Cytochrome P-450$_{cam}$ Monooxygenase can be Redesigned to Catalyse the Regioselective Aromatic Hydroxylation of Diphenylmethane

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A single-site mutant (Y96A) of the monooxygenase cytochrome P-450$_{cam}$ was found to bind diphenylmethane **5** and to catalyse its regioselective aromatic hydroxylation to *p*-hydroxydiphenylmethane **6**.

Monooxygenases catalyse the selective oxidation of non-functionalised hydrocarbons using oxygen,[1] and are therefore of great interest for potential use in organic synthesis. However, progress in this area has been hampered by the difficulty in isolating sufficient quantities of enzyme and the associated electron-transfer proteins. Despite the availability of amino acid sequences of more than 150 different cytochrome P-450 monooxygenases, to date structural data of only three are available,[2–4] and few have been successfully over-expressed in bacterial systems.[5]

One cytochrome P-450 monooxygenase, which is soluble and can be expressed in sufficient quantities, is the highly specific cytochrome P-450$_{cam}$ from *Pseudomonas putida* which catalyses the regio- and stereo-selective hydroxylation of camphor **1** to 5-*exo*-hydroxycamphor.[6] The high-resolution crystal structure of P-450$_{cam}$ has been determined,[2] and since the catalytic mechanism of this bacterial enzyme is believed to be very similar to that of its mammalian counterparts, it has been used as a framework on which models of mammalian enzymes are based.

In an attempt to design new biocatalysts, we have initiated a project which aims to redesign the active site of P-450$_{cam}$, such that it is able to carry out specific oxidations of organic molecules which are not substrates for the wild-type protein. Our initial aim was to incorporate an 'aromatic pocket' into the P-450$_{cam}$ active site, which would encourage the binding of substrates containing aromatic sidechains.

The active site of P-450$_{cam}$ is shown in Fig. 1. Protein sidechains are in close van der Waals contacts with the hydrophobic groups of camphor. In particular, three aromatic residues (Y96, F87 and F98) are grouped together and line the substrate binding pocket, with a hydrogen bond between tyrosine 96 and the camphor carbonyl oxygen maintaining the substrate in the correct orientation to ensure the regio- and stereo-specificity of the reaction. Replacement of any of these aromatic residues with a smaller, hydrophobic non-aromatic side-chain could provide the desired 'aromatic pocket'.

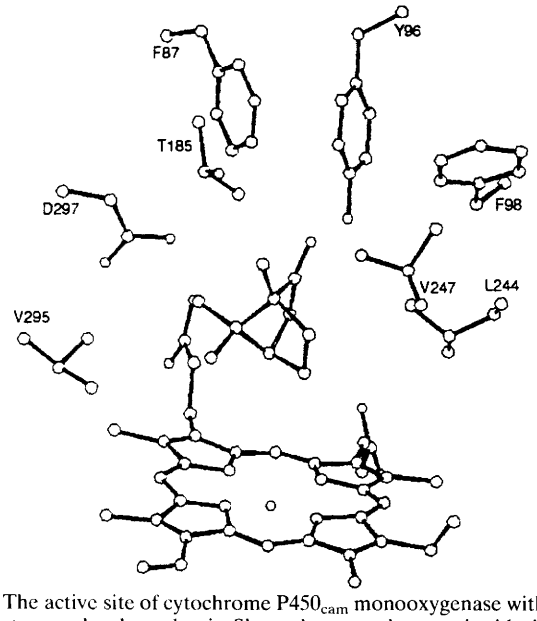


Fig. 1 The active site of cytochrome P450$_{cam}$ monooxygenase with the substrate camphor bound to it. Shown here are the protein sidechains which are in van der Waals contact with camphor. The coordinates were obtained from the Brookhaven Data Bank (2CPP).

