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Control of Stereoselectivity of Benzylic Hydroxylation Catalysed by Wild-Type Cytochrome P450BM3 Using Decoy Molecules

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The hydroxylation of non-native substrates catalysed by wild-type P450BM3 is reported, wherein "decoy molecules" , i.e., native substrate mimics, controlled the stereoselectivity of hydroxylation reactions. We employed decoy molecules with diverse structures, resulting in either a significant improvement of enantioselectivity or clear inversion of stereoselectivity in the benzylic hydroxylation of alkylbenzenes and cycloalkylbenzenes. For example, supplementation of wild-type P450BM3 with 5-cyclohexylvaleric acid-L-phenylalanine (5CHVA-Phe) and Z-proline-L-phenylalanine yielded 53% (*R*) ee and 56% (*S*) ee for indane hydroxylation, respectively, although 16% (*S*) ee was still observed in the absence of any additives. Moreover, we performed a successful crystal structure analysis of 5CHVA-L-tryptophan-bound P450BM3 at 2.00 Å, which suggests that the changes in selectivity observed were caused by conformational changes in the enzyme induced by binding of the decoy molecules.

Introduction

The cytochrome P450 (P450) family is a superfamily of haem-containing enzymes that are involved in the processes of metabolism of toxic compounds and the synthesis of bioactive substances in living cells. As most P450s catalyse the oxyfunctionalisation of organic substrates, they have been extensively studied as promising oxygenation biocatalysts for hydroxylation and epoxidation reactions.[1, 2] CYP102A1 (P450BM3) from *Bacillus megaterium* is a self-sufficient P450, wherein the haem domain is fused to the reductase domain expressed on a single peptide chain, thus facilitating efficient electron transfer from its coenzyme, NADPH.[3] This structural feature enables P450BM3 to catalyse the highly efficient hydroxylation of its native substrates, long-chain fatty acids (C12-C20).[4, 5] Moreover, P450BM3 has been shown to possess some of the highest monooxygenase activities of any P450, rendering it an attractive candidate biocatalyst.[6-8] X-ray structural analysis of palmitoleic acid-bound P450BM3 (PDB: 1FAG)[9] and mutagenic studies[10, 11] revealed that Arg47 and Tyr51 located at the entrance of the substrate access channel of P450BM3 are key residues that mediate the binding of fatty acids, thereby modulating the enzyme's substrate selectivity. Binding of the substrate elicits discoordination of an axial-ligated water molecule from the haem, followed by the formation of the haem active oxygen species (compound I, see Fig. 1 A) and concomitant oxidation of the substrate's sub-terminal ω-1, ω-2 and ω-3 positions.[4] However, the substrate specificity of P450BM3 prevents its activation by non-native substrates other than long-chain fatty acids, thus limiting the enzyme's applicability as a promising catalyst.

The mutagenesis strategies used in the construction of P450BM3 variants successfully improved its catalytic activity for non-native substrates, such as alkanes,[12-14] alkenes[15-17], and aromatics,[15, 18-22] which were converted into their corresponding alcohols or epoxides. Regio- and stereoselective biotransformations by P450BM3 variants have also been widely investigated. Mutation at Ala184 of the F87A/T235A/R471A/E494K/S1024E variant with a positively charged residue led to an inversion of stereoselectivity in styrene epoxidation, even though Ala184 is located distant from the active site of the enzyme.[23] Shehzad *et al.* successfully co-crystallised the mutant with styrene and discussed the role of substitution of Ala184 in the stereoselectivity of styrene epoxidation.[24] Work conducted by Roiban *et al*. employing the combinational active-site saturation test (CAST) achieved the alternation of regio- and stereoselectivity of the hydroxylation

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Electronic Supplementary Information (ESI) available: Experimental section including synthesis of decoy molecules, expression and purification of P450BM3, additional figures of crystal structures, the initial turnover rate of oxidation reactions, docking simulations, GC-MS analysis, and table of data collection and refinement statistics of crystal structures (PDB codes: 5XHJ). See DOI: 10.1039/x0xx00000x

