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The effect of decoy molecules on the activity of the P450Bm3 holoenzyme and a heme domain peroxygenase variant

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Highlights:

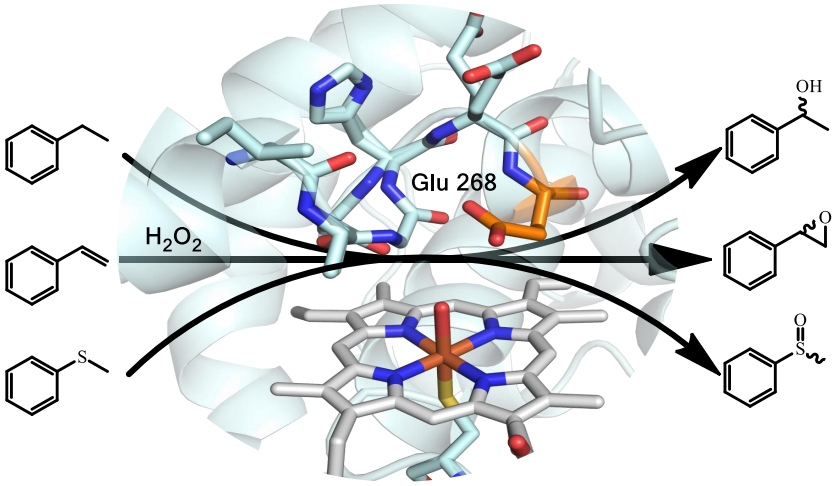
- Decoy molecules were used to increase the productivity of P450 holoenzyme variants
- Hydroxylation and epoxidation reactions were improved dramatically as was sulfoxidation
- A heme domain peroxygenase variant (Thr268Glu) was used to catalyse P450 activity with H_2O_2
- The peroxygensae catalysed sulfoxidation > epoxidation > benzylic C-H hydroxylation
- Decoy molecules did not improve peroxygenase productivity

Abstract

Perfluorinated decoy molecules based on a combination of fatty and amino acids were used to enhance hydroxylation, epoxidation and sulfoxidation reactions of P450Bm3. The combination of amino acid derived second generation decoy molecules, with the rate accelerating variant R19 (R47L/Y51F/H171L/Q307H/N319Y) displayed the highest oxidation rates. Mutation of Thr268 to Glu (Bm3TE) converted the heme domain to a H_2O_2 utilising peroxygenase. This Bm3TE variant displayed significant peroxygenase activity towards all the substrates tested with a preference for methylthiobenzene sulfoxidation. However, the addition of decoy molecules did not improve the efficiency of this variant.

Keywords: cytochrome P450 monooxygenase; hydroxylation; peroxygenase; epoxidation; sulfoxidation; biocatalysis

Graphical Abstract



Abbreviations

P450, cytochrome P450; P450Bm3, CYP102A1 from *Bacillus megaterium*; IPTG, Isopropyl β-D-1-thiogalactopyranoside; BID, Barrier discharge Ionization Detector; WT, wild-type; R19, (R47L/Y51F/H171L/Q307H/N319Y); Bm3TE, Thr268 to Glu variant of the CYP102A1 heme domain; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NADH, reduced nicotinamide adenine dinucleotide; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; PFC, perfluorocarboxcylic acid; PFC9-L-Ala, N-perfluorononanoyl-L-alanine; PFR, product formation rate.

1. Introduction

Cytochromes P450 (P450s) are a family of enzymes that carry out oxidative transformations including hydroxylations, epoxidations, sulfoxidation and other more complex reactions.[1] As a result these enzymes offer advantages over traditional methods of synthesis for carbon-hydrogen bond hydroxylation in that the reaction occurs with high regio- and stereoselectivity in a single step under mild conditions.[2] Most P450s catalyse C–H bond hydroxylation via a high-valent iron-oxo radical cation intermediate which abstracts a hydrogen from the substrate before undergoing an oxygen rebound step, labelled the radical rebound mechanism.[3, 4] The key steps of the catalytic cycle are, in order, substrate binding, first electron transfer, dioxygen binding, activation of the oxygen by delivery of a second electron and proton delivery to enable O–O bond cleavage to generate Cpd I (Figure 1). The electrons are sourced from a nicotinamide cofactor (NADH or NADPH) and delivered via specific electron transfer partners. [5] These reducing cofactors are expensive and many attempts have been made to replace them with simpler alternatives or to do away the requirement for electron transfer proteins by using hydrogen peroxide (H_2O_2) as a source of oxygen via a shunt mechanism (Figure 1).[6-12]

