfluoronorbornic (PFC9) and perfluorodecanic (PFC10) acids, with WT and KT2 P450Bm3 significantly improves the rates of product formation for cyclisation, and some known to be relatively stereospecific. Importantly the turnovers with KT2 were more active and the product profiles of the oxygenated substrates remained predominantly unchanged. The mechanisms by which decay molecules and the rate accelerating variants show enhanced unnatural substrate activity are different but work in concert to facilitate the oxidation of organic molecules. Here we investigate the effect of the rate accelerating variants and decay molecules on the stereoselectivity of alcohol and epoxide formation by utilising substrates that are known to be oxidised to products which contain a stereocentre.

Results

Ethylbenzene and n-propylbenzene

Although they do not resemble the fatty acid substrates, ethylbenzene and n-propylbenzene are known to be oxidised by WT and KT2 P450Bm3 with high selectivity (Scheme 1). They are hydroxylated at the benzyl (or α) C–H bonds with α-hydroxylation resulting as a minor product for each (Scheme 1). In addition, WT P450Bm3 is known to be relatively stereospecific in its oxidation of n-propylbenzene producing (R)-1-phenyl-1-propanol in excess over the (S) enantiomer (reported as an enantiomeric excess of: 90%). Ethylbenzene and n-propylbenzene were both oxidised by WT and KT2 P450Bm3 in the presence and absence of PFC decay molecules (Fig. 1, Table S1). n-Propylbenzene was turned over with a product formation rate (PFR) of 368 nmol nmol P450 h−1 (henceforth given as min−1). The oxidation products were identified by GC-MS coelution experiments with authentic standards where available. WT and KT2 P450Bm3 in the presence and absence of decoys primarily oxidised ethylbenzene and n-propylbenzene at the benzylic (α) position to give 1-phenyl-1-ethanol (α-Et; 83-92%) and 1-phenyl-1-propanol (α-Pr; 91-94%) as the major products (Scheme 1, Fig. 2, Table S2). Minor products for both substrates were observed as a result of α-hydroxylation on the aromatic ring and for n-propylbenzene from hydroxylation of the β-position of the alkyl chain (2-4%, Scheme 1, Table S2). No hydroxylation of the terminal alkyl carbon was observed for either substrate nor was any desaturation product identified for ethylbenzene which has been observed for some P450Bm3 variants. There was also the presence of a small amount of a ketone over-oxidation product of α-Pr in the n-propylbenzene turnovers (α-ket-Pr; 1-2%). Oxidation of either substrate at the α-position to give the hydroxylated product introduces a chiral centre (Scheme 1).

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**Figure 1**

![Diagram of substrate oxidation](image)

**Scheme 1**

Product distributions of catalysed oxidation of ethylbenzene, n-propylbenzene, styrene and trans-β-methylstyrone in the presence and absence of decay molecules. The products from ethylbenzene: 1-phenylethanol (α-Et) and 2-ethylphenol (2-Et); from n-propylbenzene: 1-phenyl-1-propanol (α-Pr), 1-phenyl-2-propanol (β-Pr), 2-propylphenol (α-Pr) and propiophenone (α-ket-Pr); from styrene: styrene oxide; and from trans-β-methylstyrene: 1-phenylpropane oxide (β-oxide). The values in italics represent the ratio of the (R)- and (S)-enantiomers of 1-phenylethanol, 1-phenyl-1-propanol and styrene oxide. For 1-phenylpropane oxide, the values in italics represent the ratio of the (R,R)- and (S,S)-enantiomers of 1-phenylpropane oxide. The values in bold are the enantiomeric excess (ee).
The chiral benzylic hydroxylation products were assigned by coelution experiments using a chiral GC column. WT and KT2 P450Bm3 were mostly selective for the (R)-enantiomer in the absence and presence of decoys (α-ET, 48% ee and α-Pr 74% ee). n-Propylbenzene was also oxidised predominantly to the (R)-enantiomer of α-Pr and the enantiomeric excess (ee) was greater those of the equivalent ethylbenzene turnovers (Fig. 2, Fig. S1, Fig. S2). WT oxidation of ethylbenzene was more enantioselective than KT2. The greatest enantiomeric excess of (R)-α-Et formed with WT P450Bm3 was 62% compared to 34% with KT2 (both using PFC10, Fig. S1). WT and KT2 both displayed similar selectivity for (R)-α-Pr but the PFC9 and PFC10 decoys resulted in slightly more selective combinations for both (Fig. 2, Fig. S1, Table S2). The maximum enantiomeric excess of the (R)-α-Pr formed was 80% for WT and 72% for KT2 when decoy molecules were used (Table S2). These results agree with previous studies which also observed the (R)-isomer being produced in large excess by WT P450Bm3 (Fig. 2, Table S2).[16a]