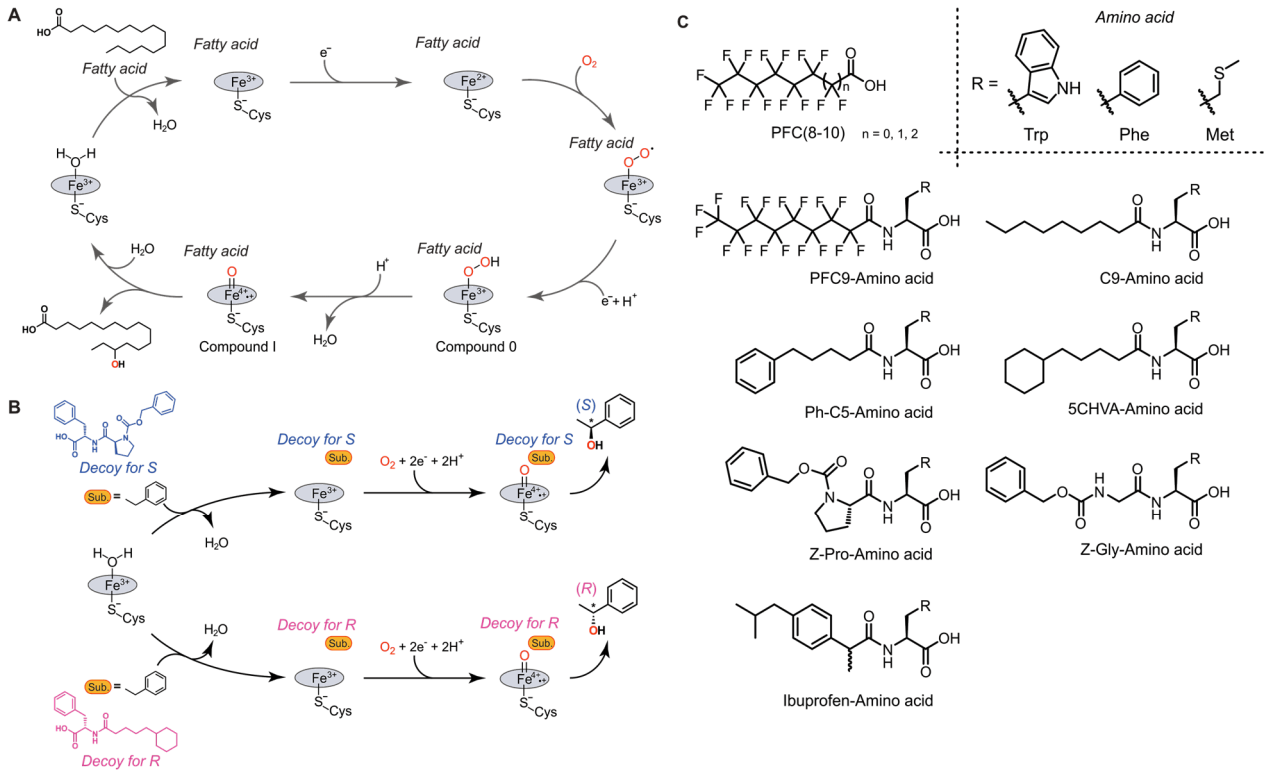


Fig. 1 (A) Catalytic cycle of P450BM3. (B) Concept of this study; the control of stereoselectivity by decoy molecules. Here "Decoy for *S*" triggered (*S*)-selectivity and "Decoy for *R*" triggered (*R*)-selectivity in ethylbenzene hydroxylation. (C) Structures of decoy molecules employed in this study.

of various compounds, such as cyclohexane,[25] ketone[26] and tetralone[27] derivatives.

