Enzyme Engineering

The Heme Monooxygenase Cytochrome $P450_{cam}$ Can Be Engineered to Oxidize Ethane to Ethanol**

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The selective catalytic oxidation of alkanes to alcohols under ambient conditions is a great scientific challenge and is of potential industrial and economic importance. Heterogeneous catalysts require elevated temperatures and pressures, and further oxidation to aldehydes and carbon dioxide is prevalent. Although biological catalysts are attractive because they operate under mild conditions, the only enzymes capable of oxidizing ethane and methane are the copper and non-heme iron methane monooxygenases.[1–5] Of the other classes of monooxygenases, the heme-dependent cytochrome P450 enzymes, which use two electrons from NAD(P)H to activate oxygen and generate a ferryl intermediate $[(Por)^{+}Fe^{IV}=O]$ to attack C–H bonds, are known to oxidize a wide range of organic molecules, but not ethane and methane (Por = porphyrin).[6,7] We have explored the engineering of $P450_{cam}$ from *Pseudomonas putida* [8] for alkane oxidation.[9,10] The approach of varying the $P450_{cam}$ active-site volume to fit a non-natural substrate was first used by Loida and Sligar for ethylbenzene oxidation.[11,12] We have used bulky amino acid substitutions in the $P450_{cam}$ active site to promote the binding and oxidation of gaseous alkanes. The mutants showed fast and efficient *n*-butane oxidation, but propane oxidation activity was modest, and no ethane oxidation was observed.[10] This approach of decreasing the active-site volume has been combined with directed evolution to engineer $P450_{BM-3}$ from *Bacillus megaterium* to oxidize propane with a turnover rate of 54 min^{-1}.[13] Herein, we report the engineering of $P450_{cam}$ to provide the first example of ethane oxidation by a P450 enzyme.

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The most active $P450_{cam}$ mutant for propane oxidation reported previously was the F87W/Y96F/T101L/L1244M/V247L mutant (the EB mutant).[10] We determined the crystal structure of the substrate-free form of the precursor mutant F87W/Y96F/V247L to a resolution of 2.1 Å to provide insight into the effects of the mutations.[14] The active-site structure is shown in Figure 1. The most interesting feature is that there

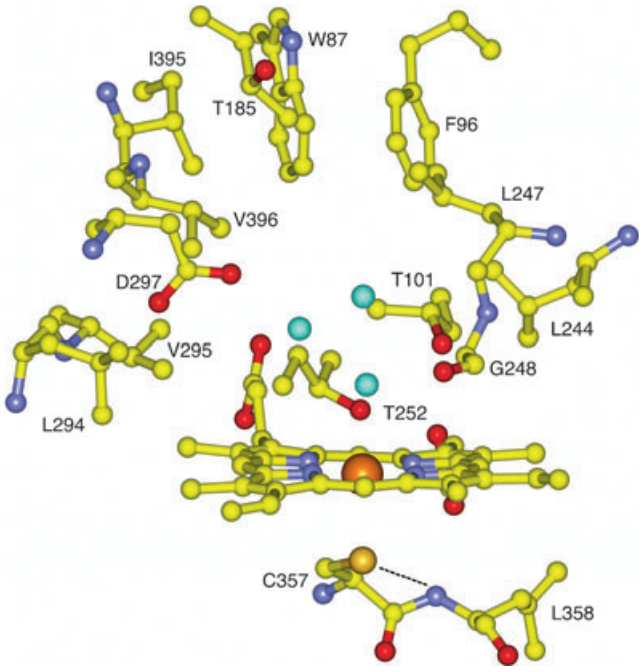

Figure 1. The active-site structure of the substrate-free form of the F87W/Y96F/V247L mutant of $P450_{cam}$ highlighting the cluster of three water molecules (light blue) in the substrate pocket and the hydrogen bond between the thiolato side chain of C357 and the main-chain amide of L358.

are only three water molecules in the active site, compared with six in the wild-type enzyme structure.[15] This probably results from the decreased active site volume, and is consistent with the increased activity of the mutant for the oxidation of small molecules such as propane. In the work discussed herein, we introduced additional mutations to the EB mutant to further decrease the size of the substrate-binding pocket. We examined the structure (Figure 1) for cavities within the active site that could bind a small molecule such as ethane away from the heme iron center. Mutations were then introduced to close such cavities. The $P450_{cam}$ active site is highly irregular in shape, and cavities were found between L294 and T252, L247 and G248, and in the A296–D297 region. The cavity between the W87, L247, and V396 side chains at the top of the active site is covered by T185. Therefore, the mutations L294M, G248A, and T185M were introduced to force ethane to bind closer to the heme iron center.