**Styrene and trans-β-methylstyrene**

Styrene has previously been reported to be a poor substrate for WT P450Bm3 but other variants epoxidise this substrate to give styrene oxide as the major product. Mutations at the Ala82 and Thr438 residues have been shown to be effective in encouraging styrene oxidation.[17] The P450Bm3 variant A82F/T438F, which contains a more restricted active site, oxidised styrene to give the (R)-enantiomer of the epoxide in an enantiomeric excess of 64%. trans-β-Methylstyrene has also been trialled as a substrate with the KT2 variant and was oxidised at the double bond to give the epoxide mostly as the (R)-isomer (reported ee: 88%).[18]

The KT2 combinations oxidised both substrates at higher activities than the equivalent WT reaction in every circumstance. Overall, both styrene substrates were oxidised at slower rates than their alkylbenzene counterparts (Fig. 1, Table S1). The addition of PFC9 generated the most efficiently coupled reactions and PFC10 induced the highest NADPH activity but a lower coupling efficiency.
Figure 2. GC-MS analyses of (a) WT (grey) and WT/PFC9 (black) with ethylbenzene; (b) WT and WT/PFC with n-propylbenzene; (c) chiral GC analysis of the 1-phenylethanol product in the turnovers with RLYF/PFC10 (solid) R19/PFC10 (dash) and R19 (dash/dot) and (d) chiral GC analysis of the 1-phenyl-1-propanol product in the turnovers with KT2 (black) KT2/PFC10 (dash) and RLYFP/PFC10 (dash/dot). The internal standard is labelled when shown (IS) as are impurities (*).

The inclusion of PFC9 in the WT-catalysed oxidation of styrene resulted in a 73-fold increase in PFR whereas PFC10 improved the rate by 91-fold due to the superior NADPH consumption rate (Fig. 1 and Table S1). The best improvement with a decoy and the KT2 variant was obtained using PFC9 increasing the PFR by almost 12-fold over KT2 alone. The overall improvement of styrene oxidation was 140-fold when using KT2/PFC9 over WT (Fig. 1, Table S1).

trans-β-Methylstyrene was oxidised at faster rates than styrene for all the combinations. For the WT enzyme/decoy molecule combinations, the greatest improvement was observed with PFC10 (41-fold increase in PFR) due to a smaller reduction in coupling efficiency, compared to PFC9 combined with the fastest NADPH activity. For KT2, PFC9 was the optimum sized decoy, and the KT2 variant was obtained using PFC9 increasing the PFR by almost 12-fold over KT2 alone. The overall improvement of styrene oxidation was 140-fold when using KT2/PFC9 over WT (Fig. 1, Table S1).

Styrene oxide, which contains a single stereocentre, was mostly generated as the (R)-stereoisomer with the enantiomeric excess ranging from 14-32% (Fig. 3, Fig. S1 and Table S2). WT P450Bm3 epoxidation was more stereoselective than KT2 (18% vs 14% ee) and the inclusion of decoys improved the enantiomeric excess of the (R)-isomer of both (up to 32% ee, Table S2). Epoxidation of trans-β-methylstyrene introduced two stereocentres. Both WT and KT2 were highly selective for the (R,R) isomer over the (S,S) and this oxidation was more stereoselective (≥70% ee) compared to that of styrene (Fig. 3, Fig S1, Table S2). The results were in general agreement with those previously reported with KT2.[18] Unlike the oxidation of styrene there was little variation in stereoselectivity between the WT and KT2 enzymes, nor did introducing decoys significantly alter the enantiomeric excess (Fig. 3d, Table S2).