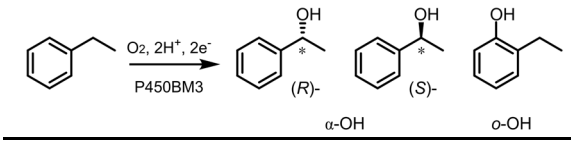
As a novel method to turn P450BM3 into a versatile biocatalyst for the transformation of small organic molecules, a substrate misrecognition system was developed by Zilly *et al*.[28] and our research group,[29] wherein dummy substrates were used to "trick" the enzyme. These dummy substrates have been termed "decoy molecules" by our group[30] and will be indicated henceforth as such. The first reported decoy molecules for P450BM3 were simple perfluorocarboxylic acids (PFCs, Fig. 1 C), which mimicked the structure of the native fatty acid substrates and, thus, similarly accommodated in the enzyme, consequently triggering the initialisation of the catalytic cycle. As PFCs are not oxidised because of the high energy of the C–F bonds, additional substrates, such as propane[28, 29, 31] and benzene,[32] are preferentially hydroxylated by wild-type (WT) P450BM3, which are not catalysed in the absence of decoy molecules. We recently reported that 2nd-generation decoy molecules, i.e., PFCs modified with L-amino acids (Fig. 1 C), significantly enhanced the catalytic activity for propane and ethane hydroxylation, showing that carboxylate modification with amino acids is effective in activating P450BM3.[33] More recently, we have demonstrated that *N*-acyl amino acids, as well as amino acid dipeptides lacking fluorination, such as *N*-nonanoyl-L-phenylalanine (C9-Phe) and Z-L-proline-L-phenylalanine (Z-Pro-Phe) strongly activate P450BM3 for benzene hydroxylation to generate phenol. This result clearly showed that diverse carboxylic acids without the requirement for fluorination could be exploited as building blocks for novel decoy molecules via the modification of their carboxylic acid with amino acids.[34] Herein, as an alternative approach, we explored the stereoselectivity of benzylic hydroxylation of non-native substrates catalysed by WT P450BM3 using a range of structurally diverse decoy molecules (Fig. 1 C).

Results and Discussion

Ethylbenzene Hydroxylation

The structures of the decoy molecules employed in this study are shown in Fig. 1 C. Initially, we investigated the stereoselectivity of benzylic hydroxylation of ethylbenzene catalysed by WT P450BM3 using perfluorinated decoy molecules. The results are presented in Table 1. Full details are listed in the Supporting Information, Table S1. Both in the presence and absence of decoy molecules, ethylbenzene was hydroxylated predominantly at the benzylic position to 1-phenylethanol with mediocre (*R*)-selectively. In addition, a small amount of *o*-hydroxylated product, 2-ethylphenol, was also detected. As expected, the addition of decoy molecules yielded higher turnovers than that observed in their absence. Enantiomeric excess (ee) was moderately affected by the chain length and amino acid modification of the decoy molecules, indicating that decoy-molecule binding affects the location and orientation of ethylbenzene at the enzyme's active site. (*R*)-selectivity was enhanced (57-68% ee) with increasing decoy chain length (PFC8-10), consistent with a previous report by Munday *et al*.[35] Among the three 2nd-generation decoy molecules reported here, PFC9-Phe exhibited the highest turnover (373 min^{-1}) and ee.

Table 1 Hydroxylation of ethylbenzene catalysed by WT P450BM3 in the presence of decoy molecules.[a]



Decoy molecule	Turnover rate [/min/P450]	ee [%] (*R*/*S*)	α-OH [%]	*o*-OH [%]
None	147 ± 11	45 (*R*)	83	17
PFC8	296 ± 13	57 (*R*)	90	10
PFC9	337 ± 9	61 (*R*)	90	10
PFC10	324 ± 23	68 (*R*)	91	9
PFC9-Trp	292 ± 16	45 (*R*)	87	13
PFC9-Phe	373 ± 46	52 (*R*)	89	11
PFC9-Met	297 ± 3	39 (*R*)	85	15
C9-Trp	285 ± 6	40 (*R*)	84	16
C9-Phe	277 ± 24	48 (*R*)	86	14
Ph-C5-Phe	276 ± 11	68 (*R*)	91	9
5CHVA-Trp	408 ± 13	80 (*R*)	92	8
5CHVA-Phe	447 ± 13	85 (*R*)	94	6
(*R*)-Ibu-Phe	321 ± 5	7 (*S*)	77	23
(*S*)-Ibu-Phe	386 ± 17	14 (*R*)	78	22
Z-Gly-Phe	211 ± 9	18 (*R*)	73	27
Z-Pro-Phe	324 ± 9	4 (*S*)	72	28
Z-Pro-Met	239 ± 6	31 (*R*)	76	24

[a] Reaction conditions: 10 mM ethylbenzene, 5 mM NADPH, 100 μM decoy molecule, 100 mM KCl and 0.5 μM P450BM3 in 20 mM Tris-HCl buffer (pH 7.4) at 25°C for 5 min.