The cytochrome P450Bm3 from *Bacillus megaterium* rapidly oxidises long chain fatty acid substrates (C12 to C16) close to the omega terminus.[13, 14] It, along with other members of the CYP102A subfamily, is unusual compared to other bacterial counterparts in that it is fused to a domain which contains the organic electron transfer cofactors FAD and FMN. This results in the enzyme being self-sufficient in that it requires no other external proteins.[14] P450Bm3 is soluble, easy to produce and sources its electrons from NADPH over NADH.[15] It has been adapted for the selective hydroxylation of a broad range of substrates through rational protein engineering.[16, 17] Directed evolution has also been used to expand the substrate range of P450Bm3 as well as increase its thermostability and to improve its activity with H_2O_2.[6, 14, 18, 19] In addition it has recently been modified to enable to it to carry out non-physiological P450 chemistry, for example isoforms

which enable the cyclopropanation, amination and aziridination of various substrates have been reported.[20]

These directed evolution studies have highlighted that P450Bm3 can be enhanced by making alterations outside of the active site. These mutations alter the conformation of the enzyme to enhance its activity but do not alter the active site architecture and so maintain the product regio-selectivity, of the WT enzyme.[21-24] The conformations adopted by these variants more closely resembles those of the fatty acid bound form of the enzyme and lengthens the heme-iron axial water bond.[22, 24] One example of such a rate accelerating mutant is the R47L/Y51F/H171L/Q307H/N319Y (R19) variant of P450Bm3, which enhanced the oxidation of hydrophobic unnatural substrates.[25, 26]

Chemically inert decoy molecules have also been used to improve the activity of P450Bm3.[27] These promote the oxidation of unnatural substrates such as benzenes, xylenes and short chain alkanes by the wild-type (WT) enzyme.[26-29] They work as the fluorinated alkyl chain of the decoy molecule is used to fill the substrate access channel of the enzyme and this leads to conformational changes. However, they are short enough that sufficient space remains in the active site of the enzyme over the heme to allow a substrate to bind.[29] One consequence of this is, as with the rate accelerating mutants described above, the regioselectivity of oxidation is largely unaffected. We have shown that it is possible to use decoy molecules, in conjugation with the rate accelerating mutants of P450Bm3 to significantly enhance the rates of product formation for cycloalkane and benzene-derived substrates and improve the productivity of regio- and stereo-selective biocatalysis reactions.[25, 26]

More recently, inspired by the H_2O_2 utilising CYP152 family of enzymes including CYP152A1 (P450BSβ) [30], CYP152A2 (P450CLA) [31] and CYP152B1 (P450SPα) [32], a single mutation at threonine 268 was introduced to alter the activity of the P450Bm3 heme domain. Threonine 268 is part of the highly conserved acid-alcohol pair used to activate dioxygen. Replacing

the threonine alcohol side chain with the acidic glutamate residue allowed the heme domain of P450Bm3 to hydroxylate fatty acids using hydrogen peroxide as the oxidant [33].

For the first time we show that the combination of second generation decoy molecules and mutated variants of the holoenzyme result in large increases in the efficiency of benzylic hydroxylation, epoxidation and sulfoxidation reactions. We also demonstrate that the threonine 268 to glutamate heme domain variant (Bm3TE) can catalyse these reactions using hydrogen peroxide but that no improvement in oxidation is observed when the decoy molecules are added.

2. Experimental

2.1 General

General reagents and organics were purchased from Sigma-Aldrich, Tokyo Chemical Industry, Chem-Supply or Fluorochem. Isopropyl-β-D-thiogalactopyranoside (IPTG) and buffer components were obtained from Astal Scientific (Australia). UV/Vis spectroscopy was performed on an Agilent Cary 60 spectrophotometer. Gas chromatography was carried out on a Shimadzu Tracera GC with Barrier discharge Ionization Detector (BID) detector using an Rt-βDEXse column (30m × 0.32mm × 0.25 um). Gas Chromatography-Mass Spectrometry was carried out on a Shimadzu GC-2010 with GC–MS-QP2010S detector. The GC methods are provided in the Supplemental section.

2.2 Protein expression and purification

Plasmids, pT7 containing the gene encoding the P450Bm3 heme domain Thr268Glu mutant (Bm3TE) or pET28 with the WT and R19 holoenzyme genes, were transformed into *E. coli* BL21 (DE3) competent cells and grown on LB plate in the presence of ampicillin or kanamycin, respectively [21, 33]. A single colony was added to 500 mL of LB media containing trace elements solution ($CaCl_2$, $ZnSO_4.7H_2O$, $MnSO_4.H_2O$, Na_2-EDTA, $FeCl_3.6H_2O$, $CuSO_4.5H_2O$, and $CoCl_2.6H_2O$) in presence of the relevant antibiotic and incubated at 37 °C and 110 rpm. After 14 h incubation the temperature was lowered to 18 °C followed by the addition of 0.02 % v/v benzyl alcohol and 2 % v/v ethanol after 30 min. IPTG (100 μM) was then added to induce protein expression. Cells were harvested after 24 h by centrifugation (5000 g, 10 min, 4 °C) and resuspended in 50 mM buffer pH 7.4 (Tris buffer for Bm3TE and phosphate buffer for holoenzymes; both contained 1 mM DTT). The cells were lysed by sonication on ice for 30 min (20 s on, 40 s off) and cell debris was removed by centrifugation (37000 g, 20 min, 4 °C). The supernatant was loaded onto a DEAE Sepharose column (XK50, 200 mm × 40 mm; GE Healthcare) and eluted using a salt linear gradient (100 - 400 mM KCl in Tris buffer for Bm3TE and 80 - 400 mM $(NH_4)_2SO_4$ in phosphate

