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Selective aliphatic and aromatic carbon–hydrogen bond activation catalysed by mutants of cytochrome $P450_{cam}$

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Abstract

The substrate range of the haem monooxygenase cytochrome $P450_{cam}$ (CYP101) has been broadened by site-directed mutagenesis. The hydroxylation selectivity of five mutants at the 96 position towards a range of substrates has been used to investigate $P450_{cam}$-substrate molecular recognition. The substrates contained aromatic and activated and unactivated aliphatic C–H bonds, as well as reactive functional groups. Diphenylmethane, diphenylether, diphenylamine, and 1,1-diphenylethylene were all hydroxylated regiospecifically at the *para* position, with no attack at the amine or the olefinic double bond. With benzylcyclohexane the activated benzylic and tertiary C–H bonds were not attacked, and the reactions catalysed by the Y96G and Y96A mutants were highly diastereoselective, with 4-*trans*-benzylcyclohexanol constituting 90% of the products. 1-Phenyl-1-cyclohexylethylene was oxidised predominantly at the 4-position of the cyclohexane ring without attack at the olefinic double bond, and approximately equal amounts of *cis*- and *trans*-4-phenylethenylcyclohexanol were formed. These results show that $P450_{cam}$ can be engineered to oxidise C–H bonds without attacking more reactive functional groups.

Keywords: Monooxygenase; $P450_{cam}$; Mutagenesis; Protein engineering; Selective hydroxylation; Unnatural substrates

1. Introduction

The design and synthesis of catalysts for the selective hydroxylation of carbon–hydrogen bonds in complex molecules containing chemically reactive functional groups is an important scientific challenge. Such monooxygenase catalysts potentially have wide applications in synthesis. Compared to molecular systems, enzymes have the possible advantage that the enzyme–substrate molecular recognition interactions could orientate a reactive functional group in a substrate away from the hydroxylation centre, such that an unactivated C–H bond can be selectively attacked. Most monooxygenases are highly substrate specific, and do not oxidise many molecules of interest in chemical synthesis. The techniques of protein engineering offer the possibility of broadening the substrate range of monooxygenase enzymes, and engineering the selectivity of C–H bond oxidation.

The most common hydroxylating enzymes are the haem-dependent cytochrome P450 superfamily of monooxygenases [1,2]. The most well-characterised of these is cytochrome $P450_{cam}$ which is isolated from the soil bacterium *Pseudomonas putida*. This stable, solu-

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ble enzyme catalyses the stereospecific hydroxylation of camphor to 5-*exo*-hydroxycamphor [3]. Since high resolution crystal structures of the $P450_{cam}$ protein are available [4–6], this is an attractive system for protein engineering studies because the side-chains which line the substrate binding pocket are known, thus enabling rational protein active site redesign.

We have shown that the substrate range of $P450_{cam}$ can be broadened by a single active site mutation, Tyr-96 → Ala (Y96A). This mutant oxidised diphenylmethane **1** regiospecifically to *para*-hydroxydiphenylmethane **2**, a reaction not catalysed by the wild-type enzyme [7]. We have also shown that the activity and selectivity of oxidation of phenylcyclohexane **3** could be improved by site-directed mutagenesis [8]. Here we report the oxidation by $P450_{cam}$ mutants of a range of compounds related to **1** and **3**. We wished to probe the selectivity of hydroxylation, to investigate if the cyclohexane ring is always preferentially hydroxylated in mixed phenyl–cyclohexyl compounds, and if the enzyme can maintain specificity for C–H bond oxidation when reactive functional groups are present. We have compared the activity of wild-type $P450_{cam}$ with mutants in which the tyrosine-96 residue was changed to glycine (Y96G), alanine (Y96A) [7], valine (Y96V), phenylalanine (Y96F) [9] and leucine (Y96L). The substrates studied contained either two phenyl groups or one phenyl and one cyclohexyl group bridged by a single carbon or heteroatom. The substrates were diphenylmethane, diphenylether, diphenylamine, benzylcyclohexane, 1-phenyl-1-cyclohexylethylene, and 1,1-diphenylethylene. The last two compounds contained an olefinic double bond at the bridge and these will test the functional group tolerance of the mutants.