Subsequently, to alter stereoselectivity, we employed next-generation decoy molecules that possessed a range of structures. The stereoselectivity of ethylbenzene hydroxylation was largely affected by these decoy molecules (Table 1). The enantioselectivity displayed in the presence of C9-Trp and C9-Phe was similar, indicating that the effect of a straight carbon chain on enantioselectivity is almost identical. Subsequently, we designed a decoy molecule possessing a phenyl ring at the terminal position as we anticipated productive π–π stacking interactions between ethylbenzene and the decoy molecule. As expected, Ph-C5-Phe yielded an enantiomeric excess (68% (*R*)) 20% higher than that of C9-Phe (48% (*R*)). To investigate whether bulky groups are effective in controlling stereoselectivity, a cyclohexyl group was introduced at the terminus of the decoy molecule. 5-Cyclohexylvaleric acid-Phe (5CHVA-Phe) displayed the most prominent improvement of (*R*)-selectivity amongst all of the decoy molecules employed in this study, reaching an ee of 85%. Conversely, 5CHVA-Trp induced a slight decrease in enantioselectivity (80% ee), suggesting that Phe modification is superior to Trp modification regarding (*R*)-selectivity. Furthermore, inversion of stereoselectivity (from (*R*)- to (*S*)-selectivity) was observed when using (*R*)-ibuprofen-Phe ((*R*)-Ibu-Phe) and Z-Pro-Phe as decoy molecules, even though the overall ee was not very high. The fact that (*S*)-Ibu-Phe gave (*R*)-selectivity indicated that the chirality of decoy molecules is also important in influencing the stereoselectivity of hydroxylation by P450BM3. In the presence of Z-Pro-Phe, P450BM3 catalysed the hydroxylation of ethylbenzene with 4% (*S*) ee; in contrast, Z-Pro-Met did not yield (*S*)-selectivity (31% (*R*) ee), indicating that, in addition to the carboxylic acid structure, the type of amino acid modification is important for the design and tweaking of decoy molecules to elicit the desired stereoselectivity. Interestingly, a minor shift in regioselectivity from benzylic to aromatic position was triggered by select decoy molecules. 5CHVA-Phe decreased *o*-hydroxylation to 6%, whereas Z-Pro-Phe increased *o*-hydroxylation to 28%, compared with the 17% recorded in the absence of any decoy molecule. Intriguingly, an apparent correlation between (*R*)- and α-selectivity was established, with a strong correlation coefficient (r = 0.93). More precisely, increased (*R*)-selectivity also yielded improved α-selectivity (Fig. 2). This result suggests that decoy molecules that enhance (*R*)-selectivity may have a tendency to limit the degree of freedom of ethylbenzene in the active site of P450BM3, potentially leading to a decrease in binding modes that favour *o*-hydroxylation. This hypothesis is supported by the reduced active site size observed in the crystal structure of 5CHVA-Trp-bound P450BM3 (details discussed below, see Crystal Structure Analysis and Docking Simulation section, Figs.3 and 4); the conformational change of P450BM3 triggered by the binding of 5CHVA-Trp is assumed to prevent the benzene ring of ethylbenzene from coming close to compound I, thus diminishing *o*-hydroxylation and, in turn, enhancing α-hydroxylation.