The WT and KT2 catalysed oxidation of both substrates was highly regioselective, with a major single product arising from both (Scheme 1, Fig. 3, Table S2). Both were epoxidised at the double bond to yield styrene oxide (phenyloxirane) and 1-phenylpropylene oxide (β-oxide), respectively. Small amounts (~1%) of a second product arose in styrene turnovers, which from coelution experiments was assigned as phenylacetalddehyde.[19] Small peaks (<1% of total product) which coeluted phenylacetone and propiophenone were observed in the P450Bm3 catalysed oxidation of trans-β-methylstyrene. No variation in the regioselectivity was observed for any of the enzyme/decoy combinations.
The internal standard is labelled when shown (IS) as are impurities (*).

Rate accelerating variants incorporating the R47L/Y51F couple

The above results show that the activity of P450Bm3 can be enhanced by adding fatty acid based decoy molecules to the WT enzyme and rate accelerating variant KT2. We wanted to assess if decoy molecules could be used to enhance the oxidation of unnatural products when combined with highly active rate enhancing variants which contain mutations at the R47 and Y51 residues. The arginine and tyrosine are known to interact with the acidic group of the fatty acids. This pair of mutations would be expected to promote the oxidation of non-natural substrates but could potentially lower the affinity for the fatty acid decoy molecules. For all four substrates the activity of product formation decreased in the order RLYFIP > R19 > KT2 > WT (Fig. 1, Table S1). In all instances both the NADPH oxidation activity and the coupling efficiency was greater for the variants containing the R47LY51F couple. The RLYFIP variant was better than R19 predominantly due to an increased NADPH oxidation rate even though its coupling efficiency was often lower (Fig. 1, Table S1). The turnovers of n-propylbenzene and trans-β-methylstyrene with R19 and RLYFIP alone were better than the optimal KT2/PFC decoy molecule combinations (Fig. 1, Table S1).

The oxidation of the smaller ethylbenzene and styrene substrates were both enhanced by adding PFC10 to the R19 and RLYFIP turnovers. In the case of ethylbenzene this was due to an increase in the coupling efficiency but the overall activity of product formation was similar to that observed with the KT2/PFC9 combination. With styrene there was an increase in both the NADPH oxidation activity and coupling efficiency when PFC10 was used as a decoy molecule (Fig. 1, Table S1). There was significant improvement over the most active KT2 turnover with the rate enhancement of the RLYFIP/PFC10 combination being 410-fold more active than the WT enzyme alone (Fig. 1, Table S1). The addition of the decoy molecule PFC10 to the turnovers of n-propylbenzene with R19 or RLYFIP did not result in a significant increase in activity with the RLYFIP/PFC10 combination. However the addition of PFC10 to R19 did enhance trans-β-methylstyrene oxidation mainly through an increase in the rate of NADPH oxidation (Fig. 1). The epoxidation activities were enhanced over the KT2 combinations and the improvements over the WT enzyme alone for the R19 and RLYFIP mutants when using PFC10 as a decoy were both greater than 130-fold.

These combinations resulted in the highest product formation rates for the different substrates ranging from 661 min⁻¹ for
The regioselectivities of styrene and trans-β-methylstyrene oxidation were unchanged compared to the WT and KT2 turnovers with epoxidation being the sole major product (>98%). The same was true for n-propylbenzene oxidation with 1-phenyl-1-propanol being the major product. More further oxidation to the α-ketone occurred in the R19 and RLYFIP turnovers, however the selectivity for the α-position was ≥ 98% (Table S2). The regioselectivity of oxidation of ethylbenzene by R19 (82% α-Et) was similar to KT2 in that both were less selective than the WT (Table S2) in generating 1-phenylethanol as the major product. By way of contrast the RLYFIP variant was marginally more selective producing 91% of 1-phenylethanol. In both instances the addition of the PFC10 decoy molecule moderately increased the selectivity for the major product (Table S2).