Morishima and co-workers recently reported the interesting effects of the L358P mutation.[16,17] The main-chain amide of L358 forms a hydrogen bond to the thiolato side chain of C357, thus modulating the donor strength of this heme-

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proximal ligand.[18] The L358P mutation removes this hydrogen bond and alters the reduction potential of the heme, but it also promotes the oxidation of non-natural substrates.[16] We therefore investigated the effect of this mutation on alkane oxidation. While this work was in progress, the crystal structure of the L358P mutant was reported.[17] Among the interesting structural changes, it was found that the heme was pushed into the substrate pocket towards the substrate; a general tightening of the pocket was also observed. These changes may promote the binding of non-natural substrates that do not fit as well into the pocket as does camphor.

As none of the previous $P450_{cam}$ mutants oxidized ethane, we first examined the propane oxidation activity of the new mutants;[19] those with the highest activity were then tested for ethane oxidation. The results are given in Table 1. All the mutants showed $>90\%$ high-spin heme content upon propane binding (data not shown). The L294M mutation increased the NADH oxidation rate for propane by 50% relative to the EB mutant, but decreased the coupling efficiency (product yield based on NADH consumed). Overall, the propane oxidation rate was slightly increased. Interestingly, the EB/L294M mutant gave readily detectable amounts of propan-1-ol, whereas all previous $P450_{cam}$ mutants gave propan-2-ol exclusively. Hence the L294M mutation decreased the mobility of propane within the active site such that the higher intrinsic reactivity of secondary C–H bonds in the radical mechanism no longer dominated over that of the larger number of the less reactive, primary C–H bonds. The T185M mutation significantly increased the coupling, resulting in another increase in the propane oxidation activity. The L358P mutation increased both the activity and coupling, in agreement with previous reports on the effects of this mutation on the oxidation of non-natural substrates. Adding the G248A mutation increased both the activity and coupling further. The fast propane oxidation rate ($500\ min^{-1}$) and excellent coupling (86%) of the EB/L294M/T185M/L1358P/G248A mutant indicated good fit between the engineered $P450_{cam}$ active site and propane.

The EB/L294M/T185M/L1358P/G248A mutant was examined for ethane oxidation. The heme shifted to $>85\%$ high spin in the presence of ethane (Figure 2). We were surprised that the NADH oxidation activity ($741\ min^{-1}$)[20] was higher than that for propane. GC analysis showed that ethane was oxidized to ethanol (no ethanal was formed) at a rate of $78.2\ min^{-1}$, which corresponds to 10.5% coupling (Figure 3).[21] There was more peroxide uncoupling (40%) than observed with propane (15%). Therefore, the oxidase uncoupling pathway accounted for $\approx 50\%$ of the NADH consumed in ethane oxidation. This pathway becomes significant if the ethane substrate is bound too far away from the ferryl oxygen atom for rapid substrate oxidation,[12] such that two-electron reduction of the ferryl to the ferric state competes with substrate oxidation. The drop in coupling from propane (86%) to ethane (10%) is surprisingly large, indicating the stringent requirement of the active site architecture to localize ethane close to the heme iron center. It also explains why the other mutants did not show readily detectable ethane oxidation activity.

The EB/L294M/T185M/L1358P/G248A mutant also had the interesting property of being $\approx 45\%$ high spin even in the absence of substrate (Figure 2), whereas all other mutants

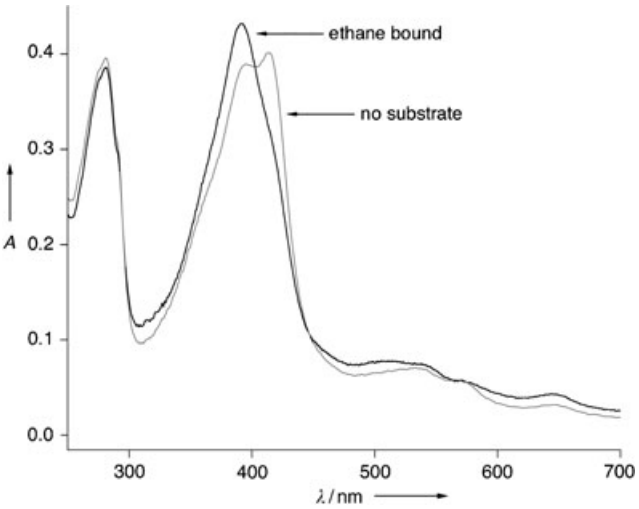

Figure 2. The Soret region of the electronic spectrum of the EB/L294M/T185M/L1358P/G248A mutant of $P450_{cam}$ showing the partial high-spin heme nature of the mutant in the absence of substrate and the $>85\%$ high-spin heme content upon binding of ethane.

