Engineering molecular recognition in alkane oxidation catalysed by cytochrome $P450_{cam}$

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The activity, selectivity and coupling efficiency of alkane oxidation by the monooxygenase enzyme cytochrome $P450_{cam}$ can be enhanced by engineering the active site topology to accommodate different alkanes: reducing the active site volume by the Y96F–V247L double mutations resulted in four-fold higher activity for the oxidation of hexane (**1**) than 3-methylpentane (**2**) while the larger active site of the Y96A–V247A double mutant gave rise to a two-fold preference for **2** over **1**.

The rational active site redesign of a monooxygenase enzyme for the substrate-specific and regioselective oxidation of simple alkanes is an interesting challenge in protein engineering and molecular recognition research, and may have industrial relevance. We have shown that the haem monooxygenase cytochrome $P450_{cam}$ can be re-engineered to increase its activity towards the oxidation of simple alkanes.[1,2] The natural substrate of $P450_{cam}$ is camphor, although the enzyme also has low activity towards the oxidation of small hydrophobic molecules such as styrene,[3,4] ethylbenzene,[5] and indeed as we showed, small alkanes such as hexane.[1] By replacing the polar residue tyrosine-96 (Fig. 1) with alanine and phenylalanine (the Y96A and Y96F mutants) which have hydrophobic side-chains, the alkane oxidation activity of $P450_{cam}$ was significantly increased, for some alkanes by two orders of magnitude.[1]

In the work described here, we have carried out rational redesign of the $P450_{cam}$ active site to investigate three important aspects of monooxygenase re-engineering, *i.e.*, to increase the alkane oxidation activity further, to impart selectivity between, for example, linear and branched alkanes, and to alter the regioselectivity of alkane oxidation. Our approach was to combine the Y96A and Y96F mutations, which had been shown to enhance the alkane oxidation activity of $P450_{cam}$,[1] with mutations at valine-247, a residue in closer proximity to the haem (Fig. 1). We sought to engineer the activity, substrate specificity and regioselectivity of alkane oxidation by varying the volume and topology of the $P450_{cam}$ active site with the combined Y96A–V247A and Y96F–V247L mutations.[6] Hexane (**1**) and 3-methylpentane (**2**) were used as model substrates.

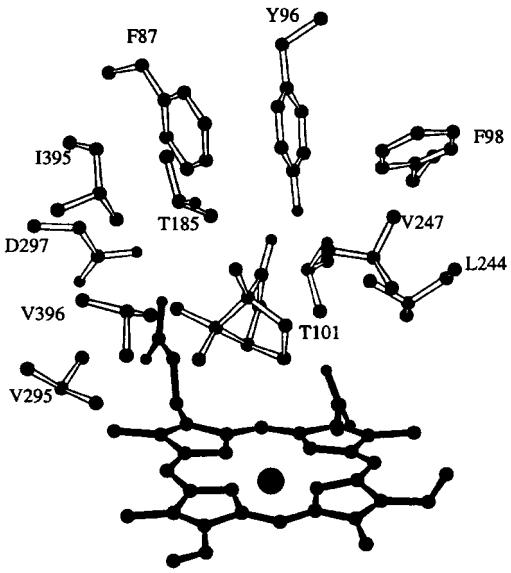

Fig. 1 Active site structure of wild-type $P450_{cam}$ with bound camphor showing the location of the Y96 and V247 side-chains

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Site-directed mutagenesis,[7] expression and purification of $P450_{cam}$,[8] and the electron-transfer proteins putidaredoxin reductase[9] and putidaredoxin[10] followed standard literature methods. Incubation mixtures (2.5 ml) contained 50 mM Tris–HCl, pH 7.4, 1 μM $P450_{cam}$, 16 μM putidaredoxin, 1 μM putidaredoxin reductase, 200 mM KCl and 40 $\mu g ml^{-1}$ bovine liver catalase. The alkane substrate (total 15 μmol) was added as a 1 M stock in ethanol. After incubation at 30 °C for 2 min, NADH was added to a final concentration of 400 μM and the absorbance at 340 nm monitored. Organics were then adsorbed onto a Varian Bond-Elut C_8 column (1 ml matrix volume) which was washed with 50 μM Tris–HCl buffer (1 ml), pH 7.4, and then dried under vacuum. The products were eluted from the column with 300 μl of $CHCl_3$. An internal standard was added and the mixture analyzed on a Fisons Instruments 8000 Series gas chromatograph using a DB-1 fused silica column (30 m × 0.25 μm i.d.) and flame ionisation detection. To obtain quantitative results, mixtures containing known concentrations of a product alcohol and all the incubation components except NADH were extracted and analyzed as described above. Linear calibration curves which passed through the origin were obtained for all the products.