2. Experimental

2.1. General

Phenylcyclohexylketone, diphenylamine, diphenylether, 1,1-diphenylethylene, methyltriphenylphosphonium bromide, and HPLC grade chloroform, hexane and isopropanol were from Aldrich Chemicals. Buffer components were from Biometra. NADH was from Boehringer Mannheim. PD10 Sephadex G25 gel filtration columns were from Pharmacia Biotech. UV/Vis spectra were recorded on a CARY 1E double-beam spectrophotometer. The cell holders were equipped with magnetic stirrers and the cell temperatures were maintained (± 0.1°C) by a Peltier temperature controller. Gas chromatographic analyses were carried out on a Fisons Instruments 8000 series gas chromatograph with a flame ionisation detector using a DB-1 fused silica column (30 m × 0.25 mm i.d.). HPLC experiments were carried out on a Gilson apparatus using analytical (5 mm i.d. × 250 mm) or semi-prep (10 mm i.d. × 250 mm) silica columns. Enantioselectivities were determined by HPLC on a Regis Technologies column (4.6 mm i.d. × 250 mm) with a 4-(3,5-dinitrobenzamido)tetrahydrophenanthrene chiral stationary phase. The eluents were monitored at 254 nm. NMR spectra were recorded at 300 MHz on a Bruker AM300 or at 500 MHz on a Bruker WH500 or a Varian Gemini 2000 spectrometer.

2.2. Recombinant protein preparation

Expression and purification of $P450_{cam}$ and the associated electron transfer proteins putidaredoxin and putidaredoxin reductase, were carried out using literature methods [10–12]. Site-directed mutagenesis was carried out by the Kunkel method [13] using the Bio-Rad Mutagene kit. General bacterial growths and DNA manipulations followed standard methods [14].

2.3. Preparation of benzylcyclohexane 8

Benzylcyclohexane **8** was prepared by Clemmensen reduction of phenylcyclohexylketone. Zinc wool (3.5 g, 65 mmol) was placed in a 50 ml round bottom flask. $HgCl_2$ (0.16 g, 0.6 mmol) was dissolved in 5 ml of dilute HCl (1:20 v/v conc. $HCl:H_2O$) and added slowly. After 5 min the liquid was decanted from the

zinc amalgam, and 20 ml of HCl (5:1 v/v conc. HCl:H_2O) and phenylcyclohexylketone (1.13 g, 6 mmol) were added. The mixture was refluxed for 2 h, with 1 ml conc. HCl being added every 30 min. After cooling to room temperature the mixture was extracted with ether (4 × 15 ml) and the organic extracts were combined. After washing with water (2 × 15 ml) and drying over $MgSO_4$, the ether was evaporated off under reduced pressure. Benzylcyclohexane was purified by silica column chromatography using hexane as the mobile phase. Fractions were collected and the product characterised by NMR spectroscopy. Yield 0.13 g.

2.4. Preparation of 1-phenyl-1-cyclohexylethylene **11**

1-Phenyl-1-cyclohexylethylene **11** was prepared by the Wittig reaction between methylenetriphenylphosphorane and phenylcyclohexylketone. A mixture of sodium amide and $MePPh_3Br$ (4 g, 9.6 mmol) was stirred in 20 ml THF under nitrogen for 2 h. Phenylcyclohexylketone (1.48 g, 8 mmol) in 10 ml THF was then added. The reaction mixture was stirred under nitrogen for 18 h and then quenched with 20 ml of degassed water. The THF was evaporated under reduced pressure, the residual aqueous layer was extracted with ethyl acetate (2 × 15 ml) and the extracts combined. After washing with 10 ml each of 1 M HCl and then water, the organic extract was dried over $MgSO_4$ and evaporated to dryness under reduced pressure. The $Ph_3P{=}O$ by-product was separated from the 1-phenyl-1-cyclohexylethylene by chromatography on a silica column using hexane as the mobile phase. The product fractions were collected and the product characterised by NMR spectroscopy. Yield 0.72 g.

2.5. NADH turnover experiments

The three proteins in the $P450_{cam}$ system were stored in 50% glycerol buffered solutions. The storage solution for the $P450_{cam}$ monooxygenase also contained 0.5 mM camphor to stabilise the protein. Immediately before experiments, glycerol and camphor were removed by gel filtration on a PD10 column.

