The oxidation of polychlorinated benzenes by genetically engineered cytochrome $P450_{cam}$: potential applications in bioremediation

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Polychlorinated aromatic compounds are persistent environmental contaminants; we describe here the redesign and engineering of the haem monooxygenase cytochrome $P450_{cam}$ to oxidise these compounds efficiently to the chlorinated phenols which are readily degraded by many micro-organisms, thus providing a basis for novel systems for biological clean-up of these inert compounds.

Polychlorinated aromatic compounds are among the most problematic environmental pollutants because of their chemical inertness, lipid solubility and toxicity.[1] Naturally occurring micro-organisms have evolved to degrade and mineralise many, but by no means all, of these compounds. For example, the dioxins and heavily chlorinated biphenyls are degraded very slowly or not at all. Similarly, numerous micro-organisms have been isolated which together degrade most of the chlorinated benzenes,[2] but the most heavily chlorinated compounds pentachlorobenzene (PeCB) and hexachlorobenzene (HCB), as well as 1,2-dichlorobenzene (1,2-DCB) and 1,3,5-trichlorobenzene (1,3,5-TCB) are particularly inert to biodegradation.[3] We noted that chlorinated phenols are readily degraded by micro-organisms.[4] We reasoned that if a monooxygenase can be prepared that can efficiently oxidise polychlorinated benzenes to the phenol derivatives, then the monooxygenase system could be introduced into chlorophenol-degrading micro-organisms such as *Pseudomonas* bacteria to enable them to degrade polychlorinated benzenes. We report here the genetic engineering of the haem monooxygenase cytochrome $P450_{cam}$ from *Pseudomonas putida* to oxidise 1,2-DCB, 1,3,5-TCB and PeCB, creating a basis for novel bioremediation systems.

The criteria for successful redesign of the $P450_{cam}$ active site for the oxidation of polychlorinated benzenes are reasonable rates of substrate turnover and tight coupling of NADH consumed to substrate oxidation. Uncoupling wastes NADH and can produce harmful hydrogen peroxide,[5] both of which will put metabolic stress on the host organisms and thus reduce their viability. We have shown that the Y96A and Y96F mutations greatly improved the activity of $P450_{cam}$ for the oxidation of a wide range of organic compounds, including simple alkanes,[6] styrene[7] and naphthalene.[8] We proposed that these mutations at Y96 (Fig. 1) improve the turnover of such hydrophobic compounds by increasing the hydrophobicity of the enzyme active site. We also noted that the oxidation by the Y96F mutant of naphthalene, which is larger than the chlorinated benzenes considered here, had a coupling of 55% compared to 17% for the Y96A mutant. Since the Y96A mutant should have a larger active site, the Y96F mutation should be our starting point. The coupling could then be increased by improving the enzyme–substrate fit and by forcing the chlorinated benzenes to bind closer to the haem. Hence the active site volume was reduced by further substitutions with amino acids with bulkier side-chains at three other active site residues. Phenylalanines 87 and 98 are at approximately the same height as Y96 above the haem and near the top of the active site (Fig. 1), and valine-247 is just below these residues and closer to the haem.[9] The new mutants F87W–Y96F, F87W–Y96F–F98W and F87W–Y96F–V247L were prepared and their activities compared to the WT enzyme.

1,2-DCB was oxidised by all the $P450_{cam}$ enzymes to 2,3-dichlorophenol (2,3-DCP) and 3,4-dichlorophenol (3,4-DCP) in 9:1 ratio (Fig 2), and 2,4,6-trichlorophenol (2,4,6-TCP) was the only product from 1,3,5-TCB. The predominant product from PeCB oxidation was pentachloro-