The possible effect of point mutations of the three aromatic residues was investigated by molecular modelling. The program GRID[7] was used to calculate the energy of interaction between an aromatic probe (sp^2 CH) and possible mutants of cytochrome P-450$_{cam}$ where these residues were changed to alanine (F87A, Y96A and F98A). Default parameters were used for the GRID calculations except for a grid spacing of 0.5 Å. The results were then examined graphically using the molecular modelling package Quanta[8] using contours of −3.0 and −4.0 kcal mol^{-1} (1 cal = 4.184 J). Both the Y96A and F98A mutants had an interaction energy of lower than −3.0 kcal mol^{-1} over the region occupied by the respective aromatic sidechain in the wild-type. For both mutants there was a small volume contour at −4.0 kcal mol^{-1} centred over one side of the wild-type aromatic ring. For F87A much less interaction energy was predicted by the GRID program. It was decided in the first instance to mutate tyrosine 96 to alanine as it is more central to the binding pocket, whereas phenylalanine 98 is in a groove to one side. Also, removal of tyrosine 96 should alter the substrate specificity of the enzyme due to the loss of the polar hydroxide group.

Based on these considerations, a mutant protein was prepared which contained alanine instead of tyrosine at position 96 (Y96A). Further experimental details on gene cloning, mutagenesis and expression will be published elsewhere.

Binding of potential substrates was investigated by spectroscopic methods. The wild-type enzyme in the absence of substrate is in the six-coordinated low-spin form with a weakly bound water occupying the sixth coordination site, and shows a characteristic Sorét absorption maximum at 417 nm.[4] Upon binding of camphor **1**, the water dissociates and the haem is converted (96%) to the five-coordinated high-spin form, with a Soret maximum at 391 nm. Binding of the substrate analogues adamantanone **2**, adamantane **3** and norbornane **4** also fully converted the haem to the high-spin form. However, diphenylmethane **5** did not give a shift in the absorption spectrum.

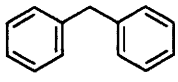
The Y96A mutant, while giving the same results as the wild-type enzyme for compounds **3** and **4**, was not fully converted to the high-spin form even when **1** and **2** were added in excess. As shown in Table 1, the apparent dissociation constants (K_{app}) for camphor and adamantanone are increased in Y96A. On the other hand, the K_{app} values for the hydrophobic substrates adamantane and norbornane are reduced, indicating that the enzyme pocket has become more selective for hydrophobic substrates. The greatest change in binding was observed with diphenylmethane, which bound poorly to the wild-type protein, but its affinity for the Y96A mutant was greatly enhanced and it induced a substantial (45%) shift of the mutant to the high-spin form.

The mutant Y96A was shown to catalyse the hydroxylation

of camphor **1**, although compared to the wild-type enzyme the reaction was slower (Table 1) and less selective, similar to the data reported for the mutant Y96F.[9] The decrease in selectivity can be attributed to the loss of the hydrogen bond between Y96 and camphor.

The monooxygenase activities of the enzymes (Table 1) were investigated by measuring the NADH turnover rates with **1–5** as substrates. In monooxygenase systems, the oxidation of one substrate molecule consumes one molecule of NADH. No NADH turnover above background was detected in incubations of the wild-type protein with **5**. On the other hand, the oxidation of **5** mediated by the Y96A protein was catalytic, with a NADH turnover rate of 6 s^{-1} at 30 °C. The organic extracts of the crude incubation mixture for this reaction† were analysed by gas chromatography. Only one major new peak was detected by GC (see Fig. 2), which had the same retention time as an authentic sample of *p*-hydroxydiphenylmethane **6**. The other potential aromatic hydroxylation products, the *ortho* and *meta* isomers, had different retention times. Further confirmation of the identity of the product as structure **6** was provided by mass spectrometry, which gave the correct mass peak at *m/z* 184. The total amount of product formed was estimated to correspond to at least 150 turnovers, showing that the reaction was clearly catalytic.