Incubation mixtures (1.5 ml) in a 2 ml cuvette contained 50 mM Tris–HCl buffer, pH 7.4, 0.05 μM $P450_{cam}$, 16 μM putidaredoxin, 0.5 μM putidaredoxin reductase and 200 mM KCl. Substrates were added as 0.1 M stocks in ethanol to a nominal final concentration of 4 mM and precipitation was observed for all solid substrates. After equilibrating at 30°C for 2 min, NADH was added to 0.25 mM and the absorbance at 340 nm monitored. The turnover rates were calculated using $\varepsilon_{340\,nm} = 6.22\ mM^{-1}\ cm^{-1}$. Since the substrates and the products do not absorb significantly at 340 nm, and the amount of NADH added would consume only approximately 5% of the total amount of substrate present, neither substrate/product absorption nor turbidity changes should interfere with the rate calculations.

2.6. Preparative scale incubations and purification and characterisation of products

The oxidation product of **1** was identified by co-elution on both GC and HPLC with an authentic sample. However, the hydroxylation products of the other substrates investigated in this work were not readily available, so preparative scale incubations were necessary in order to characterise the products by chemical methods. With the relatively high turnover activities of the mutants and stability of the $P450_{cam}$ system, it was possible to isolate and purify the enzymatic turnover products by HPLC of the organic extracts. All the reactions were catalytic, with total turnovers numbers of up to 5000. The total yields of up to 10 mg of products from 20 nmol of $P450_{cam}$ enzyme were more than sufficient for characterisation of the products by NMR spectroscopy. Where necessary the NMR spectral assignments were confirmed by 2D NMR spectra and double resonance on selected signals.

For preparative scale incubations the reac-

tions were scaled up to 5–10 ml and carried out in 25 ml flasks with stirring. The protein concentrations were 1 μM $P450_{cam}$, 10 μM putidaredoxin, and 1 μM putidaredoxin reductase. NADH was added every 20 min, with each aliquot increasing the concentration by 1 mM. After 3 h the reaction mixtures were extracted with $CHCl_3$ (50% of reaction volume) and the phases separated by centrifugation at 4000 *g* for 15 min at 4°C. The extraction was repeated twice and the combined $CHCl_3$ extracts were evaporated to dryness under a stream of nitrogen. The residue was dissolved in hexane and the substrate and products were separated by normal phase HPLC with a semi-prep (10 mm i.d. × 250 mm) silica column. The mobile phase was hexane containing isopropanol, the concentration of which was increased in a 0–5% v/v gradient over 40 min. The product fractions were combined and the solvent evaporated under a stream of nitrogen.

2.7. NMR characterising data for the turnover products

^{1}H NMR (500 MHz, in CD_2Cl_2 except for **12** and **15**, δ ppm, J in Hz):

Para-hydroxydiphenylether **6**: 7.30 [dd, $2H_{meta\text{-Ph}}$, ^3J(H–H) 8.5, 7.4], 7.03 [tt, $H_{para\text{-Ph}}$, ^3J(H–H) 7.4, ^4J(H–H) 1.0], 6.92 [dd, $2H_{ortho\text{-Ph}}$, ^3J(H–H) 8.5, ^4J(H–H) 1], 6.91 [m, $2H_{ortho\text{-PhOH}}$, ^3J(H–H) 8.9], 6.82 [m, $2H_{meta\text{-PhOH}}$, ^3J(H–H) 8.9].

Para-hydroxydiphenylamine **7**: 7.19 [t, $2H_{meta\text{-Ph}}$, ^3J(H–H) 7.5], 7.02 [m, $2H_{ortho\text{-PhOH}}$, ^3J(H–H) 8.7], 6.90 [m, $2H_{ortho\text{-Ph}}$, ^3J(H–H) 7.5], 6.81 [t, $1H_{para\text{-Ph}}$, ^3J(H–H) 7.5], 6.79 [m, $2H_{meta\text{-PhOH}}$, ^3J(H–H) 8.7], 4.70 [br. s, NH].