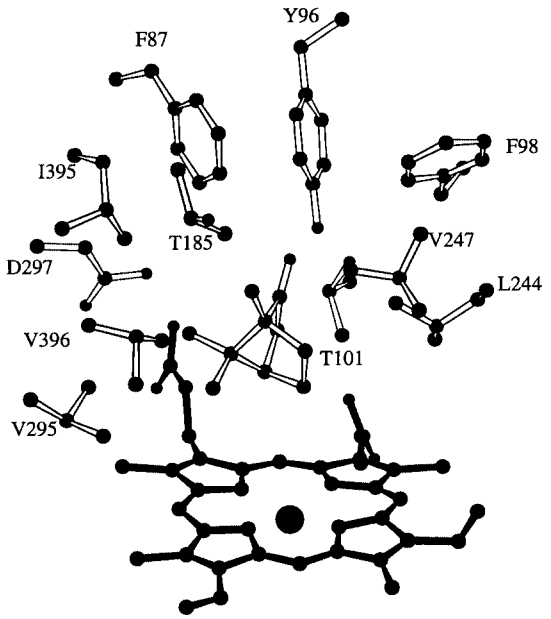

Fig. 1 The active site structure of wild-type $P450_{cam}$ with bound camphor.

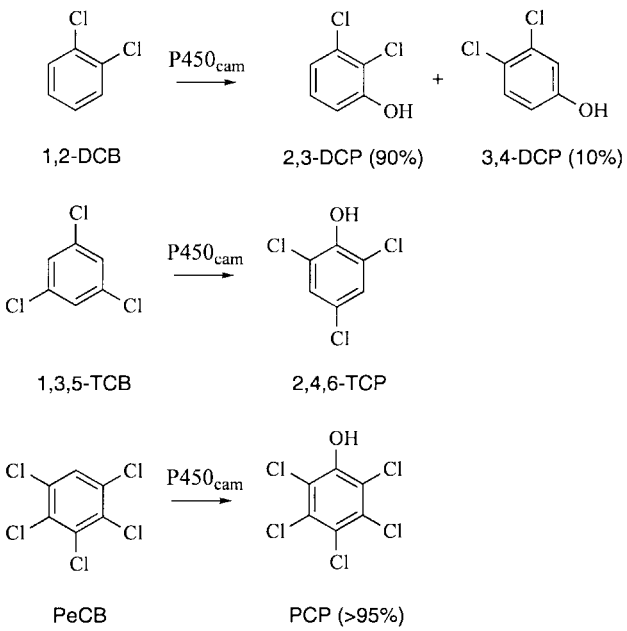

Fig. 2 The oxidation of chlorinated benzenes by wild-type $P450_{cam}$ and active site mutants.

Table 1 The activity of wild-type (WT) cytochrome $P450_{cam}$ and active site mutants for the oxidation of chlorinated benzenes. ND: no product observed by HPLC. The products of the reactions are shown in Fig. 2

	WT	F87W–Y96F	F87W–Y96F–V247L	F87W–Y96F–F98W
1,2-dichlorobenzene (1,2-DCB)				
Binding constant K_D/μM	3.0	2.0	0.9	1.2
NADH turnover rate[a]	20	408	391	158
Product formation rate (k_2)[b]	0.45	106	83	78
Coupling efficiency[c]	2.3%	26%	21%	49%
$(k_2/K_D)/M^{-1}\ s^{-1}$	2.50×10^3	8.83×10^5	1.54×10^6	1.08×10^6
1,3,5-trichlorobenzene (1,3,5-TCB)				
Binding constant K_D/μM	3.9	3.0	2.0	1.8
NADH turnover rate	6.5	224	308	121
Product formation rate (k_2)	0.07	115	175	119
Coupling efficiency	1.1%	51%	57%	97%
$(k_2/K_d)/M^{-1}\ s^{-1}$	3.00×10^2	6.39×10^5	1.46×10^6	1.10×10^6
Pentachlorobenzene (PeCB)				
NADH turnover rate	2.4	100	229	43
Product formation rate	ND	2.3	5.5	3
Coupling efficiency	—	2.3%	2.4%	7%