The trends in enantioselectivity were more complex for the R19 and RLYFIP variants. The enantioselectivity for trans-β-methylstyrene oxidation was virtually unchanged across all of the variants and decoy molecule combinations though it was marginally lower for the turnovers with RLYFIP (70% vs. 75% ee, Table S2). The enantioselectivity of the 1-phenyl-1-propanol product in the R19 and RLYFIP turnovers was similar to the KT2 combinations though slightly lower than those of the WT (68-70% vs. 74-80% ee, Table S2). We note that the higher levels of the ketone further oxidation product may affect the results by favouring the oxidation of one enantiomer of the alcohol over the other. Larger changes were observed with the smaller substrates. With ethylbenzene the preference for the R-enantiomer product with R19 (27% ee) was similar to KT2 (22% ee) while that of RLYFIP (51% ee) was more like the WT enzyme (48% ee) in having a larger enantiomeric excess (Table S2). The enantioselectivity of styrene oxide formation was lower for R19 (25% ee) while RLYFIP formed an almost equal mixture of both enantiomers (6% ee, Fig. 3c and Table S2). Importantly for both ethylbenzene and styrene turnovers the decoy molecule PFC10 increased the enantioselectivity of the turnover in line with what was observed for the WT and KT2 turnovers. This resulted in an improved ee of 62% for ethylbenzene hydroxylation by RLYFIP/PFC10 and 31% for styrene oxide formation by R19/PFC10 (Table S2).

Discussion

Overall the rates of product formation of the four prochiral substrates were significantly increased using decoy molecules and a generic rate accelerator variant. Using a combination of both methods resulted in the optimal biocatalyst in terms of product formation activity (PFR). The product formation rates for the rate accelerating mutants were higher compared to their WT equivalents due to a combination of superior coupling efficiency and NADPH activity. This presumably arises in part from them being in a 'catalytically ready' conformation. The inclusion of the perfluorinated fatty acid decoy molecules also increased the product formation rates. The decoy molecules are proposed to act in a similar fashion to the rate accelerating mutants by placing the enzyme in a more substrate-bound like conformation which enables more efficient oxidation. The decoy molecules can also help exclude water molecules from the active site, which could improve the coupling efficiency. For the substrates with lower activities, addition of the decoy molecule PFC10 resulted in improvements in variants containing the R47L/Y51F couple. This suggests that the decoy molecules can still effectively bind to these variants and facilitate substrate oxidation. It is also important in that this could be an effective method for improving the activity of related CYP102 family enzymes not all of which contain this pair of residues.

The turnovers of the substrates with the shorter side chains, ethylbenzene and styrene, were inferior to those of n-propylbenzene and trans-β-methylstyrene, respectively. This arose from a combination of lower NADPH oxidation rates and coupling efficiencies. The oxidation activity of the planar alkenes substituted benzenes were also reduced compared to the alkylbenzene equivalents. The lower activity of the alkenes was predominantly due to lower coupling efficiency. Carbon-hydrogen bond hydroxylation is more energetically challenging than epoxidation suggesting that these planar substrates must be bound in a less favourable location in the active site compared to the alkylbenzenes. Overall n-propylbenzene oxidation proceeded with the highest activity, suggesting this substrate was well positioned in the active site for efficient C–H bond abstraction. As a consequence the improvements observed with this substrate on addition of decoy molecules were reduced compared to the other substrates. The oxidation of n-propylbenzene is superior to ethylbenzene, toluene and n-butylbenzene suggesting the three carbon alkyl group is of the optimal size and fit for binding in the active site of P450Bm3.[64-66] The main product of alkyl benzene oxidation occurred at the benzylc or α-position, which contain the most reactive C–H bonds in the molecule. This contrasts with the oxidation of toluene and anisole which occurs predominantly at the ortho C–H bond on the aromatic ring. While the oxidation of the more rigid styrenes was less active than their alkylbenzene equivalents, both resulted in the formation of a major single product arising from epoxidation of the double bond. In addition to being more active and tightly coupled the oxidation of n-propylbenzene and trans-β-methylstyrene were more stereoselective than those of ethylbenzene and styrene. The longer alkyl or vinyl side chain must modify the binding orientation to place one face of the molecule significantly closer than the other and in a more favourable position for efficient oxidation. Alternatively the smaller substrates may be more mobile in the active site and bind in multiple orientations which results in the decrease in stereoselectivity and coupling efficiency. It is of note that mutating the Thr438 residue to Phe, which would decrease the size of the active site, improves the enantioselectivity of P450Bm3 styrene oxidation to give (R)-styrene oxide at 64% ee.[17]