Trans-4-benzylcyclohexanol **9**: 7.26 [m, $2H_{meta}$], 7.16 [m, $1H_{para}$], 7.13 [m, $2H_{ortho}$], 3.50 [tt, $C_1H_{ax}OH$, $^3J(H_{ax}–C_2H_{ax})$ 10.8, $^3J(H_{ax}–C_2H_{eq})$ 4.3], 2.48 [d, CH_2Ph, ^3J(H–C_4H_{ax}) 7.1], 1.91 [m, $2C_2H_{eq}$, $^2J(H_{eq}–H_{ax})$ 12.6, $^3J(H_{eq}–C_1H_{ax})$ 4.3], 1.70 [m, $2C_3H_{eq}$, $^2J(H_{eq}–H_{ax})$ 13.0, $^3J(H_{eq}–C_4H_{ax})$ 4.3], 1.49 [ttt, C_4H_{ax}, $^3J(H_{ax}–C_3H_{ax})$ 11.3, $^3J(H_{ax}–CH_2Ph)$ 7.1, $^3J(H_{ax}–C_3H_{eq})$ 3.5], 1.19 [m, $2C_2H_{ax}$, $^2J(H_{ax}–H_{eq})$ 12.6, $^3J(H_{ax}–C_3H_{ax})$, $^3J(H_{ax}–C_1H_{ax})$ 10.8], 1.04 [m, $2C_3H_{ax}$, $^2J(H_{ax}–H_{eq})$ 13.0, $^3J(H_{ax}–C_4H_{ax})$ 11.3, $^3J(H_{ax}–C_2H_{ax})$ 10.8].

1-Phenyl-1-(*para*-hydroxyphenyl)ethylene **12** (in CD_3COCD_3): 7.40–7.25 [m, 5H, Ph], 7.17 [m, $2H_{ortho\text{-PhOH}}$], 6.83 [m, $2H_{meta\text{-PhOH}}$], 5.38 [d, 1H, J(H–H) 1.3, =C*H*H], 5.30 [d, 1H, J(H–H) 1.3, =CH*H*].

4-*Cis*-phenylethenylcyclohexanol **13**: 7.35–7.25 [m, 5H, Ph], 5.14 [m, =C*H*H], 5.09 [m, =CH*H*], 4.00 [br s, $C_1H_{eq}OH$], 2.49 [m, C_4H_{ax}, $^3J(H_{ax}–C_3H_{ax})$ 11.0, $^3J(H_{ax}–C_3H_{eq})$ 5.8], 1.77 [m, $2C_3H_{ax}$, $^3J(H_{ax}–C_4H_{ax})$, $^3J(H_{ax}–C_2H_{ax})$, $^2J(H_{ax}–H_{eq})$ 11.0], 1.61 [m, $2C_2H_{eq}$ and $2C_3H_{eq}$], 1.34 [m, $2C_2H_{ax}$, $^3J(H_{ax}–C_3H_{ax})$ 11.0].

4-*Trans*-phenylethenylcyclohexanol **14**: 7.32–7.26 [m, 5H, Ph], 5.14 [m, =C*H*H], 5.01 [m, =CH*H*], 3.56 [m, $C_1H_{ax}OH$, $^3J(H_{ax}–C_2H_{ax})$ 10.5, $^3J(H_{ax}–C_2H_{eq})$ 4.5], 2.40 [m, C_4H_{ax}, $^3J(H_{ax}–C_3H_{ax})$ 11.2, $^3J(H_{ax}–C_3H_{eq})$ 3.8], 2.02 [m, $2C_3H_{eq}$, $^2J(H_{eq}–H_{ax})$ 11.0], 1.85 [m, $2C_2H_{eq}$, $^2J(H_{eq}–H_{ax})$ 13.0], 1.29 [m, $2C_3H_{ax}$ and $2C_2H_{ax}$].

3-*Cis*-phenylethenylcyclohexanol **15** (in C_6D_6): 7.51–6.97 [m, 5H, Ph], 5.25 [m, =C*H*H], 5.04 [m, =CH*H*], 3.37 [tt, $C_1H_{ax}OH$, $^3J(H_{ax}–C_2H_{ax}) = {}^3J(H_{ax}–C_6H_{ax})$ 13.0, $^3J(H_{ax}–C_2H_{eq}) = {}^3J(H_{ax}–C_6H_{eq})$ 4.5], 2.37 [tt, C_3H_{ax}, $^3J(H_{ax}–C_2H_{ax}) = {}^3J(H_{ax}–C_4H_{ax})$ 12.3, $^3J(H_{ax}–C_2H_{eq}) = {}^3J(H_{ax}–C_4H_{eq})$ 3.7], 2.04 [m, C_2H_{eq}, $^2J(H_{ax}–H_{eq})$ 12.0], 1.86 [m, C_6H_{eq}, $^2J(H_{ax}–H_{eq})$ 12.3], 1.70 [m, C_4H_{eq}, $^2J(H_{ax}–H_{eq})$ 12.5], 1.60 [m, C_5H_{eq}, $^2J(H_{ax}–H_{eq})$ 12.3], 1.10 [m, C_2H_{ax} and C_6H_{ax}], 0.98 [m, C_4H_{ax} and C_5H_{ax}].

