The Catalytic Oxidation of Linear and Branched Alkanes by Cytochrome $P450_{cam}$

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The selective catalytic oxidation of simple alkanes under mild conditions is one of the most difficult chemical reactions to achieve using conventional synthetic methods. However, the non-heme diiron methane monooxygenase enzymes from *Methylococcus capsulatus* (Bath)[1] and *Methylosinus trichosporium* OB3b[2] catalyze the hydroxylation of methane and higher homologues. Less well-characterized are the heme-dependent cytochrome P450 alkane hydroxylases found in yeast[3] and bacteria[4,5] and the non-heme iron alkane hydroxylase from *Pseudomonas oleovorans GPo1*.[6] Here we report the protein engineering of the heme monooxygenase cytochrome $P450_{cam}$ to investigate factors which promote the oxidation of simple alkanes. The natural substrate of $P450_{cam}$ is camphor,[7,8] and although the enzyme has been shown to oxidize a number of unrelated substrates,[9−12] the turnover of simple alkanes has not been described.

The high-resolution crystal structure of $P450_{cam}$ shows that the substrate-binding pocket is lined mostly with hydrophobic amino acid side chains.[13] The most hydrophilic side chain is that of tyrosine-96. Since alkanes are highly hydrophobic, we have therefore changed this residue to alanine (the Y96A mutant[14,15]) and phenylalanine (Y96F[16,17]) and investigated the catalytic activity of wild-type $P450_{cam}$ and these two mutants toward simple alkanes.

Monooxygenase activity of $P450_{cam}$ requires two electrons which are passed from NADH to $P450_{cam}$ via the electron transfer proteins putidaredoxin and putidaredoxin reductase. The NADH turnover rates of the wild-type and two mutants in the fully reconstituted system with a number of linear and branched alkanes as substrates are listed in Table 1. The alkanes were pentane (**1**), hexane (**2**) heptane (**3**), 2-methylpentane (**4**), 3-methylpentane (**5**), and 2-methylhexane (**6**). For the linear alkanes, the Y96A and Y96F mutants were up to 19 times as active as the wild-type. Smaller differences were observed with the branched alkanes. This data suggest that the increased hydrophobicity of the active sites of the mutants does indeed promote alkane oxidation.

Table 1. The NADH Turnover and Total Product Formation Rates and Coupling Efficiency of Alkane Oxidation Catalyzed by Wild-Type $P450_{cam}$ and the Y96A and Y96F Mutants

alkane substrate	NADH turnover rate[a]			product rate[b] (% coupling)[c]		
	WT	Y96A	Y96F	WT	Y96A	Y96F
1	34.8	419.6	404.8	0.9 (2.6)	60.8 (14.5)	105.7 (26.1)
2	19.5	388.7	234.4	0.4 (2.0)	89.3 (23.0)	109.8 (47.0)
3	7.9	103.4	55.9	0.2 (2.5)	25.7 (25.0)	18.3 (32.7)
4	77.1	210.9	222.0	5.9 (7.7)	86.0 (40.8)	97.0 (43.7)
5	130.4	191.2	224.0	27.7 (21.3)	99.1 (52.0)	122.6 (54.7)
6	31.6	90.7	57.8	1.7 (5.3)	18.9 (20.8)	17.9 (30.9)

[a] Given as nanomoles of NADH consumed per nanomoles of $P450_{cam}$ per minute. 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 μg mL^{-1} bovine liver catalase. Alkane substrate (15 μmol) was added as a 1 M stock in ethanol. The mixture was preincubated at 30 °C for 2 min, NADH was added to a final concentration of 400 μM, and the absorbance at 340 nm was monitored. [b] The total amount of alkane oxidation products formed (nmol) per nanomole of $P450_{cam}$ per minute. Organics from a turnover incubation were adsorbed onto a Varian Bond-Elut C_8 column (1 mL matrix volume). The column was washed with 1 mL of 50 mM Tris−HCl buffer, 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 was analyzed on a Fisons Instruments 8000 Series gas chromatograph using a DB-1 fused silica column (30 m × 0.25 mm i.d.) and flame ionization detection. To obtain quantitative results, mixtures containing known concentrations of a product alcohol and all of 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. [c] The coupling is the ratio of the total amount of products formed to the amount of NADH consumed and is expressed as a percentage.