buffer for holoenzymes). The red coloured fractions which contain the P450 enzyme were combined and concentrated by ultrafiltration (1900 g, 4 °C) using 10 and 30 kDa membranes for Bm3TE and holoenzymes, respectively, (Vivacell 100, Sartorius). Concentrated protein was desalted using a Sephadex G-25 medium grain column (250 mm × 40 mm; GE Healthcare). This was concentrated by ultrafiltration as described above to approximately 10 ml before being loaded onto a Source-Q ion-exchange column (XK26, 80 mm × 30 mm; GE Healthcare). The proteins were purified on an AKTA purifier (GE Healthcare) and eluted using a salt gradient (100 - 400 mM KCl in Tris buffer for Bm3TE and 0 - 35% 16 × phosphate buffer for Bm3 holoenzymes). Fractions with A_{418}/A_{280} > 0.5 (for both holoenzymes and Bm3TE variant) were collected and concentrated, afterwards an equivalent volume of 80% glycerol was added to the protein and filter sterilised before storage at -20 °C.

2.3 Activity assays

The peroxygenase turnover assays for the Bm3TE variant were run in 1 ml Tris buffer (pH 7.4, 100 mM) at room temperature containing 3 μM enzyme. Substrate (5 mM) and decoy molecule (100 μM if present) were added. The reaction was started by addition of 60 mM H_2O_2 and quenched after 5 min by addition of 400 μl ethyl acetate. The NADPH monnoxygenase turnover assays on the holoenzymes were carried out as reported previously (see Supplemental section).[25, 34]

2.4 Product analysis

After the NADPH consumption or hydrogen peroxide 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). These mixtures were 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 GC-coelution experiments and matching the GC-MS spectra to those of the products (styrene oxide, 1-phenylethanol and methyl phenylsulfoxide; see supplementary material). Products were calibrated

against authentic product standards using the assumption that isomeric products would give comparable responses e.g. phenylacetaldehyde and styrene oxide were presumed to give the same detector response.

3. Results

3.1 P450Bm3 catalysed oxidation of ethylbenzene, styrene and methylthiobenzene

The rates of NADPH oxidation, product formation and the coupling efficiency for different P450Bm3 variants, in the presence and absence of different polyfluorinated carboxylic acids (PFCs), were obtained for ethylbenzene, styrene and methylthiobenzene oxidation (Table 1). All the combinations tested gave rise to a single major oxidation metabolite which could be identified by GC-MS coelution experiments with authentic product standards (Scheme 1). In turnovers of styrene, there was an additional minor product which coeluted with and had a MS spectra consistent of an aldehyde (<11%) arising from a rearrangement reaction (phenylacetaldehyde, Scheme 1). There was also a small amount of a sulfone further oxidation product (<15%) in the turnovers of methylthiobenzene. In turnovers of ethylbenzene two minor products were observed and were assigned as acetophenone and 2-ethylphenol (Scheme 1).[25]

The activity of WT Bm3 for oxidation of all three substrates, as measured by the product formation rate, was low (PFR; ≤4 nmol (nmol-CYP)$^{-1}$min^{-1}; henceforth abbreviated to min^{-1}). In line with what has been reported previously for ethylbenzene and styrene the addition of first generation decoy molecules perfluorononanoic acid (PFC9) and perfluorodecanoic acid (PFC10) improved the activity (ranging from 17- to 214-fold) with all the substrates, while the regio- and stereo-selectivity was predominantly maintained (Table 1).[25, 34] Based on these previous studies, PFC9 and PFC10 were the optimal compounds in the first generation of decoy molecules, therefore longer and shorter PFCs were not examined [34].

The rate accelerating variant, R19 increased the activity of substrate oxidation over the WT enzyme (Table 1) and its combination with a first generation decoy molecules generated even higher product formation activities (466 to 1160 min^{-1} an up to 3-fold improvement over the R19 variant alone; Table 1, Figure S1). The 725-fold improvement in PFR of the WT enzyme observed with styrene oxidation by the R19 variant in combination with PFC10 was greater than those observed

for ethylbenzene and methylthiobenzene (271- and 155-fold, respectively). The regioselectivity of R19-catalysed oxidation of all substrates in the presence or absence of the decoy molecules were similar to those of the WT enzyme (Scheme 1). There were no significant changes in the stereoselectivity of styrene and methylthiobenzene oxidation. However, the R19 variant was less selective for the *(R)*-enantiomer of 1-phenylethanol compared to the WT enzyme with the enantiomeric excess (*ee*) dropping from 48-62 to 16-30% across the turnovers.