Propylbenzene, Indane and Tetralin Hydroxylation

To investigate whether the strategy of using decoy molecules for controlling stereoselectivity is applicable to other substrates, we examined the hydroxylation of propylbenzene, indane and tetralin (Tables 2 and S2). Similar to ethylbenzene,

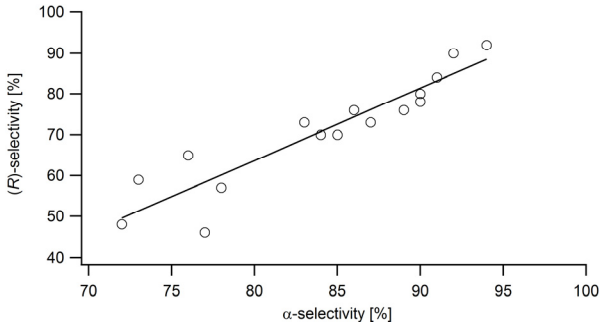
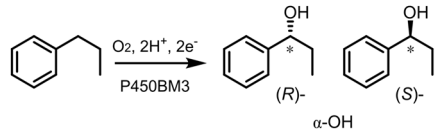
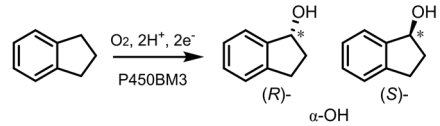
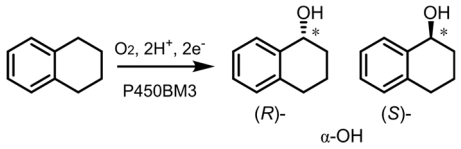


Fig. 2 (*R*)-selectivity [%] vs α-selectivity [%] for ethylbenzene hydroxylation by P450BM3 in the presence/absence of decoy molecules (listed in Table 1). The ratio of (*R*)-selectivity was calculated using the following equation: [(*R*)-enantiomer] / [(*R*)-enantiomer + (*S*)-enantiomer] × 100.

Table 2 Hydroxylation of propylbenzene, indane and tetralin catalysed by WT P450BM3 in the presence of decoy molecules.[a]

Reaction scheme	Decoy molecule	Turnover rate [/min/P450]	ee [%] (*R*/*S*)	α-OH [%]
Propylbenzene 	None	165 ± 8	89 (*R*)	>99
	PFC9-Phe	305 ± 12	94 (*R*)	>99
	5CHVA-Phe	479 ± 15	95 (*R*)	>99
	(*R*)-Ibu-Phe	308 ± 13	86 (*R*)	96
	Z-Pro-Phe	175 ± 13	81 (*R*)	97
Indane 	None	36 ± 2	16 (*S*)	>99
	PFC9-Phe	216 ± 8	6 (*R*)	96
	5CHVA-Phe	435 ± 27	53 (*R*)	97
	(*R*)-Ibu-Phe	314 ± 5	45 (*S*)	90
	Z-Pro-Phe	302 ± 10	56 (*S*)	90
Tetralin 	None	20 ± 1	55 (*S*)	>99
	PFC9-Phe	212 ± 16	69 (*S*)	>99
	5CHVA-Phe	320 ± 31	13 (*S*)	>99
	(*R*)-Ibu-Phe	332 ± 40	89 (*S*)	>99
	Z-Pro-Phe	260 ± 15	96 (*S*)	>99

[a] Reaction conditions: 10 mM substrate, 5 mM NADPH, 100 μM decoy molecule, 100 mM KCl and 0.5 μM P450BM3 in 20 mM Tris-HCl buffer (pH 7.4) at 25°C for 5 min.

these substrates have also been reported to be hydroxylated by P450BM3 at the benzylic position to generate products possessing a chiral centre.[18, 27, 36] An analysis of the reaction profile of these substrates revealed the same general tendencies as those observed for ethylbenzene; 5CHVA-Phe enhanced (*R*)-selectivity, whereas both (*R*)-Ibu-Phe and Z-Pro-Phe enhanced (*S*)-selectivity. Propylbenzene was hydroxylated to (*R*)-1-phenylpropanol with 95% ee when using 5CHVA-Phe, whereas in the absence of decoy molecules, the enzyme still exhibited an ee of 89%. Conversely, Z-Pro-Phe slightly decreased ee to 81%. The changes in enantioselectivity detected for propylbenzene hydroxylation were modest compared with those observed for ethylbenzene hydroxylation, presumably due to the longer alkyl chain, which limited the substrate's degree of freedom of motion in the active site, rendering the active conformation of the substrate less ambiguous. This may serve as an explanation for the observation that differences between individual decoy molecules were less pronounced for propylbenzene hydroxylation.