3. Results

3.1. Oxidation of diphenylmethane, diphenylether and diphenylamine

We first examined the oxidation of the closely related molecules diphenylmethane **1**,

diphenylether **4**, and diphenylamine **5**. The NADH turnover activities of wild-type $P450_{cam}$ and all the Y96 mutants towards these and the other target molecules are given in Table 1. Only the Y96A and Y96G mutants were active towards the oxidation of **1**. There was no evidence, either by GC or HPLC, of turnover by the wild-type $P450_{cam}$ and the other mutants. The Y96G and Y96A mutants of $P450_{cam}$ were also the most active catalysts for the oxidation of **4** and **5**. In contrast to **1**, the ether **4** was also oxidised by the Y96V mutant, and trace amounts of product were detected by GC in incubations of the amine **5** with wild-type $P450_{cam}$.

The oxidation of **1** gave *para*-hydroxydiphenylmethane **2** (Fig. 1) as the only product [7]. The turnover products of **4** and **5** were isolated by HPLC and characterised by NMR and mass spectroscopy. The NMR spectra showed that both **4** and **5** were regiospecifically hydroxylated at the *para* position; the only product from the oxidation of **4** was *para*-hydroxydiphenylether **6**, and **5** gave only *para*-hydroxydiphenylamine **7** (Fig. 1). The *para* isomers were readily identified by the characteristic NMR resonances of the four hydrogens in the hydroxylated phenyl ring.

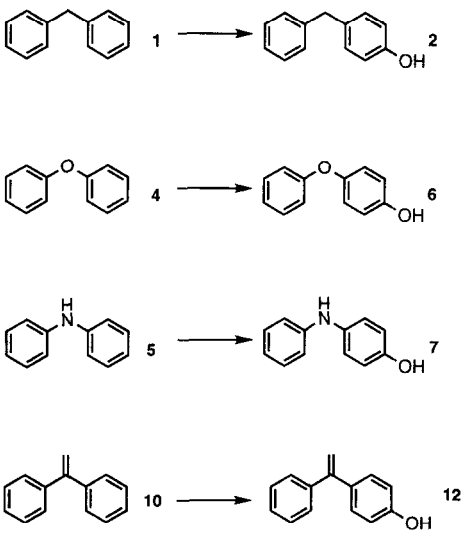
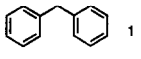
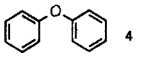
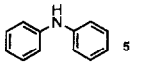
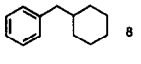
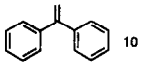
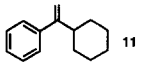


Fig. 1. The regiospecific hydroxylation of the diphenyl compounds **1**, **4**, **5**, and **10** by the Y96G and Y96A mutants of cytochrome $P450_{cam}$.

Table 1
NADH turnover rates [a,b] of wild-type and the Y96 mutants of cytochrome $P450_{cam}$ with the target substrates

Substrate	$P450_{cam}$					
	Y96G	Y96A	Y96V	Y96L	Y96F	WT
 1	70	100	–	–	–	–
 4	340	390	24	–	–	–
 5	48	80	–	–	–	– [c]
 8	70	72	– [c]	24	90	– [c]
 10	70	54	–	–	–	–
 11	160	168	40	– [c]	100	– [c]

[a] The turnover rates are given as nmol of NADH consumed per nmol of $P450_{cam}$ protein per minute.
[b] –: The turnover rates are too low to be reliably calculated.
[c] Trace amounts of products were detected by GC.

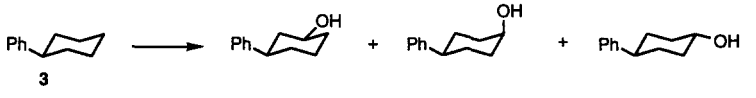
3.2. Oxidation of benzylcyclohexane 8

Benzylcyclohexane **8** was oxidised by all the Y96 mutants of $P450_{cam}$ except Y96V (Table 1). Trace amounts of products were detected by GC in the reaction catalysed by the wild-type $P450_{cam}$. In contrast to **1**, which is of comparable size to **8**, the Y96F mutant showed the highest NADH turnover activity.

