Engineering the selectivity of aliphatic C–H bond oxidation catalysed by cytochrome P450cam

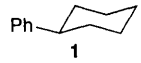
Nia E. Jones, Paul A. England, Duncan A. Rouch and Luet-Lok Wong*†

Inorganic Chemistry Laboratory, South Parks Road, Oxford, UK OX1 3QR

The regioselectivity of the catalytic hydroxylation of phenylcyclohexane 1 by cytochrome P450cam can be altered dramatically by site-directed mutagenesis: the Y96F single site mutant gives 81% *cis*-3-phenylcyclohexanol 2, with 22% enantiomeric excess (ee), while the Y96F–V247A double mutant gives 97% 2 with 42% ee and the Y96F–V247L double mutant gives 83% *trans*-4-phenylcyclohexanol 4.

The ubiquitous haem-dependent cytochrome P450 monooxygenases, which catalyse the oxidation of unactivated C–H bonds in biological systems,[1] have long been the target of rational protein redesign and engineering. The ability to engineer the selective oxidation of C–H bonds in unnatural substrates could have wide applications in synthesis. The most well-characterised P450 enzyme is cytochrome P450cam from *Pseudomonas putida*,[2] which catalyses the stereospecific oxidation of camphor to 5-*exo*-hydroxycamphor. The availability of a high resolution crystal structure,[3] and the great body of mechanistic information, makes P450cam an excellent system for protein engineering studies.

We have reported the engineering of P450cam by a single mutation (tyrosine-96 → alanine, hence the mutant Y96A) to oxidise diphenylmethane, which is not attacked by the wild-type enzyme, regiospecifically to *p*-hydroxydiphenylmethane.[4] We also showed that the wild-type P450cam and Y96A and Y96F[5] (F = phenylalanine) mutants hydroxylated phenylcyclohexane **1** exclusively on the cyclohexane ring.[6] We proposed that the selectivity for aliphatic C–H bond activation is due to preferential van der Waals interaction between the cyclohexane ring and aliphatic side-chains of amino acids (*e.g.* leucine-244, valine-247 and valine-295) in the immediate vicinity of the haem. The Y96A mutant also showed a very different selectivity pattern from the other two proteins, and we proposed that, since there is more room in the Y96A mutant, **1** is bound in a different orientation in this mutant. Here we report the effects of varying the size of the hydrophobic side-chain at the 247 position on the selectivity of the Y96A and Y96F mutants. The valine-247 → alanine (V247A) mutation decreases the size of the side-chain while the V247L (L = leucine) mutation increases it. Site-directed mutagenesis and the preparation of the recombinant forms of P450cam, and the associated electron transfer proteins putidaredoxin and putidaredoxin reductase,[7] were carried out following literature methods.



1

A major potential problem with protein engineering studies is the possibility that multiple mutations in the active site of a protein could result in significant rearrangement or even complete collapse of the active site pocket. We found, however, that the Y96–V247 double mutants had comparable stability to the wild-type P450cam protein.

Binding of substrates close to the haem in P450cam causes the dissociation of the haem iron's sixth ligand water, which results in the formation of a five coordinate high-spin haem.[8] The increase in the haem reduction potential which accompanies this spin-state change allows putidaredoxin to transfer the first electron to P450cam and for the catalytic cycle to commence.[9] The spin-state shift and NADH consumption rates observed with **1** as the substrate for the single mutants Y96A and Y96F, and the double mutants Y96A–V247A,Y96A–V247L, Y96F–V247A and Y96F–V247L are given in Table 1. The mutations at the 247 position did not affect the spin-state change with the Y96A mutant but in the Y96F mutant, the V247L mutation drastically reduced the observed shift. The turnover activities of the double mutants were significantly reduced compared to the single site mutants. As Sligar and Murray noted previously,[10] an important observation is that the extent of the spin-state shift is an indication of the likelihood of the first electron transfer, and cannot be used as a quantitative index of the overall turnover activity. This is because catalytic C–H bond activation by P450cam is a multi-step process, and unnatural substrates and different mutations may affect the subsequent steps in the catalytic cycle differently.

Preparative scale incubations[6] of **1** with all the P450cam double mutants were carried out, and the organic extracts were analysed by HPLC. The results showed that the selectivity for aliphatic C–H bond activation of **1**, previously observed with the single site mutants Y96A and Y96F, was completely maintained in all the double mutants. Furthermore, the three oxidation products previously characterised,[6] namely *cis*-3-phenylcyclohexanol **2**, *cis*-4-phenylcyclohexanol **3** and *trans*-4-phenylcyclohexanol **4**, were the only products observed with the double mutants (Table 2) except that the Y96F–V247L also gave a small amount (3%) of the *cis*-2-phenylcyclohexanol **5**, which was identified by co-elution with an authentic sample. There was no evidence of further oxidation of the alcohols at the end of the incubations, even with >95% substrate conversion.

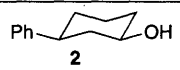
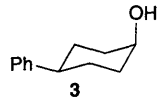
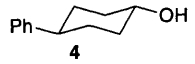
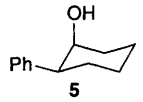
The product distributions for the single and double mutants are compared in Table 2. It is immediately evident that the mutations at the 247 position had no effect on the selectivity of the Y96A mutant, but dramatic changes were observed with the Y96F. We can infer that when the mutation at the 96 position is to an alanine, the substrate **1** is bound in such an orientation that

Table 1 Spin-state shift and NADH turnover data

Mutant	% High-spin haem[a]	NADH turnover rate[b]
Y96A	80	76
Y96F	60	55
Y96A–V247A	80	30
Y96A–V247L	80	27
Y96F–V247A	55	27
Y96F–V247L	5	14

[a] The percentage of high-spin haem in the presence of an excess of **1**. [b] Data given as nmol of NADH consumed per nmol of P450cam enzyme per minute. Incubation mixtures contained 50 mM Tris–HCl buffer pH 7.4, 1 μM P450cam, 2 μM putidaredoxin reductase, 10 μM putidaredoxin, 200 mM KCl and 200 μM of **1**, and were incubated at 30 °C for 5 min. NADH was then added to a final concentration of 500 μM and the absorbance at 340 nm was monitored.