Table 1: Propane and ethane oxidation activity of $P450_{cam}$ mutants.[a]

$P450_{cam}$ enzyme	Propane				Ethane			
	NADH rate	product rate[b]	coupling [%][c]	H_2O_2 [%]	NADH rate	product rate[b]	coupling [%][c]	H_2O_2 [%]
F87W/Y96F/T101L/V247L/L1244M[d] (EB mutant)	266	176 (<2%)	66.2	25.0	<20	–	–	–
EB/L294M	414	193 (5.8%)	46.8	38.0	<20	–	–	–
EB/L294M/T185M	302	228 (3.4%)	75.6	23.4	45	–	–	–
EB/L294M/T185M/L1358P	462	379 (3.6%)	81.9	12.4	269	<10	<4	30.0
EB/L294M/T185M/L1358P/G248A	590	505 (4.2%)	85.6	15.0	741	78.2	10.5	39.6

[a] The rates of NADH turnover and product formation are given in nmol (nmol P450)$^{-1}$ min^{-1}. The data represent the averages of at least four experiments, with all data for each parameter within 15% of the mean. [b] The dominant product of propane oxidation was propan-2-ol; the percentage of propan-1-ol is shown in brackets. Ethane was oxidized to ethanol with no evidence for ethanal formation (by GC). [c] The percentage of NADH consumed that was channeled to product formation. [d] Data from Ref. [10]. (–: The ethanol product concentration was not sufficiently high above the background to be reliably determined.)

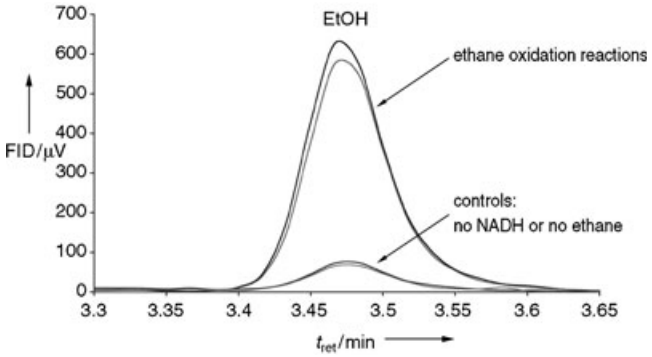

Figure 3. GC analysis of ethane oxidation assays with the EB/L294M/T185M/L1358P/G248A mutant. The controls with no ethane or no NADH added show the residual background peak from ethanol (< 1 μM) present in the water used to prepare the buffers. The apparent difference in the ethanol product peak areas for two separate incubations was corrected by the ratio to the pentan-1-ol internal standard peak. t_{ret} = retention time.

were low spin. The six-water cluster in substrate-free $P450_{cam}$ has strong hydrogen bonding, and the heme-bound water ligand is likely to have significant hydroxide ion character. Raag and Poulos noted the importance of hydrogen bonding to the heme ligand water: wild-type $P450_{cam}$ with non-natural substrates bound could have a six-coordinate heme, yet the iron center was high spin. Therefore, the presence of this sixth ligand on its own was not sufficient to bring the heme to a low-spin state.[22] Model compound studies have also shown that the P450 heme group is low spin if the axial water ligand is hydrogen bonded to other groups, but is high spin if there is no such hydrogen bonding.[23] The axial water ligand in the F87W/Y96F/V247L mutant is hydrogen bonded to two other waters, and the heme is low spin. As the EB/L294M/T185M/L1358P/G248A mutant contains five additional bulky substitutions within the active site, it is very likely that the three-water cluster observed in the F87W/Y96F/V247L mutant would be perturbed significantly, leaving only two or even one water molecule in the active site. Fewer active site water molecules and weakened hydrogen bonding allows ethane to bind more readily, and in this case, to induce a heme-group shift to > 90% high spin and a fast NADH turnover rate. Hence the EB/L294M/T185M/L1358P/G248A mutant has not only the interesting property of ethane oxidation, but its active site water structure could provide new insight into the origin of the heme spin state equilibrium in P450 enzymes.

In summary, we have engineered a cytochrome P450 enzyme to oxidize ethane to ethanol by decreasing the active site volume with bulky substitutions, and by altering the hydrogen bonding to the proximal ligand by the L358P mutation first reported by Morishima.[16,17] The high spin heme content of the EB/L294M/T185M/L1358P/G248A mutant in the absence of substrate suggests that it may also be a useful platform for structure–function studies of P450 enzymes. Finally, the high NADH oxidation rate of this mutant with ethane suggests that, as we had shown for *n*-butane and propane, fast ethane oxidation will be possible once the uncoupling pathways are suppressed by localizing ethane close to the ferryl oxygen atom.