Both cycloalkylbenzenes, indane and tetralin, were primarily converted to (*S*)-hydroxylated products in the absence of any decoy molecule, with 16% and 55% ee, respectively. Interestingly, an excellent shift in stereoselectivity was observed for indane hydroxylation depending upon the decoy molecule used; 5CHVA-Phe altered stereoselectivity to (*R*) with 53% ee, whilst Z-Pro-Phe enhanced (*S*)-selectivity with 56% ee. A CAST experiment led to the preparation of the A328F variant P450BM3 with 83% (*S*) ee for indane hydroxylation;[27] however, a variant with (*R*)-selectivity has not been reported. We successfully achieved (*R*)-selective hydroxylation by simply adding a decoy molecule to the WT enzyme. In the case of tetralin hydroxylation, although inversion of stereoselectivity was not observed, decoy molecules largely affected the enantioselectivity of the product: 5CHVA-Phe decreased enantioselectivity to 13% ee, whereas Z-Pro-Phe effectively increased (*S*)-selectivity to 96% ee. This is comparable to the 99% (*S*) ee attained using the A328F variant obtained in the CAST study.[27]

Crystal Structure Analysis and Docking Simulation

Motivated by the structural mechanism underpinning the alternation of stereoselectivity, crystallisation of P450BM3 with a decoy molecule containing the 5CHVA moiety was attempted. The crystal structure of P450BM3 bound to 5CHVA-Trp was successfully solved at resolution of 2.00 Å (Fig. 3 A, PDB: 5XHJ). Electron density revealed that 5CHVA-Trp was incorporated in the same manner as *N*-palmitoylglycine,[37]

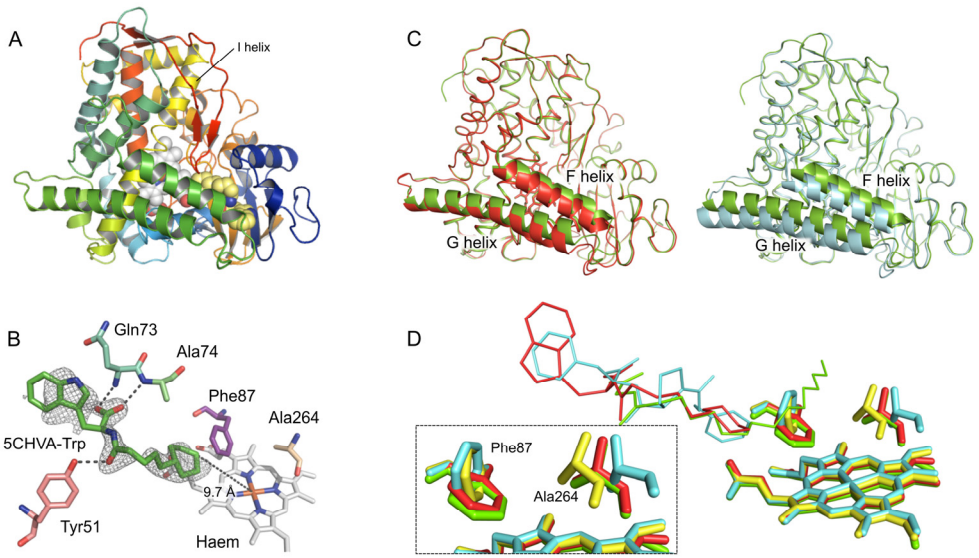


Fig. 3 (A) Overall structure of 5CHVA-Trp bound P450BM3 (PDB: 5XHJ). (B) Binding site of 5CHVA-Trp in P450BM3. The grey mesh represents an $F_o - F_c$ electron density map omitting 5CHVA-Trp contoured at 3.0σ. (C) Overlay of the overall structures of (left) 5CHVA-Trp-bound (red) and *N*-palmitoylglycine-bound (substrate-bound) (green, PDB: 1JPZ) P450BM3 and (right) Z-Pro-Phe-bound (cyan, PDB: 5XA3) and *N*-palmitoylglycine-bound (green) P450BM3. (D) Overlay of the active site structures of 5CHVA-Trp-bound (red), Z-Pro-Phe-bound (cyan), *N*-palmitoylglycine-bound (substrate-bound) (green) and substrate-free (yellow, PDB: 2HPD) P450BM3.

