Aliphatic *vs.* aromatic C–H bond activation of phenylcyclohexane catalysed by cytochrome P450cam

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Catalytic hydroxylation of phenylcyclohexane 1 by wild-type and the Y96A and Y96F mutant forms of cytochrome P450cam occurs only at the 3- and 4-positions on the cyclohexane ring, giving *cis*-3-phenylcyclohexanol 2, *cis*-4-phenylcyclohexanol 3 and *trans*-4-phenylcyclohexanol 4.

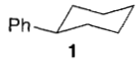
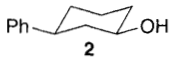
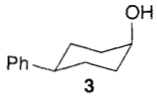
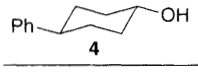
The selective catalytic oxidation of unactivated C–H bonds under mild conditions is difficult to achieve using conventional synthetic methodologies. In biological systems such reactions are carried out by monooxygenases, in most cases the haem-dependent cytochrome P450 enzymes.[1] These systems are the subject of wide interest in chemical synthesis, and microbial oxidation systems are already in use.[2] The exciting field of redesigning and engineering P450 enzymes for *in vivo* and *in vitro* oxidations of unnatural substrates has, however, not been explored extensively.[3]

We have shown that the haem-dependent monooxygenase cytochrome P450cam, which catalyses the stereoselective oxidation of camphor to 5-*exo*-hydroxycamphor,[4] can be engineered by a single mutation (tyrosine 96 → alanine, hence the mutant Y96A), to oxidise substrates such as diphenylmethane which are not attacked by the wild-type enzyme.[5] Of the four possible sites of attack, the Y96A regioselectively hydroxylates diphenylmethane at the *para* position. In order to probe further the interaction between substrates and P450cam, we have examined the oxidation of phenylcyclohexane **1** by both wild-type and Y96A and Y96F[6] (F: phenylalanine) mutants of P450cam. Details of the mutagenesis and protein expression and purification procedures, which followed literature methods,[7] will be published elsewhere. By using **1** as the substrate, it is possible not only to investigate the selectivity towards aromatic and aliphatic C–H bond oxidation, but also the effect of the mutations on the regio- and stereo-selectivities. For **1**, there are 14 possible hydroxylation products.

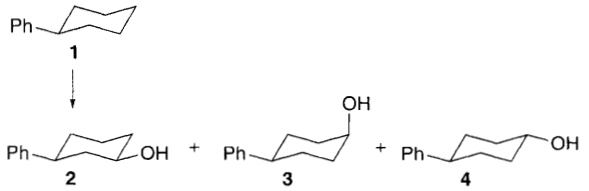
Binding of substrates close to the low-spin haem in P450cam displaces the weakly bound sixth ligand water, giving the five coordinate high-spin haem.[8] The increase in the haem reduction potential which accompanies this spin-state change is required for the catalytic cycle to commence.[9] Compared to the wild-type, the more hydrophobic active sites of the mutants Y96A[5] and Y96F[6] showed lower spin-state shifts and weaker binding with polar substrates such as camphor, but greater shifts and tighter binding with hydrophobic substrates such as adamantane,[5] and **1** (see Table 1). Although the mutant Y96A binds and oxidises diphenylmethane, neither the wild-type nor the mutant Y96F showed any activity with this substrate.

The enzymatic hydroxylation of **1** was assayed by measuring the NADH consumption rate. The turnover rate of **1** with wild-type P450cam (1.6 s^{-1}, Table 1) was much slower than that of camphor (34 s^{-1}). The single site mutations significantly increased the activity of the enzyme towards **1**, with the Y96A mutant showing the highest rate of NADH turnover (5 s^{-1}) that compared favourably with that of the natural substrate camphor (16 s^{-1}). The availability of highly purified recombinant forms of the proteins in the P450cam enzyme system[4] allowed preparative scale incubations with **1** to be carried out. With all three P450cam proteins, three oxidation products were detected and separated by HPLC. The mass spectra identified all products as hydroxylated phenylcyclohexanes, with a parent-ion peak at 176. The structures of the three products were assigned by NMR spectroscopy as *cis*-3-phenylcyclohexanol **2**, *cis*-4-phenylcyclohexanol **3** and *trans*-4-phenylcyclohexanol **4** (Scheme 1).‡ The enantiomeric forms of **2** were well-resolved on a chiral phase GC column, thus allowing the stereoselectivity to be determined (see Table 1) but the *R*/*S* identity of the enantiomers has still to be established. In incubations with the mutants, typical total isolated yields of product alcohols were 3 mg from 10 nmol of P450 enzyme, corresponding to *ca.* 1700 turnovers.§ There was no evidence of further oxidation of the

Table 1 Phenylcyclohexane **1** binding and turnover data with wild-type, Y96F and Y96A mutant cytochrome P450cam

		Wild-type	Y96F mutant	Y96A mutant
% High-spin form	Camphor	100	45	45
 **1**		35	65	85
K_{app}/μmol dm^{-3}[a]		7.0	3.9	2.3
NADH consumption[b]		1.6	3.9	5
Product		Distribution (%)		
 **2**		63 (36% ee)	81 (34% ee)	40 (racemate)
 **3**		12	13	45
 **4**		25	6	15

[a] The apparent substrate binding constant K_{app} includes contributions from the binding and spin-state equilibria.[8] [b] Given as nmol NADH consumed per nmol of P450cam per second at 303 K. Incubation mixtures (1.5 ml, KH_2PO_4 buffer, pH 7.4) contained 1 μmol dm^{-3} P450cam, 10 μmol dm^{-3} putidaredoxin, 2 μmol dm^{-3} putidaredoxin reductase, 100 mmol dm^{-3} KCl, 0.5 mmol dm^{-3} NADH and an excess of **1**.