Table 1

Substrate		K_{app}/μmol dm^{-3} [a]		NADH turnover/s^{-1} [b]	
		WT	Y96A	WT	Y96A
	**1**	6.3	12	41	17
	**2**	12	28	27	8
	**3**	8.4	1.4	7	16
	**4**	330	92	2	13
	**5**	—	73	—	6

[a] Values are the average of two independent measurements using the method of Sligar.[10] The value of K_{app} is strongly dependent on the concentration of K^+ in the buffer. At $[K^+] > 150$ mmol dm^{-3}, K_{app} for camphor is 0.6 μmol dm^{-3} for both wild-type and Y96A. Data in this table were determined at $[K^+] = 70$ mmol dm^{-3} in phosphate buffer, pH 7.4, in order to avoid salting out of substrates at higher ion concentrations. [b] Assay conditions: reaction mixtures (1 cm^3) contained 0.05 μmol dm^{-3} $P\text{-}450_{cam}$, 0.05 μmol dm^{-3} putidaredoxin reductase, 1.6 μmol dm^{-3} putidaredoxin, 100 mmol dm^{-3} KCl, 40 mmol dm^{-3} KH_2PO_4 pH 7.4, and 1 mmol dm^{-3} substrate. NADH was added to a final concentration of 250 μmol dm^{-3} and the absorbance at 340 nm monitored over 150 s (T = 30 °C).

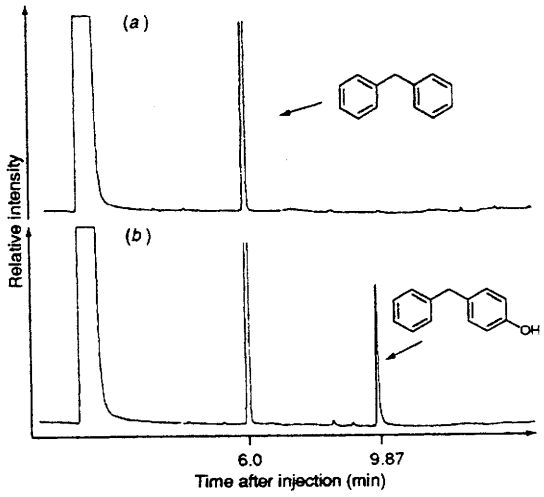


Fig. 2 Gas chromatogram of organic extracts from crude incubation mixtures† of diphenylmethane with wild-type enzyme (*a*) or mutant Y96A (*b*) (SGE 6m BP1 capillary column, 0.1 mm internal diameter, 0.10 micron coated polyimide fused silica)

In conclusion, this is the first report to show that rational redesign of cytochrome $P\text{-}450_{cam}$ substrate specificity is possible using site specific mutagenesis. By changing only one active site residue (tyrosine 96 to alanine) the affinity for camphor is reduced, but the modified substrate pocket allows tight binding of a novel substrate, diphenylmethane **5**, which is oxidised by the mutant in a regioselective manner to the *para*-hydroxy compound.

This research was supported by a British Gas plc/Biotechnology Directorate/Clean Technology Unit/DTI LINK programme to S. L. F. and L. L. W. We thank Mr R. Proctor for measuring mass spectra. L. L. W. thanks the Royal Society for a University Research Fellowship and support, and the EPA Research Fund for support. D. B. and A. C. G. W. thank British Gas plc for studentships, and D. P. N. thanks the British Council for a scholarship.

Received, 8th September 1994; Com. 4/05480F

Footnote

† *Incubation conditions for product detection by GC*: A solution containing 10 μmol dm^{-3} putidaredoxin, 2 μmol dm^{-3} putidaredoxin reductase, 1 μmol dm^{-3} cytochrome $P\text{-}450_{cam}$ monooxygenase (wild-type or Y96A mutant) and 1 mmol dm^{-3} diphenylmethane in 100 mmol dm^{-3} KCl, 20 mmol dm^{-3} KH_2PO_4 pH 7.4 was preincubated at 25 °C in a shaker for 5 min. The enzymatic reaction was initiated by firstly adding NADH to a total concentration of 2 mmol dm^{-3}. Further four aliquots of NADH (to increase the NADH concentration by 1 mmol dm^{-3} each time) were added in intervals of 5 min and the reaction quenched after 30 min by adding 0.5 cm^3 chloroform. The chloroform layer was analysed by gas chromatography.

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