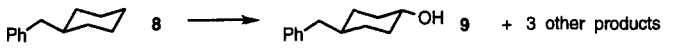
HPLC analysis of the organic extracts from turnover reactions showed that all the mutants oxidised **8** to give one major and three minor products. The minor products were not formed in sufficient quantities for detailed characterisation by NMR. The major product was readily isolated and characterised as *trans*-4-benzylcyclohexanol **9** (Fig. 2). The *trans* isomer was identified by the strong axial–axial couplings observed for the axial C_1 hydrogen. Hence the major product did not arise from attack of the aromatic ring or at the activated benzylic C– H bonds on the bridging methylene group or the tertiary C–H bond. The Y96G and Y96A mutants showed the highest selectivity, with **9** constituting 90% of the products. With larger sidechains at the 96 position, the selectivity was reduced, to 55% in the Y96L and 76% in the Y96F mutants (Fig. 2).

3.3. Oxidation of 1,1-diphenylethylene and 1-phenyl-1-cyclohexylethylene

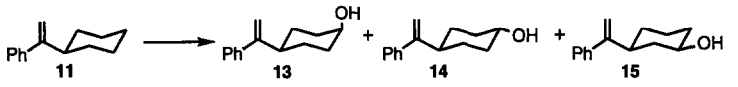
The molecules 1,1-diphenylethylene **10** and 1-phenyl-1-cyclohexylethylene **11**, contain reac-



Y96A	40%	45%	15%
Y96F	81%	13%	6%



Mutant	Y96G	Y96A	Y96V	Y96L	Y96F
% of **9** in products	90%	90%	60%	55%	74%



Mutant	Y96G	Y96A	Y96V	Y96L	Y96F
% **13**	47	48	41	-	39
% **14**	46	47	39	-	48
% **15** [a]	6 (12%)	5 (10%)	20 (10%)[b]	-	13 (40%)[b]

Fig. 2. Comparison of the regioselectivities of the hydroxylation of the related phenyl–cyclohexyl molecules **3**, **8**, and **11** by the Y96 mutants of cytochrome $P450_{cam}$. (a) Enantiomeric excess in parenthesis, (b) the enantioselectivity is in favour of the other optical isomer to that favoured by the Y96G and Y96A mutants.

tive olefinic double bonds, and these molecules therefore provide a more stringent test of the selectivity of the $P450_{cam}$ mutants.

The compound **10** showed similar behaviour to the other diphenyl derivatives. The Y96A and Y96G mutants were the only active catalysts for the oxidation of **10**, with no evidence of turnover by wild-type $P450_{cam}$ or the other Y96 mutants. Only one product was observed by HPLC, and this was characterised by NMR spectroscopy as 1-phenyl-1-(*para*-hydroxyphenyl)-ethylene **12**. Hence the reactive olefinic double bond of **10** was not attacked, and the regiospecificity of hydroxylation at the *para*-position of the diphenyl derivatives **1**, **4** and **5** was maintained (Fig. 1).

The Y96G and Y96A mutants were the most active catalysts for the oxidation of the mixed ring compound **11**. Three products were observed and isolated by HPLC and characterised by ^{1}H NMR spectroscopy as *cis*-4-phenylethenylcyclohexanol **13**, *trans*-4-phenylethenylcyclohexanol **14**, and *cis*-3-phenylethenylcyclohexanol **15** (Fig. 2). The observation of the two olefinic hydrogens at ca. 5.3 ppm in the ^{1}H NMR spectra of these products clearly indicated that the C=C double bond of **11** was not oxidised by any of the $P450_{cam}$ mutants.

The enantiomers of the chiral alcohol **15** were resolved on a chiral phase HPLC column, and the enantioselectivities are given in Fig. 2. The *R/S* identities of the enantiomers have not been established. The enantioselectivities are low, with the highest enantiomeric excess being observed in the reaction with the Y96F mutant. Interestingly, the Y96G and Y96A mutants slightly favoured the formation of one enantiomer while the Y96V and Y96F favoured the other.

3.4. Substrate conversion and uncoupling from NADH turnover

The monooxygenase reaction of $P450_{cam}$, and P450 enzymes in general, are subject to uncoupling, i.e., not all of the reducing equivalents from NADH are utilised for product formation [15]. Uncoupling of the $P450_{cam}$ catalytic cycle occurs with unnatural substrates such as styrene and ethylbenzene, whose structure differ substantially from that of camphor, where the coupling efficiency can be below 5% [16,17].