Second generation decoy molecules have been developed to explore the effect of amino acid derived compounds for small molecule oxidation by P450Bm3.[29] The oxidation activity of the P450Bm3 WT and R19 variants were tested in presence of PFC9-L-Ala, PFC9-L-Leu and PFC9-L-Phe (N-perfluorononanoyl-L-alanine, N-perfluorononanoyl-L-leucine and N-perfluorononanoyl-L-phenyalanine, see supplementary section for structures). The oxidation rates in turnovers of ethylbenzene and methylthiobenzene were found to be the greatest when the PFC9-L-Phe decoy molecule was added to the WT enzyme (Table1, Figure S1). This resulted in a 248-fold improvement with ethylbenzene (PFR 962 ± 32 min^{-1}) and a 146-fold enhancement with methylthiobenzene (PFR 502 ± 21 min^{-1}). In terms of styrene oxidation, the highest production rate with the WT was observed with addition of PFC9-L-Leu or PFC9-L-Ala (both had a PFR of 255 min^{-1}, 159-fold improvement over the WT by itself). As with the first generation decoys, the addition of these second generation molecules to the WT enzyme resulted in small changes in the stereoselectivity of the overall reactions (Table 1, Figure S2). For example, that of ethylbenzene benzylic hydroxylation was slightly reduced (Table 1).

The combination of the second generation of decoy molecules with the R19 variant was also studied. All three substrates were oxidised with their maximum product formation rates (PFR) with R19/PFC9-L-Leu or R19/PFC9-L-Ala. This arose predominantly due to a superior NADPH rate with little or no increase in the coupling efficiency over the R19 variant alone or the WT enzyme with the decoy molecules. The greatest PFR was found for the PFC9-L-Leu/R19 combination for

the oxidation reaction with ethylbenzene (PFR 1860 ± 100 $min^{-1;}$ 490-fold improvement over WT alone). For styrene epoxidation PFC9-L-Ala/R19 was the optimal combination (PFR 1580 ± 100 min^{-1}; 980-fold improvement). The coupling efficiency of methylthiobenzene oxidation was not enhanced to the same degree as the other two substrates but its oxidation rate increased 300-fold with the PFC9-L-Leu/R19 combination to yield a PFR of 905 ± 11 min^{-1}. As with the other decoy molecule turnovers the combination of the second generation of decoy molecules maintained the regio- and stereo-selectivity of the turnovers (Table 1).

3.2. Peroxygenase activity of the Thr268Glu variant of the heme domain of P450Bm3

The single mutation of threonine 268 to glutamic acid converts the heme domain of P450Bm3 (Bm3TE) into a H_2O_2 utilizing peroxygenase variant.[33] The activity of Bm3TE variant was tested for the oxidation of ethylbenzene, styrene, and methylthiobenzene. The P450Bm3 holoenzyme displayed little peroxygenase activity with these substrates (Figure 2). With styrene and ethylbenzene the Bm3TE variant generated 640 nmoles of styrene oxide and 199 nmoles of α-EtOH after addition of H_2O_2. Analysis of the methylthiobenzene turnovers was more complex as H_2O_2 by itself can lead to sulfoxidation. However in the presence of the Bm3TE variant a significant increase in sulfoxidation was observed. The oxidation of methylthiobenzene produced of 914 nmols of methyl phenyl sulfoxide (versus 80 nmols in the no P450 control). In these peroxygenase turnovers the addition of the first or the second generation of decoy molecules showed little to no improvement on the oxidation of the substrates. For styrene and ethylbenzene the highest productivity was observed in the absence of decoy molecules and significant decreases in the amount of product were found when the second generation decoy molecules were used. Small increases in product formation were observed in certain instances with methylthiobenzene (Table S1).

While the regioselectivity of the peroxygenase turnovers were similar to those of the holoenzyme monoxygenase activities differences in metabolite formation were observed (Scheme

1). In the turnovers of ethylbenzene with Bm3TE variant 15% of the further oxidation product acetophenone was detected which was higher than the monooxygenase reactions (0-4%; Scheme 1). Changes were observed in the stereoselectivity of the peroxygenase reactions. Oxidation of styrene with Bm3TE was less in favor of the *R*-enantiomer (10-20% *ee*) compared to the holoenzymes (15-32%). The stereo-selectivity on oxidation of methylthiobenzene with T268E variant towards *R* enantiomer increased up to 20% (Table S1). The most dramatic change was observed for the hydroxylation of ethylbenzene where the peroxygenase reactions favoured the (*S*)-enantiomer in contrast to the holoenzyme monooxygenase turnovers which were more (*R)*-selective (Figure 3).

4. Discussion

The rates of product formation for ethylbenzene, styrene and methylthiobenzene oxidation with P450Bm3 holoenzymes were significantly increased by using decoy molecules. The highest activities were observed with the second generation of decoy molecules with the R19 variant. The regioselectivity and the stereoselectivity was maintained across both variants in the presence of the decoy molecules.

