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Butane and propane oxidation by engineered cytochrome $P450_{cam}$

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The haem monooxygenase cytochrome $P450_{cam}$ has been engineered to oxidise the gaseous alkanes butane and propane to butan-2-ol and propan-2-ol, respectively, by the use of bulky amino acid substitutions to reduce the volume of the substrate pocket and thus improve the enzyme–substrate fit: the F87W/Y96F/T101L/V247L mutant oxidises butane with a turnover rate of 750 min^{-1} and 95% yield based on NADH consumed while the wild-type enzyme has an activity of 0.4 min^{-1} with 4% yield.

The selective oxidation of simple alkanes to alcohols under ambient conditions, without further oxidation to aldehydes/ketones or deep oxidation to carbon dioxide, is a reaction of great industrial importance. The oxidation of methane to methanol is of course a prime objective of catalysis research, but oxidation of the other gaseous alkanes to the alcohols could also afford new feedstocks for chemical synthesis. Solid state heterogeneous catalysts require elevated temperatures and pressures. On the other hand biological catalysts such as haem[1] and non-haem monooxygenases[2,3] catalyse alkane oxidation under mild conditions and, as such, are potential candidates for environmentally friendly, green chemistry alkane oxidation catalysts.

Certain yeast strains can grow on long-chain (C_8–C_{16}) linear alkanes as the only carbon and energy source by utilising the haem-dependent cytochrome P450 monooxygenase for the initial alkane oxidation step.[4] However, the oxidation of gaseous alkanes by any P450 enzyme has not been reported. We have explored the protein engineering of cytochrome $P450_{cam}$ from *Pseudomonas putida* [5] for the oxidation of a range of organic compounds,[6–8] including pentane and hexane.[9,10] We proposed and showed that the specificity of $P450_{cam}$ could be biased towards linear alkanes against the branched isomers by reducing the active site volume with bulky amino acid substitutions such as Val → Leu.[10] Here we report the further engineering of $P450_{cam}$ for the oxidation of butane and propane.

Our starting point for $P450_{cam}$ engineering is the Y96F/V247L double mutant which favours the oxidation of hexane over 3-methylpentane.[10] We reasoned that butane and propane oxidation could be promoted by further reducing the active site volume to improve the enzyme–substrate fit and to constrain these small molecules to bind closer to the haem. Substrate binding close to the haem expels the water molecules in the active site of substrate-free $P450_{cam}$, which not only converts the haem to high spin and thus give rise to faster rates of NADH turnover but also suppresses the uncoupling pathway that forms hydrogen peroxide.[11,12] Substrate binding close to the haem increases the probability of C–H bond oxidation by the ferryl intermediate, thus suppressing the oxidase uncoupling pathway.[12]

The active site structure of wild-type $P450_{cam}$ with bound camphor is shown in Fig. 1.[13] Both the Y96 and V247 side-chains are located high in the active site pocket. In order to constrain butane and propane close to the haem, we replaced phenylalanine-87 with tryptophan (F87W) which has a sterically more demanding indole side-chain. In addition, we attempted to decrease the space available in the vicinity of the haem by the threonine-101 → leucine (T101L) mutation. The butane and propane oxidation activity of wild-type $P450_{cam}$ and the mutants Y96F, F87W/Y96F, Y96F/V247L, F87W/Y96F/T101L, F87W/Y96F/V247L and F87W/Y96F/T101L/V247L were determined.

In analysing the products of butane and propane oxidation, we found that extraction with an organic solvent such as chloroform,[14] or solid-phase techniques[9] were unsatisfactory. The aqueous incubation mixtures were therefore injected directly onto a SPB-1 column (0.5 mm ID × 60 m). This column tolerated water, and clearly resolved all the C_1–C_4 alcohols, aldehydes and ketones. We found that wild-type $P450_{cam}$ and all the active site mutants oxidised butane to butan-2-ol, and propane to propan-2-ol, with no evidence for the formation of the terminal alcohols or the further oxidation products butan-2-one and acetone.

The butane and propane oxidation activity and coupling efficiency (defined as the percentage of NADH consumed that lead to product formation, *i.e.* the yield based on NADH) for the $P450_{cam}$ enzymes are given in Table 1. The wild-type enzyme has low but detectable activity. As observed previously for pentane and hexane,[9] the Y96F mutant is two orders of magnitude more active than the wild-type for butane and propane oxidation. The data show that, in general, reducing the active site volume by bulky amino acid substitutions increases the alkane oxidation activity, consistent with our design rationale. Butane oxidation is found to be faster than propane, with both higher NADH turnover rates and coupling efficiencies observed for the larger butane substrate. These trends probably reflect the lower complementarity between the mutants and the smaller propane molecule. We were surprised by the high alkane oxidation activities conferred by just two