The NADH turnover rates are however not the same as the product formation rates because the P450 catalytic cycle is susceptible to uncoupling (*i.e.*, not every molecule of NADH consumed leads to substrate conversion).[18,19] Therefore, the actual alkane oxidation activity can only be assessed by quantitation of the products formed. In these experiments we found that hydrogen peroxide generated through uncoupling[19] gradually inactivated the enzyme system. The inclusion of catalase in the reactions to remove the peroxide[10,20,21] allows the quantitation of products even from highly uncoupled reactions, enabling the effects of mutations on the catalytic activity of the $P450_{cam}$ enzyme to be accurately assessed.

The total product formation rates and derived coupling efficiencies of alkane oxidation by all three proteins are listed in Table 1. With the linear alkanes, the Y96A and Y96F mutants showed much higher product formation rates (*ca*. 100-fold) than the wild-type. These arose from both higher NADH turnover rates and increased coupling efficiencies of the mutants. With the branched alkanes, the differences were smaller (*ca*. 10-fold), particularly for **5** for which the mutants were only approximately three times as active as the wild-type.

For all the alkanes, the coupling efficiencies of the three enzymes were in the order Y96F > Y96A ≫ wild-type. The

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Table 2. Product Distributions for the Oxidation of Hexane and 2-Methylpentane

substrate/ protein	product/distribution (%)[a]		
hexane	2-hexanol	3-hexanol	2- and 3-hexanone
WT	43	57	
Y96A	47	53	
Y96F	37	60	3
2-methyl-pentane	2-methyl-2-pentanol	2-methyl-3-pentanol	4-methyl-2-pentanol
WT	60	16	24
Y96A	60	11	29
Y96F	78	7	15

[a] Values were obtained from GC peak area integrations.

primary uncoupling pathway has been proposed to occur when the ferric−peroxo intermediate is accessible to water molecules, leading to protonation of the heme-bound oxygen and the release of hydrogen peroxide.[9] The wild-type $P450_{cam}$ substrate-binding pocket is optimized for camphor, therefore water is excluded from the active site and the reaction shows a coupling of 100%.[22] In contrast, the size and shape of simple alkanes do not match the substrate pocket, thus it is likely that there is sufficient room for water to enter the active site during the catalytic cycle, resulting in uncoupling. Among the limited range of alkane substrates studied so far, no clear pattern emerges, although the best protein−substrate match appears to be with **5**. The order of coupling efficiency for the enzymes could arise in part from differences in the hydrophobicity and size of the active site pockets. The more hydrophobic active sites of the Y96A and Y96F mutants may exclude water more effectively than the wild-type protein, and thus the reactions are much more coupled. It is likely that the Y96A mutant has a larger substrate pocket than the Y96F which could explain the lower coupling with the Y96A mutant.

The product distributions for hexane and 2-methylpentane oxidation are shown in Table 2. For all of the alkanes the order of preference for C−H bond attack was tertiary > secondary, with no attack at primary C−H bonds. This is consistent with the radical rebound mechanism proposed for C−H activation by P450 enzymes[23,24] and suggests that the alkanes are somewhat mobile within the active site, such that the ferryl species can attack the more activated tertiary or secondary C−H bonds. In this respect the wild-type and Y96 mutants of $P450_{cam}$ behave very differently to the heme[3,4,5] and non-heme alkane hydroxylases,[6] which give mainly terminal C−H bond oxidation even with branched alkanes. It is possible that the natural alkane hydroxylases have more channel-like active sites with less space in the vicinity of the hydroxylation center such that only the terminal methyl group can approach sufficiently close to the iron center for attack.

In summary, we have shown that cytochrome $P450_{cam}$ catalyses the oxidation of simple alkanes and that the activity can be greatly enhanced by mutations at a single active site residue which increase the hydrophobicity of the substrate pocket. However, since the $P450_{cam}$ substrate pocket is optimized for camphor binding, a single mutation is not sufficient to bind the alkane molecule in one specific conformation, and thus the proteins exhibit little regioselectivity of alkane C−H bond oxidation. It could be envisaged that further mutations at strategic locations in the active site could lead to binding of alkanes in specific conformations, resulting in regioselective alkane hydroxylation.

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