The Bm3TE heme domain variant was capable of oxidising all of the substrates using H_2O_2 instead of molecular dioxygen and NADPH. This variant was more active for the sulfoxidation of methylthiobenzene over the epoxidation of styrene and benzylic hydroxylation of ethylbenzene. This contrasted with the holoenzyme turnovers which were most effective for ethylbenzene oxidation suggesting a potential change in the rate determining step for these reactions. The coupling efficiency of methylthiobenzene sulfoxidation, the most reactive functional group to be tested, was lowest for the monooxygenase turnovers using the holoenzyme. The addition of decoy molecules induced no improvement in the oxidation efficiency of Bm3TE. This infers that there may be a change in the rate determining step and the pathway taken during the hydrogen peroxide driven turnovers is different from that of the monoxygenase catalytic cycle of the holoenzyme. It is hypothesised that the decoy molecules enhance the activity by binding to the enzyme together with the substrate overcoming the substrate gate, inducing a conformation change which triggers the first electron transfer, subsequent oxygen binding and faster turnover of the catalytic cycle. The second generation of decoy molecules have been shown to bind tightly to P450Bm3 and to alter the conformation of the enzyme. The crystal structure of PFC9-L-Trp-bound P45Bm3 highlights modification in the position of F and G helices, the G/F loop and the I helix.[29]

The switch in in the stereoselectivity of ethylbenzene hydroxylation towards the *(S)*-enantiomer for the peroxygenase mutant compared to the monooxygenase turnovers of the holoenzyme is intriguing. This change could be due to a change the binding orientation of the

substrate in the mutant, though the regioselectivity of the reaction was not altered. In addition minimal changes were observed for the epoxidation of styrene and sulfoxidation of methylthiobenzene inferring their positions relative to the heme iron may not be altered. The double bond in styrene may provide a more rigid structure leading to less opportunity for movement.

Another potential explanation for the modified metabolite distribution between the peroxygenase and the holoenzyme could be that there may be a change in the relative proportion of putative reactive oxygen-bound heme species in the peroxygenase turnovers of the Bm3TE variant. Sulfoxidation has been proposed to be able to occur via a hydrogen peroxide bound heme intermediate and if this is the case could be a reason as to why methylthiobenzene is the optimal substrate for the peroxygenase variant.[35] In contrast benzylic hydroxylations are hypothesised to proceed via radical rebound reaction with Cpd I are not as favoured. However further work would be required to fully understand these differences.

Conclusion

The results highlight the important role that decoy molecules can play in accelerating the productive oxidation of non-physiological substrates by P450Bm3. They also back up the hypothesis that these decoy molecules work by inducing conformational changes which boost the monooxygenase catalytic cycle by enhancing the rate of electron transfer. The peroxygenase variant could also efficiently oxidise the substrates. Further optimisation of this system could result in simpler and larger scale green biocatalytic oxidation processes.

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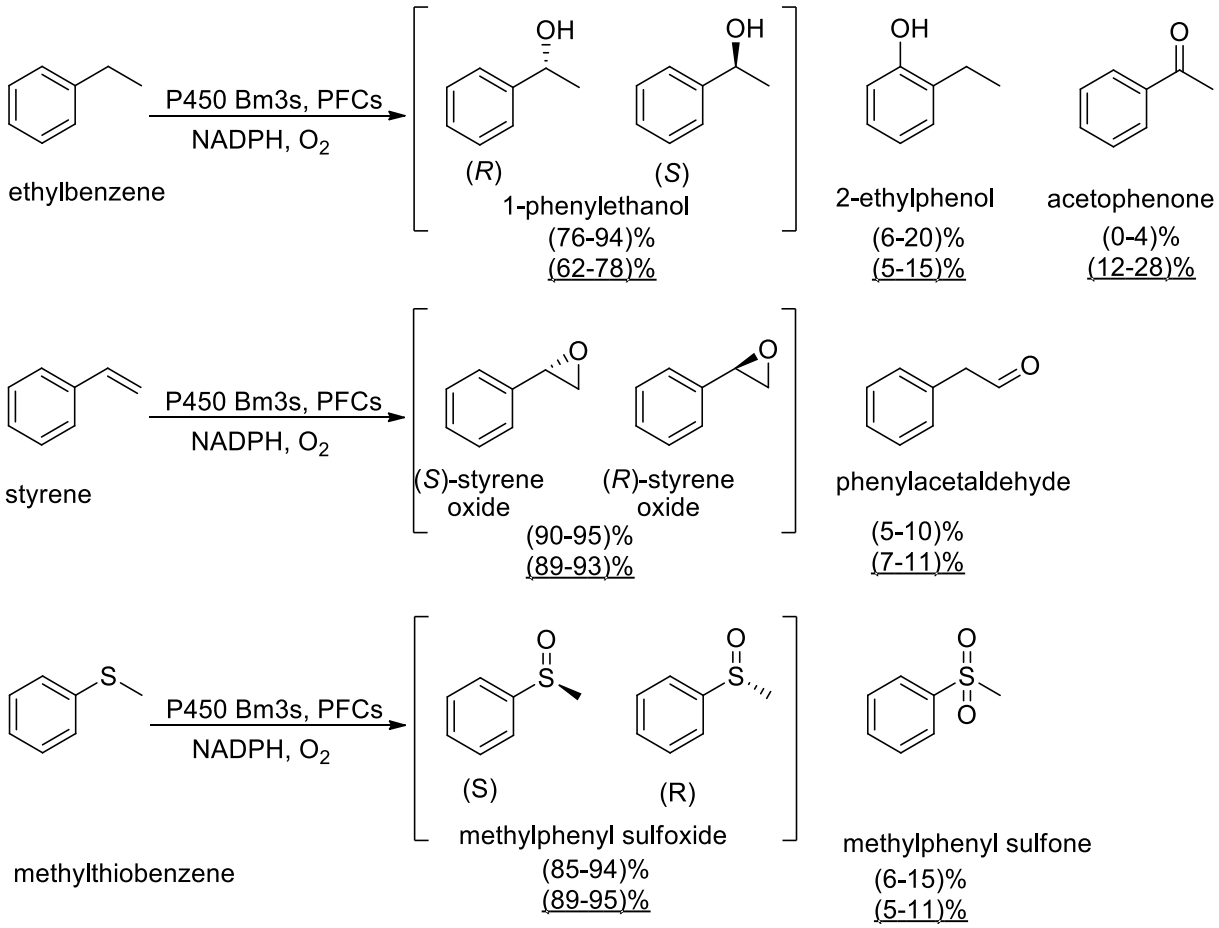
Tables