Scheme 1

alcohols at the end of the incubations, even with >95% substrate conversion.

The P450cam enzymes selectively oxidised **1** at the cyclohexane ring, while chemical systems would be expected to oxidise the aromatic ring or the benzylic C–H bond. This suggests strongly that **1** is not highly mobile inside the P450cam substrate pocket, and that the cyclohexane ring is bound near the haem, with the 3- and 4-carbons closest to the iron centre. In the absence of crystallographic data we speculate that this is due to van de Waals interactions between the cyclohexane ring and the aliphatic side chains of valine-244, leucine-247 and valine-295, and/or stacking interactions between the phenyl group of **1** and the aromatic side chains at positions 87, 96 and 98.

There was significant but not total selectivity in the hydroxylation of **1**, with only 4 out of the 14 possible isomeric alcohols being formed. The mutant Y96F showed the highest regioselectivity, giving 81% of **2** with significant enantiomeric excess (34% ee) and the smallest proportion of **4** (6%). The wild-type gave the highest proportion of **4** (25%) but showed comparable stereoselectivity with the mutant Y96F in the formation of **2** (36% ee). Since the proportion of **3** generated in the two reactions are the same, we infer that the Y96F mutation resulted in movement of the substrate such that the regioselectivity but not the stereoselectivity of attack at C_3 in **1** is altered. Moreover, the enantiomers of **2** are generated by attacks at the two chemically equivalent C_3 carbons, and it may be possible to use further mutations at amino acids closer to the haem to move one of these carbons into closer proximity of the iron centre so as to improve the stereoselectivity. In the case of the mutant Y96A, the product distribution is different, and the observation of a racemate for **2** suggests that **1** is bound in a slightly different orientation.

In summary, we have shown that site specific mutagenesis can be used to alter the regioselectivity and to increase the activity of the hydroxylation of **1** by P450cam. In contrast to chemical systems, the enzymatic oxidation occurred exclusively on the aliphatic ring. The enzyme system was sufficiently stable and active to allow preparative scale incubations to be carried out, and for the commercially unavailable hydroxylation products to be isolated by HPLC and characterised by chemical methods.[10] There was almost total substrate conversion without further oxidation of the products. The P450cam system is therefore excellent for studying the redesign of monooxygenases for the oxidation of organic compounds.

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Footnotes

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‡ ^{1}H NMR data (500 MHz, all in CD_2Cl_2, J values in Hz, resonances due to the phenyl hydrogens are not included, ax. and eq. denote axial and equatorial hydrogens respectively on the cyclohexane ring). For **2**: 3.70 [m, 1 H, C$^1H_{ax}$OH, $^3J(H_{ax}–H_{ax})$ 12], 2.60 [m, 1 H, C$^3H_{ax}$Ph], 2.12 [m, 1 H, C$^2H_{eq}$], 2.01 [m, 1 H, C$^6H_{eq}$], 1.85 [m, 1 H, C$^5H_{eq}$], 1.80 [m, 1 H, C$^4H_{eq}$], 1.45 [m, 1 H, C$^5H_{ax}$], 1.42 [m, 1 H, C$^2H_{ax}$], 1.32 [m, 1 H, C$^4H_{ax}$] and 1.24 [m, 1 H, C$^6H_{ax}$]. For **3**: 4.08 [br s, 1 H, C$^1H_{eq}$- OH], 2.54 [tt, 1 H, C$^4H_{ax}$Ph, $^3J(H_{ax}–H_{ax})$ 11.7, $^3J(H_{ax}–H_{aq})$ 3.3], 1.87 [m, 4 H, C$^3H_{ax}$ and C$^3H_{eq}$] and 1.66 [m, 4 H, C$^2H_{ax}$ and C$^2H_{eq}$]. For **4**: 3.65 [m, 1 H, C$^1H_{ax}$OH], 2.50 [tt, 1 H, C$^4H_{ax}$Ph, $^3J(H_{ax}–H_{ax})$ 12, $^3J(H_{ax}–H_{eq})$ 3.2], 2.05 [m, 2 H, C$^2H_{eq}$ and C$^6H_{eq}$, $^2J(H_{ax}–H_{eq})$ 13], 1.90 [m, 2 H, C$^3H_{eq}$, and C$^2H_{eq}$, $^2J(H_{ax}–H_{eq})$ 13], 1.53 [m, 2 H, C$^2H_{ax}$ and C$^6H_{ax}$, $^3J(H_{ax}–H_{ax})$ 12, $^2J(H_{ax}–H_{eq})$ 13] and 1.39 [m, 2 H, C$^3H_{ax}$ and C$^5H_{ax}$, $^3J(H_{ax}–H_{ax})$ 12, $^2J(H_{ax}–H_{eq})$ 13].

§ *Incubation conditions*: The reaction mixture (10 ml, 50 mmol dm^{-3} Tris, pH 7.4) contained 1 μmol dm^3 P450cam, 4 μmol dm^{-3} putidaredoxin, 1 μmol dm^{-3} putidaredoxin reductase, 200 mmol dm^{-3} Kcl, 14 mmol dm^{-3} NADH, and an excess of **1**, and was incubated at 303 K. After 2 h the mixture was extracted with chloroform, centrifuged and the organic extract was evaporated to dryness. The residue was dissolved in hexane and analysed on a normal phase silica HPLC column using a hexane–isopropanol gradient. The product phenylcyclohexanols were obtained by evaporation of the solvent under a stream of nitrogen.

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