Since the oxidation products of the substrates investigated in this work are not readily available, except for **2**, it was not possible to determine quantitatively the product formation rates and hence the coupling efficiencies. However, by carrying out the reactions under identical conditions and noting the quantity of NADH consumed compared to the GC and HPLC peak areas of the products, it was possible to obtain the relative coupling efficiencies. For all the substrates the Y96A and Y96G mutants gave more products than the other mutants. Comparing between the substrates, those containing cyclohexyl rings showed higher coupling than the analogous diphenyl derivatives. In the preparative scale incubations, benzylcyclohexane and 1-phenyl-1-cyclohexylethylene were nearly completely converted to products under the reaction conditions stated, which corresponds to approximately 4000 turnovers and $> 50\%$ coupling efficiencies. The coupling efficiencies of the oxidation of the diphenyl substrates were estimated to be $< 20\%$ from the lower substrate coversions observed.

4. Discussion

The mutations at Tyr-96 create new $P450_{cam}$ substrate pockets of different sizes which are more hydrophobic than that of the wild-type enzyme. The substrate molecules investigated in this work were chosen to probe the selectivity of C–H bond oxidation. Diphenylmethane **1** and benzylcyclohexane **8** probe the selectivity for aliphatic versus aromatic C–H bond activation and the effect of a flexible cyclohexane ring in **8** against the rigidity of the two rings in **1**. The selectivity for C–H bond oxidation in the pres-

ence of reactive functionalities was also investigated. The compounds diphenylamine **5**, 1,1-diphenylethylene **10** and 1-phenyl-1-cyclohexylethylene **11**, closely related to **1** and **8**, have reactive functional groups at the bridging atom between the two rings. Comparison of the activity of wild-type $P450_{cam}$ and the Y96 mutants towards this range of substrates reveals some interesting aspects of $P450_{cam}$-substrate recognition.

The identical regioselectivity observed in the oxidation of **1** [7], **4**, **5** and **10** (Fig. 1) suggests that these molecules are bound in very similar orientations in the active sites of the Y96A and Y96G mutants. The wild-type $P450_{cam}$ does not show significant activity towards the oxidation of these molecules. This is most likely due to the large sizes of these substrates compared to camphor. With the Y96G and Y96A mutants, and to a lesser extent the Y96V and Y96L, a hydrophobic 'aromatic pocket' is generated by replacing the aromatic side-chain of Y96 with a smaller, aliphatic side-chain. It appears that the larger aromatic pockets generated by the Y96G and Y96A mutations, but not the smaller pockets in the Y96V and Y96L mutants, could accommodate one of the phenyl groups of these substrates while the second phenyl group points towards the haem iron. Since the Y96 side-chain is in the upper part of the $P450_{cam}$ active site and far-removed from the haem, this proposed common binding orientation will position the bridging group away from the highly reactive ferryl intermediate, such that the activated benzylic C–H bonds in **1**, the amine group in **5**, and the olefinic double bond in **10** are not attacked. This binding orientation presumably also places the *para*-C–H bond of the second phenyl group closest to the ferryl intermediate, resulting in the regiospecific C–H bond activation observed. However, we note that the oxidation reaction could also proceed by an indirect, NIH shift mechanism [18] via the formation of an unobserved epoxide intermediate which ring-opens with hydrogen shifts to give the final alcohol product. Whatever the mechanism of the $P450_{cam}$-catalysed oxidation, a molecular catalyst would be expected to oxidise the amine group in **5** and the olefinic double bond in **10**. Thus specific substrate binding enables the $P450_{cam}$ mutants to oxidise a C–H bond in the presence of a reactive functionality in the substrate.

Diphenylmethane **1** and benzylcyclohexane **8** have very similar overall sizes, as do **10** and **11**. However, while **1** and **10** were only oxidised by the Y96A and Y96G mutants, **8** and **11** were also oxidised by the Y96L and Y96F. Indeed, although the Y96F mutant is inactive towards all the diphenyl compounds, it is the most active of the Y96 mutants towards the oxidation of **8**. None of these substrates was oxidised by wild-type $P450_{cam}$. The active site hydrophobicity and the structure and rigidity of the substrate may be important factors.

Increased active site hydrophobicity should promote the oxidation of the non-polar substrates investigated in this work. Thus, we have reported the oxidation of phenylcyclohexane **3** by wild-type $P450_{cam}$, and showed that the activity is increased in the Y96A and Y96F mutants [8]. In the case of the wild-type, the polar phenol side chain of Y96 presumably disfavours the close approach of the hydrophobic phenyl groups of **8** and also the olefinic double bond in the case of **11**, and the protein is inactive towards these substrates. The polar hydroxy group is absent in the Y96F mutant, and substrate binding may become possible. Since **3** is smaller than **8** and **11**, there may be sufficient room in the active site so that its phenyl ring does not have to approach as closely to the side chain of Y96, and **3** is oxidised by wild-type $P450_{cam}$.