PFC9-Trp[33], and Z-Pro-Phe (PDB: 1JPZ, 3WSP and 5XA3, respectively), with the amino acid carboxylate interacting with the backbone of Gln73 and Ala74, and the carbonyl oxygen atom forming a hydrogen bond with Tyr51 (Fig. 3 B). The distance between the terminal carbon atom of 5CHVA-Trp and haem iron was 9.7 Å, thus creating adequate space to accommodate an additional substrate at the haem distal side. F and G helices and the F/G loop located above the haem resembled the conformation of the substrate-bound (SB) form observed with palmitoleic acid (1FAG)[9] and *N*-palmitoylglycine (1JPZ) (Fig. 3 C, left).[37] This stands in contrast with these helices and loops in 3WSP and 5XA3, which possess structural characteristics between the substrate-free (SF) form (PDB: 2HPD)[38, 39] and the SB form. Intriguingly, in the 5CHVA-Trp-bound form, the Phe87 situated near the active site adopted an orientation that was quasi-parallel to the porphyrin plane (Fig. 3 D). This conformation is consistent with the SB form seen in 1FAG and 1JPZ, while the orientation of Phe87 in 3WSP and 5XA3 was rotated perpendicular to the haem, as seen in the SF form (Fig. 3 D). As revealed by extensive mutagenesis studies[16, 21, 25, 36] and molecular dynamics simulation,[40] Phe87 is the key residue governing regio- and stereoselectivity. Rotation of Phe87 in the SB form, as induced by decoy molecules, may be pivotal in evoking (*R*)-selectivity. This assumption is supported by the improvement of (*R*)-selectivity in ethylbenzene hydroxylation with increasing PFC chain length. Longer chains resemble the native substrate and are more likely to generate an active site architecture akin to the SB form.

To understand the behaviour of non-native substrates in the active site, docking simulations of 5CHVA-Trp- and Z-Pro-Phe-bound P450BM3 with indane were conducted using AutoDock FR.[41] Prior to the simulations, compound I structures were modelled manually into each crystal structure, where the oxygen atom was placed at a distance of 1.65 Å from the iron atom, perpendicularly to the haem plane, as assessed based on the experimental evidence.[42, 43] Docking simulations of indane resulted in the placement of the benzylic hydrogen of indane at a distance of 2.2 Å and 2.9 Å from the oxygen atom of compound I in the structures of the 5CHVA-Trp- and Z-Pro-Phe-bound enzyme, respectively (Fig. 4 B). Interestingly, the benzene ring of indane was docked at different positions, namely L zone and M zone (discussed below). Each simulation gave a reasonable conformation, which corroborated the experimental results; a simulation performed using 5XHJ

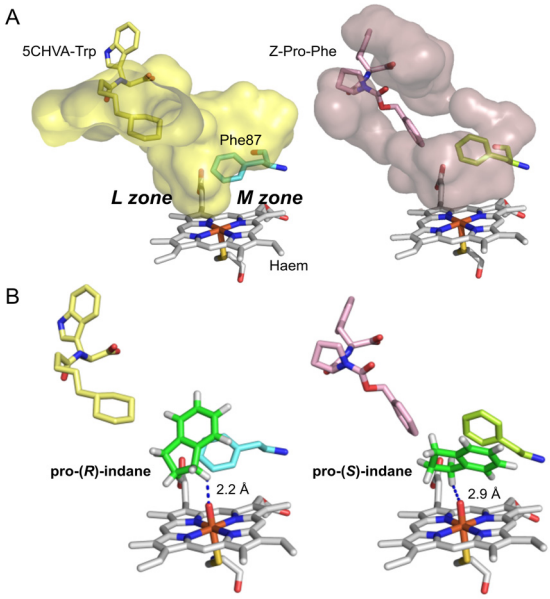


Fig. 4 (A) Cavities of the active site (depicted by a surface model) of (left) 5CHVA-Trp-bound and (right) Z-Pro-Phe-bound P450BM3. (B) Results of the docking simulations of indane into the crystal structure of P450BM3 with (left) 5CHVA-Trp and (right) Z-Pro-Phe.

showed a pro-(*R*) orientation, while docking with 5XA3 showed a pro-(*S*) orientation.