The alkane oxidation activity and the coupling efficiency to NADH consumption for the oxidation of **1** and **2** by the double mutants are compared with the values for native $P450_{cam}$ and the single-site Y96 mutants in Table 1. With **1**, the alkane oxidation activities of both double mutants were higher than those of the Y96 single-site mutants, with the Y96F–V247L being by far the most active. With **2**, however, the Y96A–V247A double mutant was twice as active as the single-site mutants and approximately seven times as active as native $P450_{cam}$, whereas the activity of the Y96F–V247L double mutant was much lower, being comparable to the native enzyme. Therefore, our results show that although the Y96 single-site mutants showed comparable rates for the oxidation of **1** and **2**, the Y96F–V247L mutant showed four-fold higher activity towards the linear alkane **1** while the Y96A–

Table 1 The NADH turnover and total product formation rates, and coupling efficiency of alkane oxide catalyzed by wild-type and genetic variants of cytochrome $P450_{cam}$

	NADH turnover rate[a]					Total product formation rate[a] (% coupling)[b]				
Alkane	WT[c]	Y96A[c]	Y96F[c]	Y96A–V247A[d]	Y96F–V247L[d]	WT[c]	Y96A[c]	Y96F[c]	Y96A–V247A[d]	Y96F–V247L[d]
1	19.5	388.7	234.4	402.1	267.6	0.4 (2.0)	89.3 (23.0)	109.8 (47.0)	122.2 (30.2)	176.1 (66.8)
2	130.4	191.2	224.0	315.4	91.8	27.7 (21.3)	99.1 (52.0)	122.6 (54.7)	199.0 (63.6)	42.3 (50.6)

[a] All rate data given as nmol (nmol $P450_{cam}$)$^{-1}$ (min)$^{-1}$. [b] Coupling efficiency in brackets is the ratio of the total amount of product to the amount of NADH consumed in substrate dependent turnover expressed as a percentage. [c] Data from reference 1. [d] This work.

V247A showed approximately two-fold higher activity for the branched substrate **2**. Hence the mutations at V247 resulted in $P450_{cam}$ mutants which have significant selectivity between linear and branched alkanes.

In order to achieve high alkane oxidation activity it is advantageous to have a high coupling efficiency. The coupling efficiency, defined as the percentage of NADH consumed in substrate turnover which leads to the formation of product (*i.e.*, similar to the yield of a chemical reaction), is a measure of the complementarity between a substrate and the enzyme active site.[2,5,11] Camphor oxidation by native $P450_{cam}$ is totally efficient (100% coupling), presumably due to the evolutionally optimised enzyme–substrate fit. With the smaller substrate **1**, the Y96F–V247L mutant, which is expected to have the smallest active site of the enzymes studied here, showed the highest coupling efficiency of 67%, which is 33-fold higher than the efficiency of native $P450_{cam}$, and a significant improvement over the 47% coupling observed for the Y96F single-site mutant (Table 1). The V247A mutation only had a slight effect on the Y96A mutant with this linear substrate. With the sterically more demanding substrate **2**, however, the Y96A–V247A mutant showed the highest coupling of 64%. It may be noted that the oxidation of 1-methylnorcamphor, a close analogue of camphor, by wild-type $P450_{cam}$ shows a coupling efficiency of 45%.[12] Therefore the data show that the $P450_{cam}$ active site can be tailored to improve the fit for alkanes of different sizes and shapes, thus leading to increased coupling efficiency of substrate oxidation.

The selectivity of alkane oxidation is controlled by the orientation of substrate binding in the active site. Since C—H bond oxidation by P450 enzymes follows a radical mechanism, substrate mobility will result in attack at the most activated C—H bonds.[1,2] This is exemplified by the oxidation of **2** where all the $P450_{cam}$ enzymes studied preferentially attacked the tertiary C—H bond, giving 60–63% 3-methyl-3-pentanol (data not shown). Similarly, only attack at the secondary carbons was observed for the linear alkane **1**. However, while native $P450_{cam}$ and the Y96 single-site mutants gave approximately equal amounts of 2- and 3-hexanol,[1] the Y96F–V247L mutant showed slightly increased selectivity for attack at C3 (67%) over C2 (33%). These data suggest that **1** is being constrained somewhat in the smaller active site of Y96F–V247L. Consistent with this argument, the Y96A–V247A mutant, which should have a larger substrate pocket, showed virtually no selectivity. Inspection of the $P450_{cam}$ active site structure (Fig. 1) suggests that while V247 is closer to the haem than Y96, even the V247L mutation is likely to leave sufficient space to allow substrate mobility, especially conformational mobility. Therefore, fine tuning by mutations at residues even closer to the haem, *e.g.*, V295 and V396, should significantly alter the regioselectivity of alkane oxidation.

In conclusion we have shown that the $P450_{cam}$ active site can be manipulated by protein engineering to accommodate a particular substrate; in this instance the smaller active site of Y96F–V247L is more complementary to the linear hexane molecule, but is too small to accommodate 3-methylpentane readily. Conversely the larger active site of Y96A–V247A is better suited to the bulkier 3-methylpentane. High coupling efficiencies (64 and 67%) were observed, but the regioselectivity of alkane oxidation was low. These results suggest that the coupling efficiency is a global measure of the achievement of good enzyme–substrate complementarity, and that local interactions between the substrate and side-chains, especially those close to the active centre, will control the regioselectivity of substrate oxidation. Our approach of moving from residues far away from the haem (*e.g.*, Y96) to those which are increasingly close to the iron has given rise to mutants which have significant selectivity between different alkanes, high alkane oxidation activity and coupling efficiency. Further fine tuning of the selectivity between alkanes and the regioselectivity of alkane oxidation will therefore be based on a platform of mutants with these desirable properties.

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