Table 1. Enzyme turnover and coupling efficiency data for WT and the R19 variant of P450Bm3 with ethylbenzene, styrene and methylthiobenzene. Activities are expressed as the NADPH oxidation rate (N) and were measured using a concentration of 0.2 μM CYP enzyme, 50 mM Tris, pH 7.4). The coupling efficiency (C) is the percentage efficiency of NADPH utilization for the formation of organic products. N and the product formation rates (PFR) are reported as mean ± S.D. ($n \geq 3$) and given in nmol(nmol-CYP)$^{-1}$min^{-1}. [a] These results are in line with what was reported previously for these substrate/enzyme/decoy combinations.[25]

	ethylbenzene			
	N	**C**	**PFR**	***ee** (R)*
WT[a]	71 ± 4	5.3 ± 1.5	3.8 ± 1.2	48
WT/PFC9[a]	1106 ± 19	74 ± 2	814 ± 33	56
WT/PFC10[a]	1311 ± 13	49 ± 2	645 ± 33	62
WT/PFC9-Phe	1560 ± 8	61 ± 2	962 ± 32	39
WT/PFC9-Leu	1040 ± 24	58± 2	563 ± 13	28
WT/PFC9-Ala	947 ± 17	51 ± 1	482 ± 4	38
R19[a]	702	52	366	16
R19/PFC10[a]	1460	71	1030	30
R19/PFC9-Phe	2240 ± 85	61 ± 3	1420 ± 26	12
R19/PFC9-Leu	2830 ± 69	66 ± 6	1860 ± 100	12
R19/PFC9-Ala	2760 ± 96	63 ± 5	1720 ± 180	20
	styrene			
	N	**C**	**PFR**	***ee** (R)*
WT[a]	41 ± 1	4.0 ± 0.6	1.6 ± 0.2	18
WT/PFC9[a]	457 ± 11	32 ± 1	146 ± 4	32
WT/PFC10[a]	691 ± 7	17 ± 1	117 ± 7	32
WT/PFC9-Phe	932 ± 25	21 ± 3	194 ± 28	15
WT/PFC9-Leu	665 ± 10	38 ± 5	255 ± 31	18
WT/PFC9-Ala	601 ± 14	43± 4	255 ± 14	18
R19[a]	976	63	611	22
R19/PFC10[a]	1530	76	1160	28
R19/P9-Phe	1620 ± 20	63 ± 4	1030 ± 70	18
R19/P9-Leu	2210 ± 130	67 ± 3	1480 ± 20	18
R19/P9-Ala	2020 ± 65	78 ± 1	1580 ± 42	26
	methylthiobenzene			
	N	**C**	**PFR**	***ee** (R)*
WT[	39 ± 1.4	12 ± 2	3 ± 1	2
WT/PFC10	267 ± 18	22 ± 3	58 ± 6.6	0
WT/PFC9-Phe	1770 ± 18	28 ± 1	502 ± 21	1
WT/PFC9-Leu	1410 ± 23	30 ± 2	423 ± 24	0
WT/PFC9-Ala	934 ± 31	23 ± 1	213 ± 13	10
R19	1060 ± 75	31 ± 2	317 ± 15	0
R19/PFC10	1440 ± 40	32 ± 2	466 ± 27	0
R19/P9-Phe	2080 ± 14	32 ± 1	660 ± 29	0
R19/P9-Leu	2880 ± 62	32 ± 0.5	905 ± 11	0
R19/P9-Ala	2330 ± 3.5	36 ± 3	831 ± 72	2

Figures



Scheme 1. Product distributions for the catalysed oxidation of ethylbenzene, styrene and methylthiobenzene in the presence and absence of decoy molecules. The products from ethylbenzene: 1-phenylethanol and 2-ethylphenol, from styrene: styrene oxide and phenylacetaldehyde and from methylthiobenzene: methylphenyl sulfoxide and methylphenyl sulfone. Values in in black represent the percentage of products with WT and R19 variant. The values obtained with the Bm3TE variant are underlined.