[a] Given as nanomoles of NADH consumed per nanomole of $P450_{cam}$ per minute and the average of at least 3 experiments with all the data within 10% of the means. Incubation mixtures (1.70 ml) contained 50 mM Tris.HCl, pH 7.4, 1 μM $P450_{cam}$, 10 μM putidaredoxin, 2 μM putidaredoxin reductase and 200 mM KCl. Both 1,2-DCB and 1,3,5-TCB (200 μM) were added as a 0.1 M stock in ethanol. The mixture was incubated at 30 °C for 2 min after the addition of NADH (100 μM) and the reaction initiated by the addition of substrate. NADH absorbance at 340 nm was monitored over the course of the reaction. [b] The total amount (in nanomoles) of chlorinated phenol products formed per nanomole of $P450_{cam}$ per minute. After the addition of 100 μl of an internal standard to a turnover incubation, organics were extracted by solid phase methods using Varian Bond-Elut columns and products were analysed by reverse phase HPLC. To obtain quantitative results, mixtures containing known concentrations of a product and all of the incubation components except NADH were extracted and analyzed as described above. Linear calibration plots that passed through the origin were obtained for all of the products. [c] The coupling efficiency is the ratio of the total amount of products formed to the amount of NADH consumed and is expressed as a percentage.

phenol (PCP), but two small peaks (*ca.* 2% each) were ascribed by co-elution experiments to 2,3,5,6- and 2,3,4,5-tetrachlorophenols formed by oxidative dehalogenation.

As shown in Table 1, the wild-type (WT) had low rates (< 0.5 min^{-1}) and couplings (1–2%) for the oxidation of the chlorinated benzenes compared to the totally coupled (100%) camphor oxidation rate of 1050 min^{-1} under identical conditions. The mutants all had 2–3 orders of magnitude faster chlorinated benzene oxidation rates than the WT. The NADH turnover rates were up to 400 min^{-1}, and the coupling efficiencies were also much higher. The 50% coupling for 1,2-DCB oxidation by the F87W–Y96F–F98W mutant is a dramatic improvement, and the near total coupling for 1,3,5-TCB oxidation is truly remarkable because the structure of this molecule is completely different from that of camphor. Very importantly, the results also showed that the low solubility of PeCB in water was not a problem, in that a very reasonable NADH turnover rate of 229 min^{-1} could be attained although the coupling was low. The data show that the rationale for active site redesign, whilst empirical and qualitative in nature, was very successful indeed.

The strength of binding and catalytic efficiency of the $P450_{cam}$ enzymes for 1,2-DCB and 1,3,5-TCB oxidation were investigated. The dissociation constants (K_D) in Table 1 show that, as expected, the binding of these compounds was strengthened by the mutations, but by no more than a factor of three. On the other hand, the substrate oxidation rates (k_2) showed 2–3 orders of magnitude increases. There was no direct correlation between the values of K_D and the NADH turnover rates or, notably, the coupling efficiency, which is a stringent measure of the enzyme–substrate fit. Nevertheless, it is instructive to compare the relative specificity (k_{cat}/K_M) of the mutants. It has been suggested that the k_2/K_D ratio is a fair approximation for the k_{cat}/K_M ratio for $P450_{cam}$.[10] The k_2/K_D ratios in Table 1 again highlight the very dramatic accelerating effects achieved by the mutations although they are well short of the near-optimal ratio for camphor oxidation by the WT enzyme ($K_D = 0.27$ μM, $k_2 = 1050$ min^{-1}, $k_2/K_D = 6.5 \times 10^7$ M^{-1} s^{-1}). However, we conclude that the F87W–Y96F–F98W mutant represents an excellent compromise between reasonable rate and tight coupling, especially for the *in vivo* oxidation of polychlorinated benzenes, where very fast turnover could lead to the build up of polychlorinated phenols to toxic levels.

All the chlorophenol products in Fig. 2 are known to be degraded by various micro-organisms,[4] and therefore the mutants can be the basis of novel bioremediation systems for polychlorinated benzenes by genetically introducing the genes encoding the three proteins of the $P450_{cam}$ system into chlorophenol-degrading micro-organisms such as *Pseudomonas* bacteria. The F87W–Y96F–F98W mutant can be used for the particularly inert 1,2-DCB and 1,3,5-TCB. The coupling for PeCB and HCB can be improved by further active site mutations, and then even these highly inert compounds can be efficiently degraded.

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