As observed previously the regioselectivity of the oxidation reactions were predominantly unchanged. The largest deviation in the regioselectivity was observed with ethylbenzene where oxidation at the benzylic C-H bond (as opposed to the ortho aromatic site) varied from 82-94%. This infers that the substrates must be positioned in similar orientations in the enzyme active site in the presence of the decoy molecules. Changes were observed on the stereoselectivity of oxidation on using the rate accelerating mutants and the addition of the decoy molecules. The decoy molecules caused some turnovers to be slightly more stereoselective while the rate accelerating variants sometimes decreased the enantioselectivity. However the variation in the enantioselectivity for each product was generally small with the smaller substrates, ethylbenzene and styrene, showing greater changes. With trans-β-methylstyrene no substantial changes were observed in the enantioselectivity across the turnovers.
Overall these observations on the relative amounts of each enantiomer show that while the generic rate accelerator mutants and the decoy molecules do not seem to alter the binding orientation of the substrate enough to modify the regioselectivity of the reactions, they can induce changes in the enantioselectivity. This must arise from shift in the location of the molecule in the active site relative to the reactive iron-oxygen species. Chiral decoy molecules, which have been tested with P450 peroxxygenase enzymes, may have the potential to be used for generate larger changes in the enantioselectivity of the products.\footnote{21
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Conclusions

The improved activity and efficiency shows that the decoy molecule combined with rate accelerator variants have the potential to improve the productivity of regio- and stereo-selective biocatalysis reactions. In some instances the enantiomeric excess was improved by the use of the decoy molecule and this could therefore be used as a strategy to improve the stereoselectivity of C–H bond oxidations or alkene epoxidations. The combination of a rate accelerating mutant and a decoy molecule could also be used in conjunction with other active site mutations which are known to reverse the enantioselectivity of certain reactions.

Experimental Section

General

Production and purification of full-length P450Bnm3 variants for in vitro use was carried out as described previously.\footnote{22,23} General reagents and organics were from Sigma-Aldrich, TCI, Acros or VWR. Suffer components, NADPH, and isopropyl-D-thiogalactopyranoside (IPTG) were from Astral Scientific and Biovectra, (Scimac, Australia). UV/Vis spectroscopy was performed on Varian Cary 5000 or Agilent Cary 60 spectrophotometers. 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. GC and chiral chromatography were performed on a Shimadzu Tracera GC coupled to Barrier discharge Ionization Detector (BID) detector using a RTB-BDExse chiral silica column (Restek; 30 m x 0.32 mm x 0.25 μm) or a Supelcowax column (30 m x 0.32 mm x 0.25 μm) and helium as the carrier gas. Retention times and methods are given in supplementary material (Table S2).

Activity assays

NADPH turnovers were run in 1200 μL of 50 mM oxygenated Tris, pH 7.4 at 30 °C, containing 0.2 μM enzyme and 120 μg bovine liver catalase. Assays were held at 30 °C for 1 min prior to the addition of the decoy molecule (100 μM) and the substrate (1 mM substrate from a 100 mM stock in DMDSO). Finally NADPH was added, from a 20 mg mL⁻¹ stock solution in DMSO. The reaction was allowed to elapse after NADPH addition before the 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 E₅₃₀ = 6.22 mM⁻¹ cm⁻¹.

Product analysis

After the NADPH consumption assays were completed, 990 μL of the reaction mixture was mixed with 10 μL of an internal standard solution (trans-4-phenyl-3-buten-2-one or p-cresol, 20 mM stock solution in DMDSO). 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 identified by coelution with authentic product standards or matching the GC-MS mass spectra to those expected for the standards (supplementary material). Products were quantitated using standards using the assumption that isomeric products would give comparable responses e.g. 1-phenylethanol (α-Et) and 2-ethylphenol (ο-Et) were presumed to give the same detector response.

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Keywords: Biocatalysis • Cytochrome P450 monoxygenases • epoxidation • C–H bond oxidation • asymmetric catalysis • enzyme catalysis • oxidation

References


P450Bm3 mutants and decoy molecules (PFC) are used to improve the catalytic oxidation of prochiral substrates whilst preserving the regioselectivity. The stereoselectivity was also maintained for larger substrates. The decoy molecules marginally improved the enantioselectivity for smaller substrates.

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