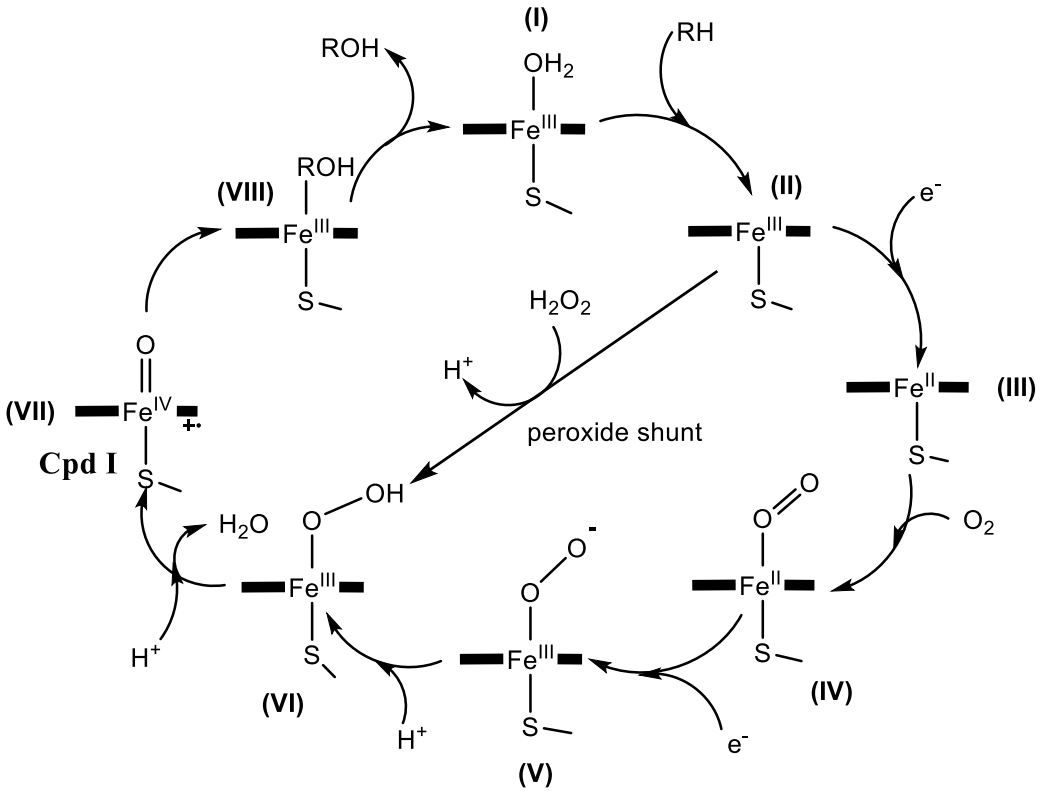


Figure 1. Catalytic cycle of P450 monooxygenases including the H_2O_2 shunt pathway