Table 2 Selectivity of the hydroxylation of **1** by mutants of cytochrome P450cam

Products	Product distribution (%)[a]					
	Y96A[b]	Y96A–V247A	Y96A–V247L	Y96F[b]	Y96F–V247A	Y96F–V247L
 **2**	47 (2% ee)	45 (2% ee)	44 (2% ee)	81 (20% ee)	97 (42% ee)	9 (29% ee)
 **3**	34	37	38	13	0	8
 **4**	19	18	18	7	0	83
 **5**	0	0	0	0	3	0

[a] Determined by HPLC peak area integration. [b] Data from ref. 6.

the cyclohexane ring is sufficiently far-removed from the side-chain of valine-247 that even the V247L mutation does not alter the selectivity. If there were rearrangements of the local structures in the vicinity of the 247 position in the Y96A–V247A and Y96A–V247L double mutants, they were not sufficient to affect the selectivity of the oxidation of **1**.

The effects of the mutations at the 247 position in the Y96F mutant were dramatic. When the size of the side-chain at the 247 position was reduced with the V247A mutation, the substrate **1** must have moved significantly in relation to the haem iron such that the chiral alcohol *cis*-3-phenylcyclohexanol **2** constituted 97% of the products. A small amount (3%) of the chiral *cis*-2-phenylcyclohexanol **5** was also formed but, unlike the Y96F single site mutant, hydroxylation at the 4-position in **1** was not observed. The stereoselectivity of the formation of **3** was also improved, from 20% enantiomeric excess (ee) in the Y96F single site mutant to 42% ee in the Y96F–V247A double mutant. The same enantiomer was preferentially formed with both mutants, but whether it is the *R* or *S* form has not been established. In the case of the Y96F–V247L double mutant the substrate must have moved in a different direction such that *trans*-4-phenylcyclohexanol **4** was the major product (83%), while approximately equal amounts of **2** and **3** were also formed. We can conclude that the cyclohexane ring of **1** is in contact with the side-chain of valine-247 in the Y96F mutant, such that by varying the size of the side-chain at the 247 position it is possible to place a particular C–H bond closer to the haem iron centre. The results are also consistent with the earlier suggestion that the binding orientations of **1** in the Y96A and Y96F single site mutants are different.[6]

In conclusion, we have shown unequivocally that it is possible to manipulate the regioselectivity, and so far to a lesser extent the stereoselectivity, of the oxidation of unactivated C–H bonds by rational protein redesign. Furthermore, since the enantiomers of **2** are generated by attacks at the two chemically equivalent C_3 carbons in **1**, it should be possible to use other mutations to improve the stereoselectivity. The results described here should also constitute a useful set of benchmarks for computer modelling work aimed at understanding protein–substrate interactions.

L. L. W. thanks the Royal Society, the E.P.A. Research Fund and the Nuffield Foundation for equipment grants. P. A. E. thanks the EPSRC for a studentship.

Footnote

† E-mail: LUET.WONG@ICL.OX.AC.UK

References

1 *Cytochrome P-450: Structure, Mechanism and Biochemistry*, ed. P. R. Ortiz de Montellano, Plenum, New York, 2nd edn., 1995.
2 I. C. Gunsalus and G. C. Wagner, *Methods Enzymol.*, 1978, **52**, 166.
3 T. L. Poulos, B. C. Finzel and A. J. Howard, *J. Mol. Biol.*, 1987, **195**, 687.
4 S. M. Fowler, P. A. England, A. C. G. Westlake, D. A. Rouch, D. P. Nickerson, C. Blunt, D. Braybrook, S. West, L.-L. Wong and S. L. Flitsch, *J. Chem. Soc., Chem. Commun.*, 1994, 2761.
5 W. M. Atkins and S. G. Sligar, *J. Biol. Chem.*, 1988, **263**, 18 842.
6 P. A. England, D. A. Rouch, A. C. G. Westlake, S. G. Bell, D. P. Nickerson, M. Webberley, S. L. Flitsch and L.-L. Wong, *Chem. Commun.*, 1996, 357.
7 B. P. Unger, I. C. Gunsalus and S. G. Sligar, *J. Biol. Chem.*, 1986, **261**, 1158; J. A. Peterson, M. C. Lorence and B. Amarneh, *J. Biol. Chem.*, 1990, **265**, 6066; T. Yasukochi, O. Okada, T. Hara, Y. Sagara, K. Sekimizu and T. Horiuchi, *Biochim. Biophys. Acta*, 1994, **1204**, 84.
8 S. G. Sligar, *Biochemistry*, 1976, **15**, 5399.
9 S. G. Sligar and I. C. Gunsalus, *Proc. Natl. Acad. Sci. USA*, 1976, **73**, 1078.
10 S. G. Sligar and R. I. Murray, in *Cytochrome P-450: Structure, Mechanism and Biochemistry*, ed. P. R. Ortiz de Montellano, Plenum, New York, 1st edn., 1986.

Received, 12th August 1996; Com. 6/05597D