The increased active site hydrophobicity alone cannot account for the behaviour of the Y96 mutants towards the substrates, because the Y96F mutant does not oxidise **1** or **10**. Another factor which may be important is the flexibility of the substrate molecules. The cyclohexane rings of **8** and **11** can adopt many different conformations distinct from the most stable chair conformation. This could enable these sub-

strates to avoid steric hindrance between the cyclohexane (and/or the phenyl) ring and active site side-chains, thus promoting substrate binding. These ring distortions would be particularly important in the Y96F and Y96L mutants because these have less room in the active site, and for the binding of **11** which has an extra olefinic double bond compared to **8**. The different behaviour of the diphenyl molecules **1**, **4**, **5**, and **10** is likely to be due to the rigidity of the phenyl groups. The Y96A and Y96G mutants, on the other hand, have sufficient room in the active site to bind all the substrates.

It is instructive to compare the regioselectivity of the Y96 mutants towards the oxidation of **3**, **8**, and **11**, all of which contain both aromatic and aliphatic groups. The hydroxylation of all three molecules occurred exclusively on the aliphatic ring (Fig. 2), and in the case of **11** there was no oxidation of the olefinic double bond. Two factors may be important. For the Y96A and Y96G mutants, the aromatic pocket could accommodate the phenyl groups in these substrates. Secondly, the presence of aliphatic side chains such as those of Leu-244, Val-247 and Val-295 in the immediate vicinity of the haem may give rise to stronger van der Waals interactions with the cyclohexane ring than with the planar phenyl group. A combination of these effects could lead to binding of **11** with the olefinic double bond orientated away from the haem iron. The regioselectivity of attack at the cyclohexane rings of **3**, **8**, and **11** is then determined by the positioning of the ring in relation to the ferryl intermediate.

Wild-type $P450_{cam}$ and the Y96 mutants attack **3** at C_3 and C_4 (Fig. 2). When there is an extra carbon between the two rings, as in the compounds **8** and **11**, the attacks occur predominantly at C_4. In the oxidation of **8** the Y96A and Y96G mutants show high diastereoselectivity, giving 90% of the *trans* diastereoisomer **9**, which suggests that the equatorial region at C_4 is closest to the oxygen of the ferryl intermediate. The reduced diastereoselectivity observed with the Y96L and Y96F mutants could arise from the ring distortions required for substrate binding which could bring other ring positions into close proximity of the ferryl intermediate. The presence of the olefinic double bond in **11** changed the orientation of substrate binding such that although the regioselectivity for attack at C_4 was $> 80\%$ for all the Y96 mutants, and the high diastereoselectivity observed with **8** was completely lost. The steric requirement of the double bond must have caused **11** to move in relation to the haem iron, or induced distortions of the cyclohexane ring, thus bringing both the *cis* and *trans* C–H bonds at C_4 close to the ferryl oxygen for hydroxylation. Hence, there is a balance between the size of the substituent on the cyclohexane ring and the selectivity of ring hydroxylation, and the $P450_{cam}$ active site completely controls the selectivity of hydroxylation. It should be possible to alter the orientation of the cyclohexane ring relative to the haem iron by further amino acid substitutions at positions close to the haem and thereby improve this selectivity.

Finally, it is important to note that the catalytic hydroxylation of all the substrates studied gave only the alcohol products; there was no evidence of further oxidation to diols or ketones even with $> 95\%$ substrate conversion. This property of the $P450_{cam}$ system could be invaluable in any synthetic applications.

5. Conclusion

The results show that the active site pockets of $P450_{cam}$ proteins both control and maintain the hydroxylation activity and selectivity. The observation of C–H bond activation by mutant $P450_{cam}$ proteins in molecules such as **5**, **10** and **11** which also contain reactive functionalities is an important step towards utilising monooxygenases in synthesis. It appears that the $P450_{cam}$ active site is pre-disposed to the binding of a cyclohexane ring close to the haem, and the active site will tolerate substituents of different sizes on the ring. This is particularly encour-

aging in the context of the synthesis of polyfunctionalised cyclohexanes which are versatile synthetic intermediates.

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