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[14] We had reported earlier that one molecule in the *P*1 asymmetric unit in the X-ray crystal structure of the F87W/Y96F/V247L mutant complexed with 1,3,5-trichlorobenzene had low substrate occupancy, and that the density above the heme could be modeled with three water molecules, but their locations could not be refined (X. Chen, A. Christopher, J. P. Jones, S. G. Bell, Q. Guo, F. Xu, Z. Rao, L. L. Wong, *J. Biol. Chem.* **2002**, *277*, 37519). The structure of the substrate-free form of the mutant was therefore determined. Procedures for crystallization of the mutant at 291 K by the hanging drop vapor diffusion method and data collection and refinement were as reported previously. Crystals of the F87W/Y96F/V247L mutant belonged to the space group $P2_1$, with unit-cell dimensions: $a = 66.8$ Å, $b = 62.1$ Å, $c = 94.9$ Å, $\alpha = 90°$, $\beta = 90.5°$, $\gamma = 90°$. A total of 236028 reflections were measured, with R_{merge} of 7.6% for 45978 unique reflections and 99.9% completeness (50–2.1 Å). Data were collected to 100% completeness in the highest resolution shell. The structure was solved by molecular replacement based on the crystal structure of wild-type $P450_{cam}$ (PDB code: 2CPP), but with the camphor removed. After initial refinement, the difference Fourier map for both molecules in the unit cell showed well-defined triangle-shaped electron density above the heme group that was modeled by three water molecules. The final refinement parameters were $R_{work} = 19.1\%$ and $R_{free} = 24.3\%$. Full details will be published elsewhere.
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[19] General methods for mutagenesis, enzyme expression and purification, activity and GC analysis for propane were carried out as described previously.[10]
[20] The ethane oxidation assays required extra precautions. Ethanol was present in varying concentrations in most components of an activity assay, even in the ultra pure water obtained from water purification systems. Only water containing <500 nM ethanol (GC) were used for buffer preparation. Buffer solutions were then analyzed by GC to ensure that there was no ethanol in the chemicals used. Commercial NADH contains ethanol as a stabilizer. This ethanol was removed by washing with diisopropyl ether: 0.5-mL aliquots of a NADH solution (20 mg mL^{-1}) were mixed with 2 mL diisopropyl ether, vortexed and then centrifuged at 3000 rpm for 5 min. The organic layer was removed, and the wash repeated a further four times. The resulting solution of NADH was analyzed by GC to determine the ethanol concentration, and then lyophilized. This procedure gave <1 μM ethanol background in the reaction mixtures. NADH turnover incubations were carried out at 30 °C in 4-mL capacity cuvettes equipped with screw caps and Teflon septum seals. Incubation mixtures contained Tris (50 mM, pH 7.4), KCl (200 mM), $P450_{cam}$ (1 μM), putidaredoxin (16 μM), and putidaredoxin reductase (1 μM). In a typical reaction, the volume of buffer required to take the final volume to 2.5 mL was $\approx$1.6 mL. Half of this required volume of buffer was saturated with ethane, and the other half was a buffer solution saturated with oxygen. A sample was taken (the no-NADH control), and the cuvette sealed with a Teflon-backed screw cap. The mixtures were equilibrated at 30 °C for 2 min, and the reaction was initiated by the addition of NADH through a hypodermic syringe needle pushed through the Teflon septum. NADH was added as a stock solution (20 mg mL^{-1}) in Tris (50 mM, pH 7.4) to $\approx$200 μM (final A_{340} $\approx$1.2), and the absorbance at $\lambda = 340$ nm was monitored. The rate of NADH consumption was calculated from the slope of the time-course plot by using $\varepsilon_{340} = 6.22$ mM^{-1} cm^{-1}. A total of five experiments were carried out for each mutant tested. For each mutant, a control incubation with no substrate was also carried out to ensure that the ethane contained no ethanol.
[21] A 90-μL aliquot of a reaction mixture or control was added to a 10-μL aliquot of an aqueous solution of pentan-1-ol (200 μM) as internal standard. A 2-μL aliquot of each mixture was injected directly onto the SPB-1 GC column (0.5 mm × 60 m). The column temperature was held at 40 °C for 4 min and then increased at 5 °C min^{-1} to 100 °C. The retention times were: ethanol, 3.50 min; propan-2-ol, 4.80 min; propan-1-ol, 6.50 min; pentan-1-ol, 16.0 min. The concentration of product in a reaction mixture was determined by calibrating the FID response to the product.[10] Peroxide uncoupling was determined by a standard horseradish peroxidase/phenol/4-aminoantipyrine assay.
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