Yang *et al*. discussed the stereoselectivity of alkane hydroxylation based on the distinction of P450BM3's active site into an L zone and M zone.[44, 45] As Phe87 is located at the M zone, substrates possessing a benzene ring are assumed to interact with Phe87 in the M zone through π–π stacking. The displacement of Phe87 and Ala264 in I helix observed in the 5CHVA-Trp-bound structure occludes the M zone, thus decreasing its accessibility (Figs 3 D and S3). It follows that the conformational change induced by binding of 5CHVA-Trp decreases the overall population of substrates present in the M zone, creating a pro-(*R*) reactive environment, as shown in the docking simulation of indane into P450BM3 with 5CHVA-Trp. As for the simulation with 5XA3, the benzene ring of indane was placed in the M zone with a parallel orientation to Phe87. Indane's 5-membered ring, considered to be bulkier than its aromatic ring, was positioned in the wider L zone, although the hydrogen atoms of the ligand were not taken into account in the simulations. The closest approach was the pro-(*S*) hydrogen of the indane molecule, at a distance of 2.9 Å from the iron-oxo species of haem.

Conclusions

Stereoselective benzylic hydroxylation catalysed by WT P450BM3 was investigated using various decoy molecules, which resulted in the enhancement of enantioselectivity or the inversion of stereoselectivity. We demonstrated that tailoring the enzyme active site with decoy molecules represents a promising tool for controlling asymmetric oxidation by P450BM3. Crystal structure analysis and docking simulations indicated that the conformational change induced by decoy molecules affects the enantioselectivity of non-native substrates. A recent study by the Raner research group indicated that long-chain fatty aldehydes access the active sites of the P450BM3 F87G variant simultaneously with 4-fluorophenol, thereby enhancing defluorination.[46, 47] These results hint at existence of numerous more potential P450BM3 activators, in addition to our decoy molecules. Designing new decoy molecules for substrate selective oxidation is a challenging task, although further investigations of the relationship between stereoselectivity and the structure of decoy molecules are still needed. We believe that computational methods, such as docking and molecular dynamics simulations employed in the field of drug discovery,[48] are effective for the design of novel decoy molecules. Important information can be obtained regarding the behaviour of the ligands in the binding site based on parameters such as the root mean square deviation (RMSD) and binding free energy by simulating the complex formed between decoy molecules, non-native substrates, and P450BM3. Moreover, many non-physiological reactions catalysed by P450BM3 variants have been reported thus far,[49-51] all of which may be promising targets for utilisation with the decoy molecule system. For hydroxylation, as described by Munday *et al*.,[35, 52] a combinatorial approach, employing both mutagenesis and decoy molecules, has shown great promise. Thus, by combining activators of P450BM3 with other strategies, such as computational analysis, mutagenesis, and modification of substrates with chemical auxiliaries,[53, 54] P450BM3 may be converted into an unparalleled versatile catalyst with excellent turnover efficiency and impeccable chemical selectivity.

Acknowledgements

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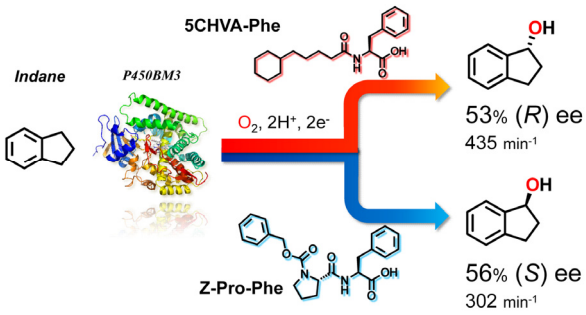

One sentence of text: The benzylic hydroxylation of non-native substrates was catalysed by cytochrome P450BM3, wherein "decoy molecules" controlled the stereoselectivity of the reactions.