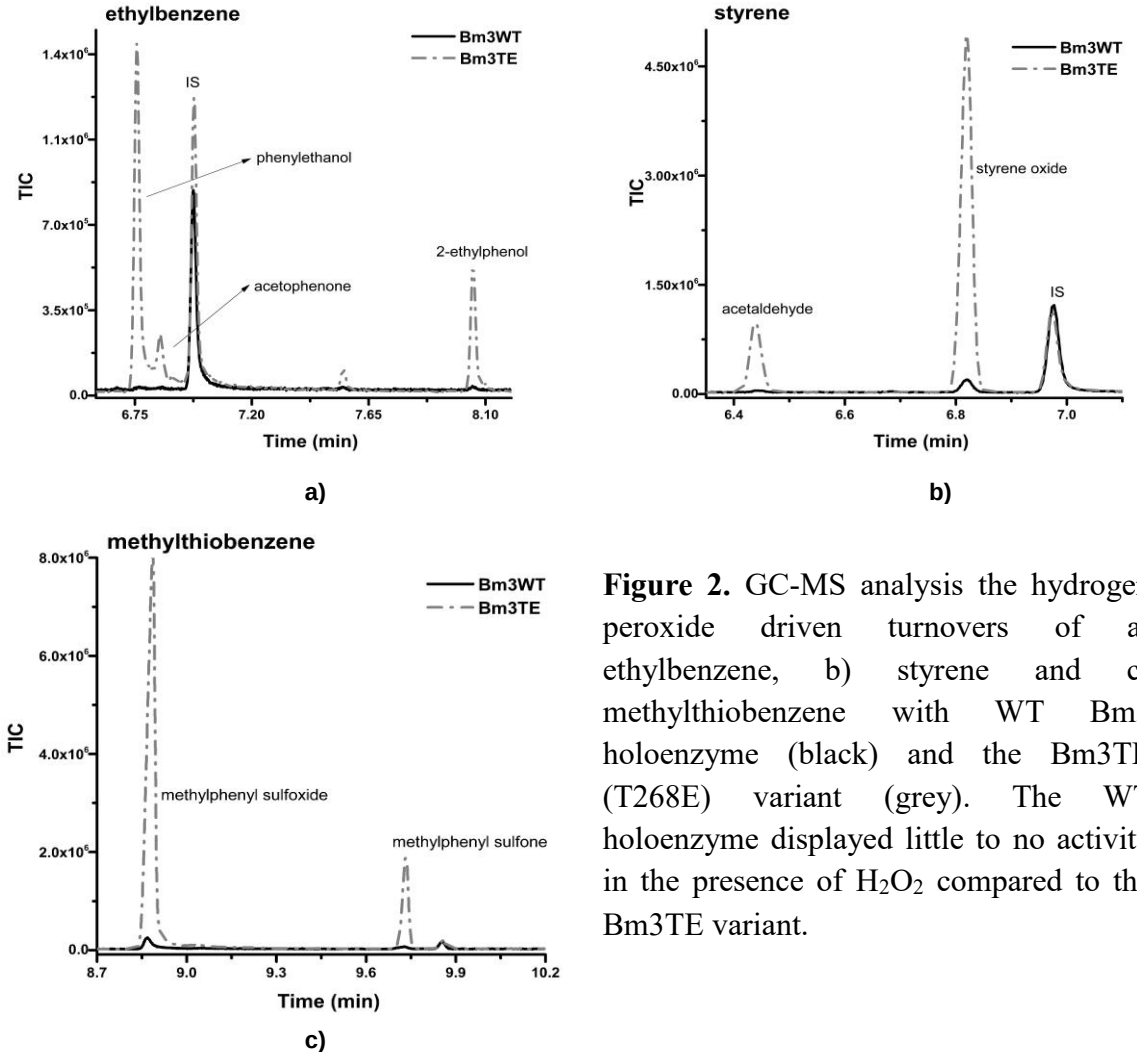


Figure 2. GC-MS analysis the hydrogen peroxide driven turnovers of a) ethylbenzene, b) styrene and c) methylthiobenzene with WT Bm3 holoenzyme (black) and the Bm3TE (T268E) variant (grey). The WT holoenzyme displayed little to no activity in the presence of H_2O_2 compared to the Bm3TE variant.

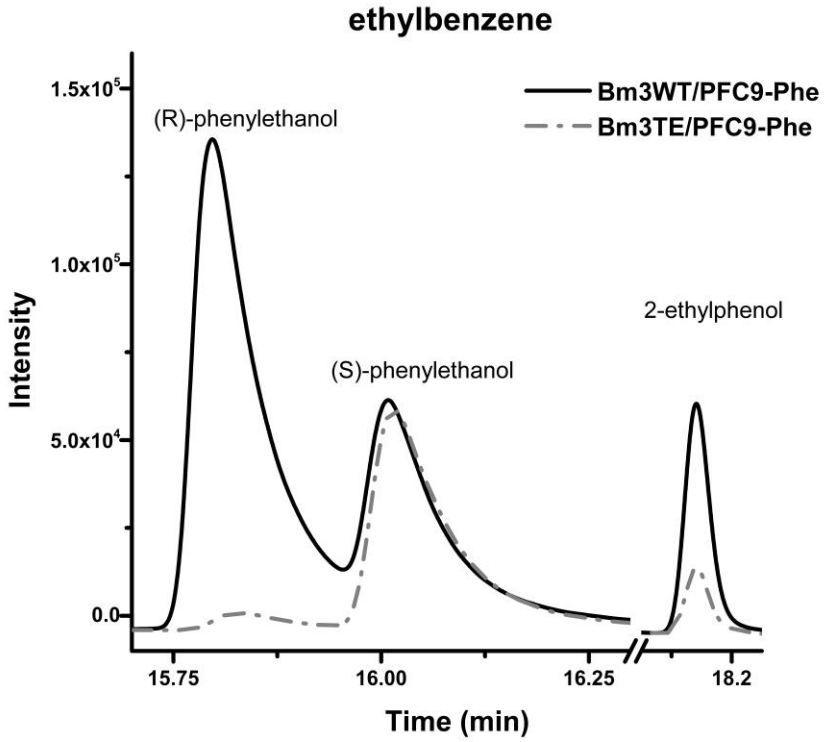


Figure 3. Chiral GC analysis of ethylbenzene oxidation illustrating a change in stereo-selectivity of the Bm3TE variant versus the holoenzymes for 1-phenylethanol. While WT and R19 variant are more (*R*) selective the Bm3TE variant is more (*S*) selective.

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