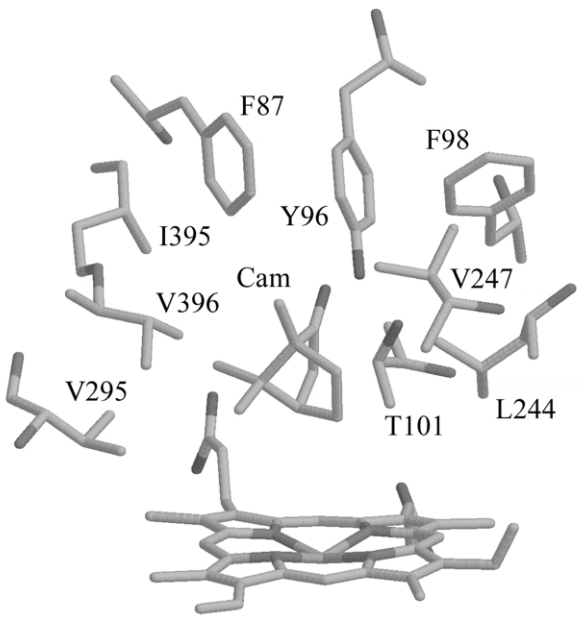

Fig. 1 The active site structure of $P450_{cam}$ with bound camphor.

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Table 1 The NADH oxidation and alcohol product formation rate and coupling efficiency of butane and propane oxidation catalysed by wild-type cytochrome $P450_{cam}$ and active site mutants. Rates are given in nmol (nmol $P450_{cam}$)$^{-1}$ (min)$^{-1}$. The coupling efficiency is defined as the proportion of NADH consumed which is utilised by the enzyme for product formation and is given as a percentage. All values are means of at least 6 separate experiments, with all data within 15% of the respective means

	Butane			Propane		
	NADH oxidation rate[a]	Butan-2-ol formation rate[b]	Coupling efficiency	NADH oxidation rate[a]	Propan-2-ol formation rate[b]	Coupling efficiency
Wild-type	10	0.4	4%	8	0.08	0.9%
Y96F	100	42	42%	18	2.2	12%
F87W/Y96F	200	132	66%	60	12	20%
Y96F/V247L	250	186	75%	110	43	39%
F87W/Y96F/T101L	540	460	85%	198	51	26%
F87W/Y96F/V247L	263	88	34%	82	13.7	17%
F87W/Y96F/T101L/V247L	795	750	95%	346	110	32%

[a] Incubations were carried out in cuvettes equipped with a screw cap and a Teflon septum. Mixtures (1.5 mL) contained 50 mM Tris pH 7.4, 1 μM $P450_{cam}$, 16 μM putidaredoxin, 1 μM putidaredoxin reductase, 200 mM KCl and 50 μg mL catalase. The mixture was oxygenated and the alkane substrate was added as a saturated solution in 50 mM Tris, pH 7.4. The mixture was incubated at 30 °C for 2 min and NADH added to 400 μM by piercing the septum with a syringe. The absorbance at 340 nm was monitored and the NADH turnover rate calculated using $\varepsilon_{340} = 6.22\ mM^{-1}\ cm^{-1}$ [b] A 90 μL aliquot of an incubation mixture was mixed with 10 μL of a 200 μM solution of the internal standard pentan-1-ol in water. The mixture was injected directly onto a SPB-1 gas chromatography column. The column temperature was held at 40 °C for 4 min and then raised at 5 °C min^{-1} to 100 °C. The retention times were: propan-2-ol, 4.35 min, butan-2-ol 6.53 min, pentan-1-ol 12.2 min.

mutations, *viz*. the F87W/Y96F and Y96F/V247L double mutants. The very significant effects of the T101L mutation most likely arise from both decreased active site volume and increased active site hydrophobicity. Of the mutants studied in this work the F87W/Y96F/T101L/V247L quadruple mutant has the highest activity. The butane turnover rate of 750 min^{-1} (2000 times faster than the wild-type enzyme) is quite phenomenal because it is comparable to the camphor oxidation rate by the wild-type enzyme (1200 min^{-1}) under identical conditions, and the 95% coupling efficiency is no less significant. Whilst the propane turnover rates are lower, it is important to note that the activity trend for propane mirrors that for butane, suggesting that high propane oxidation activity may be achieved with additional mutations that further reduce the active site volume.

Butane and propane are oxidised exclusively at the secondary C–H bonds. The absence of attack at the primary C–H bonds reflects substrate binding orientation and mobility which allow the ferryl intermediate to select the more activated secondary C–H bonds.[9] Molecular dynamics simulations of camphor oxidation by wild-type $P450_{cam}$ suggested that the camphor C5 and C9 are at the same distance from the ferryl oxygen but only the more activated, secondary C–H bond at C5 is attacked.[15] Hence primary C–H bond oxidation would only be observed if the substrate is constrained such that only primary C–H bonds are close to the haem.

In conclusion we have used rational protein engineering to generate, for the first time, a P450 enzyme capable of rapid and efficient oxidation of gaseous alkanes. We also attempted ethane and methane oxidation with the mutants but the activity was barely detectable by gas chromatography. We note from the data in Table 1 that the alkane oxidation activity for each mutant dropped by approximately an order of magnitude from butane to propane. It is likely then that the ethane oxidation activity will be at least an order of magnitude lower than for propane for the mutants studied here, but equally we expect the activity to increase when other appropriate mutations are introduced. The exciting prospect remains of engineering $P450_{cam}$ into a methane monooxygenase.

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